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EXPERIMENTAL ARTICLES

Influence of Soil Pollution on the Composition of a Microbial Community

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Abstract—The abundance dynamics and composition of indigenous soil microbial communities were studied in soils polluted with naphthalene, dioctyl phthalate, diesel fuel, and crude oil. DGGE analysis of the 16S rRNA genes amplified from the total soil DNA revealed that the bacterial community of uncontaminated soil was more diverse and included no dominant species. In the soil samples polluted with the crude oil, diesel fuel, or dioctyl phthalate, *Pseudomonas* became the dominant bacteria since the third day of the experiment. In the soil polluted with naphthalene, two genera of bacteria (*Pseudomonas* and *Paenibacillus*) were dominant in population on the third day of the experiment, while on the 21th day of the experiment *Arthrobacter* became dominant. During the experiment, the average number of indigenous bacterial degraders increased approximately by two orders of magnitude. While the key genes of naphthalene catabolism, *nahAc* and *nahH*, were not detected in the pristine soil, they were found in a significant amount on the third day after naphthalene addition. Three degrader strains harboring the plasmids of naphthalene biodegradation (IncP-9 group) were isolated on the third day from the soil polluted with naphthalene. Two of these plasmids, although isolated from various degraders, were shown to be identical.

Keywords: naphthalene, DGGE analysis, *Pseudomonas, nahAc* and *nahH* genes

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Due to the increasing environmental pollution, soil microorganisms are permanently subjected to various stable toxic organic compounds. However, the dynamics of microbial communities and the changes in their composition induced by specific pollutants are insufficiently studied. Investigation of the laboratory model soil systems (microcosms) makes it possible to determine the response of bacterial communities to specific pollutants and the patterns of their functioning. In the present work, common multicomponent environmental pollutants (crude oil and diesel fuel) were used, as well as naphthalene and dioctyl phthalate. Dioctyl phthalate is a toxic liquid used as a plasticizer of vinyl polymers, rubbers (to obtain frost-resistant rubbers), cellulose ethers, and polystyrene, and also as a hydraulic liquid and a dielectric liquid in capacitors. Naphthalene is a low-molecular aromatic hydrocarbon, which is wide-spread contaminant and is therefore frequently used as a model compound in the studies of the degradation of polycyclic aromatic hydrocarbons (PAH).

To detect the changes in the composition of bacterial communities in different ecosystems under various stress factors, denaturing gradient gel electrophoresis (DGGE) is used [1]. This approach is used to investigate the 16S rRNA genes of all members of a bacterial community in order to determine their systematic relations.

In this work DGGE analysis was used to monitor the changes occurring in microcosms in the presence of frequently used pollutants.

MATERIALS AND METHODS

Subjects of the study. Grey forest soil collected near Pushchino, Moscow region, was used in the work. The soil composition was 91.00% ash (SiO_2, 72.50%; C, 2.89%; H, 1.05%; N, 0.25%; P, 0.06%; Ca, 0.48%; Mg, 0.14%; Fe, 1.20%; K, 2.47%); pH of the aqueous extract was 5.5. In laboratory experiments the pollutants were added to the soil in the following concentrations: naphthalene, 1 mg/g; crude oil, 2 μL/g; diesel fuel (DF), 2 μL/g; dioctyl phthalate (DOP), 2 μL/g. Experiments were carried out using petri dishes containing 20 g of soil each. The plates were incubated at 28°C. Soil moisture was maintained at the level of 40% by adding distilled water. An experiment was carried out for 21 days. The samples were collected on days 0, 3, 10, and 21. Each variant was repeated three times. Indigenous bacteria isolated from soil were grown at 28°C on LB agar [2] and on solid salt–mineral Evans medium [3]. Naphthalene and diesel fuel were applied to the cover of the inverted dish. Sodium salicylate, dioctyl phthalate, and crude oil were added to the

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PCR primers used in the study

Gene	Primers	Nucleotide sequence, (5' → 3')	Size of PCR product, bp	Reference
16S rRNA for DGGE analysis	U968GC	[CGCCCGGGGCGCGCCCCGGGCG-GGGCGGGGGCACGGGGG] CAACGCGAACCTTAC	440	[6]
	L1401-1378	CGGTGTGTACAAGGCCCGGGAACG		
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	1465	[7]
	1492R	GGYTACCTTGTTACGACTT		
	BoxA1R	CTACGGCAAGGCGACGCTGACG	–	[8]
repA (IncP-7)	RepAP7F1	GCCCATGCCGAAAAAGGTGTC	412	O.V. Volkova (IBPM RAS)
	RepAP7R1	GAATCGTTGATAGGCATCCGAC		
repAB (IncP-9)	repF	CCAGCGCGGTACWTGGG	398	[9]
	repR	GTCGGCAICTGCTTGAGCTT		
nahAc	Ac149f	CCCYGGCGACTATGT	865	[10]
	Ac1014r	CTCRGGCATGTCTTTTTC		
nahG	shc1_up	CGGCKTTHGGTGARGTCGGTGC	893	[11]
	shc1_lo	GGCGAGGAARTAGGCGTCCTCAAG		
nahH	23DOF	ATGGATDTDATGGGDTTCAAGGT	721	[12]
	23DOR	ACDGTCADGAADCGDTCGTTGAG		
nahR	nahR_1f	ATGGAACTGCGTGACCTGG	585	[11]
	nahR_585r	GCCGTAGGAACAGAAGCG		

medium in concentrations of 1, 1, and 10 mL/L, respectively. Conjugation transfer of the plasmids was carried out using the plasmid-free *P. putida* KT2442 (*gfp*, Km^r, Rif^r) strain as recipient. The strain was kindly supplied by K. Smalla, Germany.

Variants of microcosms. Variant I: soil + naphthalene; variant II: soil + DF; variant III: soil + crude oil; variant IV: soil + DOP. At each sampling point, the samples (250 mg) were taken for isolation of the total soil DNA. Serial dilutions of the soil samples were plated on LB agar (for determination of the total number of microorganisms) and on mineral Evans medium containing naphthalene, diesel fuel, dioctyl phthalate, or crude oil (for enumeration of indigenous degraders).

Isolation of total DNA from the soil. Total DNA was isolated from 500 g of the soil sample using Fast DNA® SPIN Kit for soil (Q-Biogene, United States) according to the manufacturer's protocol.

Isolation of plasmid DNA. Plasmid DNA was isolated by alkaline lysis [2] with modifications.

Conjugational transfer of the plasmids was carried out as described [4].

Exopolysaccharides were isolated as described [5].

Polymerase chain reaction was carried out in a Mastercycler Gradient cycler (Eppendorf, Germany). Oligonucleotide primers used in this study are listed in the table. Amplification reactions were carried out under standard conditions; the volume of the reaction mixture was 25 μL. For DGGE analysis, the reaction mixture contained *Taq* DNA polymerase 1× buffer (Promega, United States), 1.5 mM of $MgCl_2$, 5% of DMSO, 20 pM of each primer, 2 U of *Taq* DNA polymerase, 200 μM of deoxyribonucleosidetriphosphates, 25–100 ng of DNA. For DGGE-analysis, 16S rRNA amplification conditions were as follows: 94°C for 5 min; 35 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 30 s; additionally 72°C for 10 min. In case of Box-PCR reaction, the mixture contained 5× Gitcher buffer, 1% of DMSO (Sigma, United States), 0.3 μg of primer, 0.4 U of *Taq* DNA polymerase (Promega, United States), 1.25 mM of deoxyribonucleosidetriphosphates, 0.4 mg of BSA, and 25–100 ng of DNA. In other cases, it contained 1× *Taq* DNA polymerase buffer (Promega, United States), 1.5 mM of $MgCl_2$, 5% of DMSO, 20 pM of each primer, 2 U of *Taq* DNA polymerase, 200 μM of deoxyribonucleosidetriphosphates, and 25–100 ng of DNA.

DGGE-analysis of the amplified 16S rRNA genes was carried out using a Dcode Universal Mutation Detection System (BioRad, United States). The PCR product (300–500 ng) was applied to the 9% polyacrylamide gel [13] with gradients 40–80% (100% denaturing gel contained 7 M urea and 40% deionized formamide). DGGE was carried out in 1× *Tris*–acetate–EDTA buffer at 220 V and at 60°C for 6.5 h. The

resulting gel was stained with silver [14]. The major bands were excised from the gel, washed with 20 μL of 0.5% potassium ferricyanide, $K_4[Fe(CN)_6]$, then washed twice with 500 μL of deionized sterile water, and frozen. After several freeze-thaw rounds, the fragments were reamplified and sequenced.

DNA sequencing. Sequencing was performed using the ABI PRISM® BigDye™ Terminator v. 3.1 Kit with subsequent analysis of the reaction products on an ABI PRISM 3730 automatic sequencer (Applied Biosystems, United States). Nucleotide sequences were analyzed using the DNAStar software package and the BLAST N server [NCBI, http://www.ncbi.nlm.nih.gov/BLAST].

Restriction analysis. The following enzymes were used: for 16S rRNA gene restriction, *Rsa*I and *Msp*I (*Hpa*II); for plasmid restriction, *Eco*RI. The treatment of DNA with restriction endonucleases was carried out at 37°C for 2 h according to the manufacturer's recommendations (all enzymes were produced by Fermentas, Lithuania).

DNA–DNA hybridization. The samples were separated by electrophoresis in a horizontal agarose gel and transferred to the Hybon N+ nylon filters (Amersham, United Kingdom) in 0.4 M NaOH solution. The transfer was carried out for 3–4 h. Then filter was neutralized by soaking in the solution of 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 5 min. Prehybridization and hybridization with DNA probes amplified with the primers for *nahAc* and *nahH* and labeled with dioxygenin (DIG) using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany) were carried out at 62°C (20% formamide) in a hybridization oven (Binder BFED53, Germany) with subsequent washing of the membrane. The membrane was washed twice at room temperature in 2× SSC, 0.1% SDS, for 5 min, and twice at 68°C in 0.1× SSC, 0.1% SDS, for 15 min (according to the manufacturer's protocol).

RESULTS

Dynamics of indigenous strains in the soil. Plating from serial dilutions on LB medium showed that the soil used in the experiment contained 2×10^6 CFU (colony-forming units) of microorganisms per 1 g of soil that could be cultivated on this medium. The total number of aerobic bacteria increased in all variants by 21 day of the experiment and was 10^7–10^8 CFU/g soil (Fig. 1). Before an experiment the absence in the soil of indigenous strains able to degrade naphthalene, salicylate, DOP, DF, and crude oil as a single source of carbon and energy was demonstrated using direct plating. The number of these microorganisms was probably less than 10^2 CFU/g soil and, therefore, they could not be detected by the standard microbiological methods [15]. In response to pollution, the number of Nah^+ strains in variants I, II, III, and IV increased by more than two orders of magnitude at the third day, reaching 10^3 CFU/g (Fig. 1). At day 21 the number of naphthalene degraders in variants II and IV remained at the same level, while in variant III it increased up to 10^4 CFU/g, and in variant I, up to 10^5 CFU/g. The number of DF, DOP and crude oil degraders also increased in all variants during the experiment, although at day 21 it did not exceed 6×10^3 CFU/g.

Changes in the composition of the soil microbial community during the experiment. DGGE analysis showed that the bacterial community in non-polluted soil was characterized by high species diversity and by the absence of dominant groups (Fig. 2). The major bands on the DGGE profile indicated 16S rDNA of bacteria prevailing in the population at the moment of sampling. Sequencing of DNA isolated from the dt1, o1, and dof1 major bands (Fig. 2) revealed that from the third day of the experiment until its end in soil samples polluted with the crude oil, DF, and DOP predominated bacteria related to the genus *Pseudomonas* (the amplified 16S rDNA fragment and the 16S rDNA of *Pseudomonas* sp. CL1.82 shared 98% of identity). In microcosms polluted with naphthalene two genera of bacteria dominated at the third day: *Pseudomonas* (n1 band, 98% identity to the 16S rDNA of *Pseudomonas* sp. CL1.82) and *Paenibacillus* (n2 band, 99% identity to the 16S rDNA of *Paenibacillus* sp. La1). Nevertheless, by day 21 of the experiment the genus *Arthrobacter* became dominant (n3 band, 93% identity of the amplified 16S rDNA fragment to the *Arthrobacter* sp. S6).

Isolated strains and their plasmids. Five different strains were isolated from microcosms, variant I, at the third day of experiment by direct plating (NZ3.1, NZ3.2, NO1, NO2, NO3). Strains NZ3.1, NZ3.2 and NO2 were able to grow both on liquid and agarized media with naphthalene and salicylate. Strains NO1 and NO3 also grew both on the agar-containing media with naphthalene and salicylate and on the pure agar mineral culture medium. Therefore, these strains utilized agar as a source of carbon and energy; aromatic hydrocarbons were not toxic in this case. These strains did not grow in liquid culture media containing naphthalene and salicylate. Strains NO1 and NO3 synthesized exopolysaccharides in concentrations of 1 and 5 g/L, respectively. The species position of the strains was determined using ARDRA (amplified ribosomal DNA restriction analysis) of the 16S rDNA amplification products and using *Rsa*I and *Msp*I (*Hpa*II) enzymes. *Pseudomonas putida*, *P. fluorescens*, *P. chlororaphis*, *P. aeruginosa*, and *P. aureofaciens* were used as the controls. All naphthalene degraders were shown to belong to *P. fluorescens*. NO1 and NO3 restriction profiles differed from those of pseudomonads while demonstrating high similarity to each other. To determine the species position of these strains, the PCR fragments of their 16S rDNA (838 bp) were sequenced. The partial sequence of the NO1 16S rRNA gene was submitted to GenBank under acces-

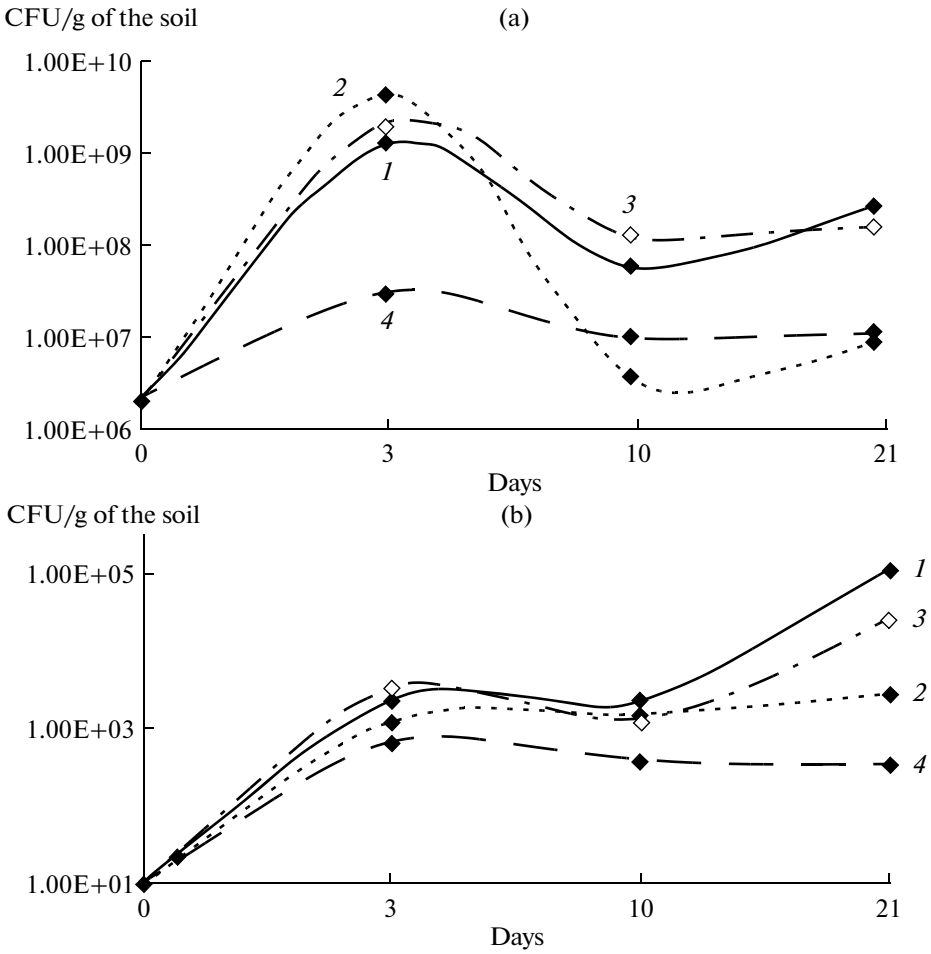

Fig. 1. Total numbers of: aerobic bacteria in all variants of microcosms at 21 day of experiment (a); and aerobic naphthalene-degrading bacteria in all variants of microcosms at 21 day of experiment (b). Soil + naphthalene (*1*); soil + DF (*2*); soil + crude oil (*3*); soil + DOP (*4*).

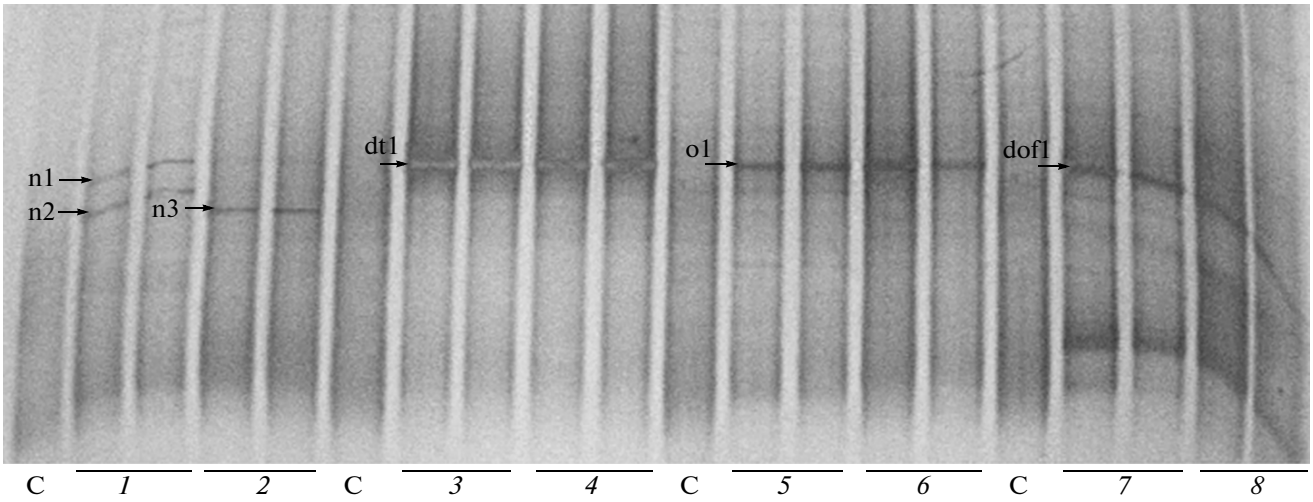

Fig. 2. DGGE of 16S rRNA genes amplification products from the total soil DNA. C, soil (day 0); soil + naphthalene (day 3) (*1*); soil + naphthalene (day 21) (*2*); soil + DF (day 3) (*3*); soil + DF (day 21) (*4*); soil + crude oil (day 3) (*5*); soil + crude oil (day 21) (*6*); soil + DOP (day 3) (*7*); soil + DOP (day 21) (*8*).

sion number JF438971. The sequence of PCR products shared 99% identity with the corresponding *Paenibacillus* sp. La1 16S rRNA gene sequence; therefore the strains were classified within the genus *Paenibacillus*. Box-PCR demonstrated all isolated strains to be different from each other.

The plasmids of the P-9 incompatibility group were isolated from *P. fluorescens* strains NO2, NZ3.1, and NZ3.2. Determination of the differences between these plasmids was carried out using RFLP-analysis (restriction fragment length polymorphism) with *Eco*RI. The plasmids pNO2 and pNZ3.2 were shown to be identical; the restriction profile of pNZ3.1 was different. The plasmids were transferred by conjugation to *P. putida* KT2442 with defective system of foreign DNA restriction and carrying the *gfp* gene (green fluorescent protein) in the chromosome. Expression of *gfp* made it possible to differentiate the colonies of this strain on agar media from the colonies of other bacteria [16]. Obtained transconjugants were able to grow on mineral culture media with naphthalene. To analyze the key genes of naphthalene biodegradation, the genes *nahAc, nahG, nahH*, and *nahR* coding for the big subunit of naphthalene 1,2-dioxygenase, salicylate 1-hydroxylase, catechol 2,3-dioxygenase, and the regulatory protein, respectively, were selected within the plasmids under study. PCR using the plasmids isolated from transconjugants as templates demonstrated that pNO2, pNZ3.2, and pNZ3.2 contained all the key genes for naphthalene utilization.

The *nahAc* and *nahH* occurrence in polluted soil. Analysis of the total DNA from soil samples was carried out to reveal the presence of the key genes of naphthalene catabolism: *nahAc* coding for the big subunit of naphthalene 1,2-dioxygenase, and *nahH* coding for catechol 2,3-dioxygenase and usually located on plasmids. PCR with specific primers and subsequent hybridization with the corresponding probes were used. The samples were collected at 0, 3, 10, and 21 days. PCR and hybridization results are shown on Fig. 3. Before addition of naphthalene to the soil, the key genes of naphthalene biodegradation were not revealed in any of the experimental variants. Three days after naphthalene addition in variant I, significant amounts of *nahAc* and *nahH* genes were detected.

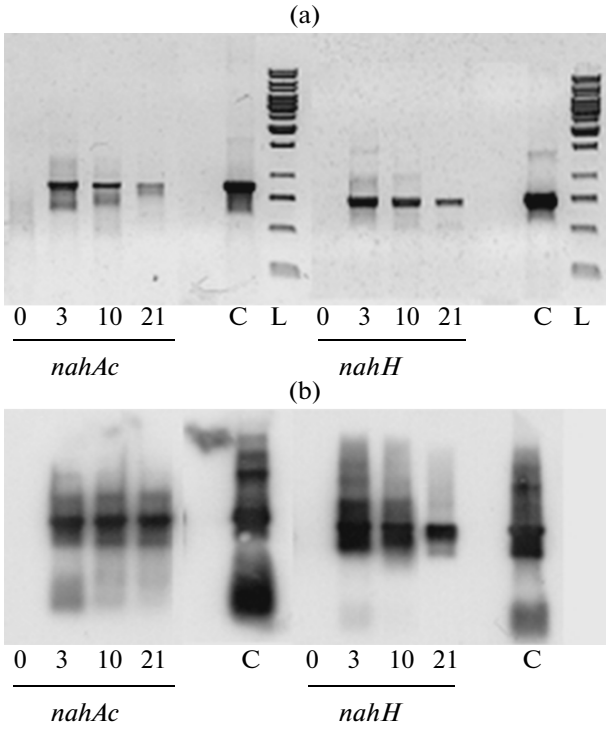

Fig. 3. Electrophoresis of amplification of the *nahAc* and *nahH* genes (a) from the total soil DNA taken at days 0, 3, 10, 21 of experiment from microcosm I (soil + naphthalene). (b) DNA–DNA-hybridization of amplification products with DIG-labeled samples of the *nahAc* and *nahH* genes. C, pNF142.

DISCUSSION

DGGE analysis of the 16S rDNA amplified from the total soil DNA revealed changes in the composition of soil bacterial populations under effect of the investigated substances. Plating of the soil samples from the control points demonstrated that the presence of pollutants (naphthalene, crude oil, diesel fuel, and dioctyl phthalate) resulted in increased total bacterial number, including an increase in abundance of bacterial degraders. Similar results were reported by Del Panno et al [17] in the work with soil polluted with sludge: maximum of the total numbers of bacteria and of degrader bacteria was observed in the period before the 40th day of the experiment. In the current work, in spite of an increase of the population abundance, a simultaneous decrease of species diversity was observed (Fig. 2). These data correlate with the results of a few works regarding the dynamics of bacterial communities of polluted soils [18, 19]. However, Feris et al. [20] demonstrated that pollution of river water with crude oil components, BTEX (mixture of benzene, toluene, ethylbenzene, and xylenes) and MTBE (methyl *tert*-butyl ether) did not result in decreased bacterial diversity in the silt, although the species structure of bacterial communities changed.

Since the third day of the experiment in microcosm variants II, III, and IV, predominance of gammaproteobacteria (*Pseudomonas* sp.) in population was shown. In variant I at the third day of the experiment bacteria of the genus *Pseudomonas* and gram-positive bacteria *Paenibacillus* prevailed. Three naphthalene-degrading strains of *P. fluorescens* and two strains of *Paenibacillus* sp. were isolated from the soil at the third day of the experiment by direct plating. Most paenibacilli are known to synthesize phytohormones [22], antibiotics [23, 24], and to degrade aromatic hydrocarbons [25]. Nevertheless, *Paenibacillus* sp. strains isolated in this work did not utilize naphthalene but

survived in the polluted environment and were able to grow using agar as a single source of carbon and energy. At day 21 of the experiment, drastic changes in the community composition were observed: the genus *Arthrobacter* became dominant, although it did not replace proteobacteria completely since slight bands (lines no. 2) in the DGGE profile were detected (Fig. 2).

Pollution with naphthalene resulted in an increase of the number of naphthalene-degrading bacteria in soil. The content of the naphthalene catabolism key genes (namely, *nahAc* and *nahH*) increased simultaneously (Fig. 3). All *nah*-like genes (*nah, ndo, pah*, and *dox*) constitute a conservative group and occur in fluorescent pseudomonads [26]. In non-polluted soil, the *nahAc* gene is usually not detected [26, 27]. Park et al. demonstrated that, 6 days after soil contamination with naphthalene, the number of *nahAc* copies increased from 5×10^3 copies/g soil up to 10^7 copies [27]. In the soils polluted with oil products, the amount of *nahAc* correlated with the ability of microbial community to degrade naphthalene. We also registered an increase of the concentration of the gene for the big subunit of naphthalene 1,2-dioxygenase, which is typical of the genus *Pseudomonas* (Fig. 3). Increase of *nahAc* amount correlated with the increase of *nahH* amount. The *nahH* gene determines the synthesis of the first enzyme of the catechol utilization *meta*-pathway, catechol 2,3-dioxygenase (Fig. 3). This fact indirectly suggested the increase in the concentration of both catabolic genes to be related to the presence of plasmid-carrying strains, since the genes for the *meta*-pathway are usually located on the plasmids of biodegradation. The genes of the "upper" operon of the naphthalene utilization pathway including the *nahAc* gene may be located both on plasmids and on a chromosome [28]. The genes *nahAc* and *nahH* were detected at day 21, when the strains of the genus *Arthrobacter* were shown to dominate in the soil; these strains are known to carry the genes for catabolism of aromatic hydrocarbons not homologous to those of pseudomonads. Thus, at the end of experiment actinobacteria did not completely replace *Pseudomonas* sp. in the soil community (Fig. 2, lines no. 2).

Three different *Pseudomonas* strains carrying plasmids of the P-9 incompatibility group (containing all the necessary genes for naphthalene utilization—*nahAc, nahG, nahH,* and *nahR*) were isolated from microcosm I. Two plasmids were identical. Detection of identical plasmids in two distinct strains suggested horizontal plasmid transfer in the soil bacterial community. The possibility of transfer of the biodegradation genes from these pseudomonads to other bacterial species within the soil community by conjugation plasmids and transposons could therefore not be excluded. Conjugation plasmids and transposons usually control catabolism of xenobiotics, including naphthalene. Therefore, increase of the concentrations of the catabolic genes in the soil polluted with naphthalene may occur due to high competitiveness of plasmid-bearing strains or to horizontal plasmid transfer to the suitable recipients.

Other genes that control isofunctional enzymes synthesis but are unrelated to the *nah* genes are also known. These include the genes *nag* (*Ralstonia* sp.), *nar* (*Rhodococcus* sp.), *phd* (*Comamonas testosteroni*), and *phn* (*Burkholderia* sp.) [26]. The primers used in this work did not allow for the detection of either the genes unrelated to the *nah*-genes of fluorescent pseudomonads including the isofunctional genes of the *Betaproteobacteria* and *Actynomycetes*, or the genes of anaerobic degradation of aromatic hydrocarbons [29]. The presence of other naphthalene degraders containing naphthalene 1,2-dioxygenase genes, nonhomologous to the *nahAc*, in the studied soil should not be ruled out.

DGGE analysis of the 16S rRNA genes together with PCR analysis of the total soil DNA aimed to detect the major genes for aromatic hydrocarbon degradation indicated that bacterial communities in the polluted environment were less diverse and their composition and the level of diversity depended on the type of pollutant and on duration of its action. The current study of the microbial communities of polluted ecosystems revealed predomination of the microorganisms utilizing toxic pollutants (*Pseudomonas* sp., *Arthrobacter* sp.) or at least able to survive under their effect (*Paenibacillus* sp.). Isolated paenibacilli produced significant amounts of exopolysaccharides, natural carriers enhancing the level of survival of both paenibacilli and pseudomonads under conditions of pollution with toxic substances [30].

The consequences of pollution with various substances obviously depend not only on the type and amount of the pollutant but also on the type of soil and its initial metabolic potential, i.e. on the microorganisms present in the soil prior to the pollution and carrying the genes for biodegradation. After addition of the pollutant, we observed an increase of the total number of soil microorganisms, including bacteria unable to degrade it. Probably xenobiotics stimulated bacterial utilization of the soil organic substances, as was shown previously [31].

Based on the data obtained and according to the previous studies [32–34] one could suggest that pollution of the environment (water, soil, silt etc.) with crude oil, diesel fuel, BTEX, benzene, or dioctyl phthalate, usually led to predominance of proteobacteria. In locations polluted with naphthalene, in most cases actynobacteria became dominant in the population, replacing proteobacteria in the course of time. The plasmids carrying the genes for naphthalene utilization may play an essential role in its degradation.

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