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RESEARCH PAPER

BIOREMEDIATION OF SOIL OF THE KOLA PENINSULA (MURMANSK REGION) CONTAMINATED WITH DIESEL FUEL

Maria V. Korneykova[1,2*], Vladimir A. Myazin[1], Nadezhda V. Fokina[1], Alexandra A. Chaporgina[1]

[1]Institute of North Industrial Ecology Problems - Subdivision of Federal Research Centre "Kola Science Centre of Russian Academy of Science", 14A Akademgorodok, 184209, Apatity, Russia

[2]Peoples' Friendship University of Russia (RUDN University), 6 Miklukho-Maklaya St, 117198, Moscow, Russia

***Corresponding author:** korneykova.maria@mail.ru

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ABSTRACT. This work focuses on the creation and use of associations of hydrocarbon-oxidizing microorganisms. Bioremediation of soils with the help of mixed cultural and associations of microorganisms provides wider adaptive possibilities than individual species. This is especially important in conditions of short northern summer. The results of field experiments showed that microbial associations based on indigenous microorganisms (bacteria *Pseudomonas fluorescens, P. putida, P. baetica, Microbacterium paraoxydans* and fungi *Penicillium commune, P. canescens st. 1, P. simplicissimum st. 1*) with mineral fertilizers reduced the content of total petroleum hydrocarbons in the Hortic Arthrosol soil of the Kola Peninsula by 82% over 120 days. Also, the microbial associations with mineral fertilizers had a positive effect on the physical properties of the soil, increasing its humidity. The bacterial-fungi associations changed the number, abundance and structure of the indigenous community of microorganisms. *Penicillium canescens*, which was included in the composition of fungi association, became dominant. During the rapid decomposition of hydrocarbons are released to the soil toxic intermediates or metabolites of the microbial oxidation of hydrocarbons. Hydrocarbon oxidizing microfungi suppressed the germination of test plant seeds to one degree or another. *Penicillium commune* fungal metabolites inhibited seed germination only by 29% for *Lepidium sativum* L. and 24% for *Triticum aestivum* L. This species can be used for bioremediation of petroleum contaminated soils.

KEY WORDS: diesel fuel, petroleum-contaminated soil, bioremediation, hydrocarbon-oxidizing bacteria, microfungi, phytotoxicity

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INTRODUCTION

The constantly increasing anthropogenic impact results in the high accumulation of various xenobiotics in ecosystems. Mineral oil and petroleum products are major pollutants among all environmental contaminants because of their danger, quick spreading capacity, and slow decomposition in the environment. The petroleum hydrocarbons in soil lead to a loss in the soil's fertility, causing soil structure damage, a change in water–air and redox conditions, a loss of infiltration capacity, the appearance of anaerobiosis, and a decrease of biogeochemical activities (Das and Chandran 2011; Adams et al. 2015).

Potentially hazardous sources of contamination with petroleum products in the Kola region include oil tank farms, fuel and power facilities, major industrial plants with their own vehicle fleets, petrol filling stations, and military facilities that use various petroleum products.

Purification of petroleum-contaminated areas is a challenge for environmental remediation. Thus, there is an urgent need to develop methods for reducing the negative impact of petroleum hydrocarbons on the natural ecosystem. This investigation is especially important for the North Kola region. The self-purification and self-recovery rate of petroleum contaminated soil depends on soil type, petroleum composition, contamination rate, solar radiation intensity, concentration of macronutrients, temperature and oxygen concentration (Grotenhuis et al. 1998; April et al. 2000).

Mechanical remediation of contaminated soil leads to the disturbance of the soil profile as well as to removal of the topsoil layer. The use of various sorbents and solutions creates a danger for secondary environmental pollution. Thereby, the effective removal of hydrocarbons from the soil by only one of methods (mechanical, physical, and chemical) is almost impossible. Consequently, the successful remediation of petroleum contaminated soil can be based on complex approaches only. Biological method is more advanced and promising for the final elimination of hydrocarbon and other types of pollution (McGill 1977; Koronelli 1996; Pinedo-Rivilla et al. 2009; Adams et al. 2015; Korneykova et al. 2019). The method is based on introducing

hydrocarbon-oxidizing microorganisms isolated from the contaminated soils or genetically modified. The biological method also involves the stimulation of indigenous hydrocarbon-oxidizing microorganisms combined with agrotechnical methods (cultivation, fertilizer application, watering etc.) (Heath 1993; Ghazali et al. 2004; Joo et al. 2008; Nilanjana and Chandran 2011). The main advantages of this method are efficiency, profitability, ecological safety, operation flexibility, and lack of secondary pollution (Kireeva et al. 2005).

Bacteria are essential for the purification and remediation of contaminated soil, especially the hydrocarbon-oxidizing strains of genera *Acinetobacter, Agrobacterium, Arthrobacter, Bacillus, Corynebacterium, Enterobacter, Flavobacterium, Microbacterium, Micrococcus, Mycobacterium, Nocardia, Pseudomonas, Rhodococcus,* and *Stenotrophomona* (Myazin and Evdokimova 2012). Microfungi significantly contribute to the self-purification of soil from hydrocarbons (Bilaj and Koval 1980; Field et al. 1992; April et al. 2000; Cerniglia and Sutherland 2001; Tigini et al. 2009; Samson et al. 2010; Khabibullina and Ibatullina 2011; Evdokimova et al. 2013; Korneykova et al., 2019), especially in acidic soil with unfavorable conditions for bacterial growth. The most active hydrocarbon-oxidizing fungi strains mainly refer to the genera *Aspergillus, Penicillium, Fusarium,* and *Trichoderma* (Glyaznetsova and Zueva 2013).

The biological methods for soil remediation can lead to some problems related to the interaction of introduced associations with native soil microbial communities. The increase of species with phytotoxic activity is possible due to rearrangements in the complex of microorganisms. According to Kireeva et al. (2009), the hydrocarbon-oxidizing microfungi are less sensitive to petroleum products, but they can increase soil phytotoxicity. Therefore, the possible microbial toxicosis of petroleum contaminated soils should also be taken into account when using biological preparations.

The main goal of research was to study the influence of biological treatment (with microbial associations) on the rate of remediation of Kola Peninsula soils contaminated with diesel fuel under a field condition.

MATERIALS AND METHODS

Soil

The soil was Hortic Arthrosol on sandy lake-glacial sediments. The samples were collected on each plot at 10-cm depth after 1, 3, 12, 15 months. Stones and root were removed; the soil was passed through a 1 mm sieve and thoroughly mixed for chemical analysis. That is an arable soil with 3.38% organic matter, nitrogen - 0.3%, calcium - 2.26 mg-eq/100 g, magnium - 0.41 mg-eq/100 g and pH 5.5.

Contaminant

The summer diesel fuel was used to contaminate the soil. The diesel fuel brand is L-0.2-62, it meets the requirements of GOST 305-82 (density with 20°C – 835 kg/m^3; viscosity with 20°C – 5.11 mm^2/s; fractional composition, 96% - not more 359°C; cetane number – 47; resin concentration – 4.6 mg/100 cm^3; mass fraction of sulfur – 0.16%).

Microbial associations

At first hydrocarbon-oxidizing bacteria (HOB) (*Pseudomonas fluorescens, P. putida, P. baetica,* and *Microbacterium paraoxydans*) and microfungi (*Penicillium canescens st. 1, P. commune,* and *P. simplicissimum* st. 1) were grown separately. These microorganisms' strains were taken from collection of the INEP KSC RAS. Previously, these strains were isolated from petroleum contaminated soil on Kola Peninsula.The 16S ITS gene regions obtained from those bacterial strains were deposited in the NCBI GenBank database under the registration numbers KM288708, KM288709, and KM 216318. A bacteria was grown in meat-peptone broth in the laboratory fermenter (BIOSTAT® A plus, Sartorius, Germany) under 27°C and aeration for 3 days. Bacterial suspension density was 10_8–10_9 cells/L. The fungal suspension was incubated in Erlenmeyer flasks in Czapek's liquid medium at a temperature of 27°C for 10 days. The number of fungi in suspension was 10_5–10_6 colony-forming units (CFU)/L. To prepare the bacterial-fungal suspension, the bacterial and fungal ones were mixed in equal proportion in meat-peptone broth.

Experiment design

The microfield experiment was performed at the Polar Experimental Station of Apatity Branch of the Vavilov All-Russian Research Institute of Plant Industry (Apatity, Murmansk region, coordinates 67°32'57", 33°22'30"). The size of each experimental plot is 1 m^2. The experiment continued for 15 months: from beginning of June 2018 until the end of September 2019. The experiment design is given in Table 1. Each variant was in triplicate.

Diesel fuel was added once on June. Just after the soil contamination it was amended with a complex mineral fertilizer (N = 16%, P_2O_5 = 16%, K_2O = 16%) in an amount of 60 g/m^2 as well as the microbial associations were inoculated there in the amount of 1.2 L/m^2. The top layer of the soil in all variants was mixed to a depth of 5 cm. After 1 month the soil amendments were repeated.

Determination of the total petroleum hydrocarbons content (TPH) in soil

The content of TPH in the soil was determined by IR spectrometry, using the AN-2 analyzer. The method

Table 1. The experiment design

Soil sample	Treatment	Disel fuel dose, l/m^2	Total amount of inoculated microorganisms, CFU/L	Mixing and fertilizer application	Total $N_{16}P_{16}K_{16}$, (g N,P_2O_5,K_2O/m^2)	
					1stmonth	2ndmonth
PS	Background (pure) soil	-	-	+	-	-
DF	Diesel fuel	10	-	+	9.6	9.6
DF+B	Bacteria		10^8–10^9	+		
DF+F	Fungi		10^5–10^6	+		
DF+BF	Bacteria+Fungi		10^6–10^7	+		

is based on the extraction of TPH from the soil with tetrachlorcarbon, separation of oil products from the polar hydrocarbons in the column filled with aluminum oxide, and further spectrophotometric identification of hydrocarbon content, according to absorption intensity of infrared radiation at fixed wavelengths (Drugov and Rodin 2007). The effect of microbial preparations was evaluated through analysis of the residual content of TPH in soil and decomposition rate.

Number and species diversity of culturable soil microfungi

The number of culturable microfungi was determined by plating method on the Czapek's nutrient media with lactic acid (4 ml/L of medium for bacteriostatic effect). Morphological characteristics were evaluated using an optical microscope Olympus CX-41 (Japan) with a camera Jenoptik ProgRes CT3 (Germany). Species were identified according to classical identification guides (Raper and Thom 1968; Klich 2002; Domsh et al. 2007; Seifert et al. 2011). Microfungi names were checked according to CABI Bioscience Databases (http://www.indexfungorum.org).

Phytotoxicity of hydrocarbon-oxidizing microfungi (HOM)

The level of HOM toxicity for plants was evaluated using the seeds of *Triticum aestivum* L. and *Lepidium sativum* L. The HOM were grown in Czapek's liquid medium for 10 days. The cultural liquid was separated from mycelium by filtering. The experimental seeds (30 seeds in triplicate) were placed into a Petri dish with filter paper and 10 ml of a fungi cultural liquid. Instead of fungi cultural liquid, water and sterile nutrient medium was used in the control. The Petri dishes were kept in the thermostat for 24 h at 25–26°C and then the number of germinated seed was counted. The fungi were considered toxic for plants at the reduction of seed germination by 30% in comparison with the control (water) (Bilaj and Koval 1980).

Determination of physical-chemical properties of soil

Soil humidity was measured by drying soil samples at 105°C to a constant weight.

The actual soil acidity was determined by the potentiometric method with a Radelkis OP-300 laboratory pH-meter with a combined pH electrode in the 1:2.5 water extracts from soil.

Statistical analyses

The data were statistically analyzed by the Statistica 6.0 and Microsoft Excel 2007 applied software. The reliability of the calculated coefficients was determined to use Student test.

RESULTS AND DISCUSSION

Influence of microbial associations on physical-chemical properties of soil

A positive effect of biological treatment on soil humidity was noted. This trend was also found at the beginning of the second vegetation period. However, by the end of the second vegetation period, there was no significant difference between the treatments (Fig. 1). The positive impact of biological treatment on the humidity content of contaminated soil will allow using plants for further bioremediation in more favorable conditions.

The microbial associations with mineral fertilizers resulted in a pH decrease of soil by 0.1 to 0.2 units. At the same time, despite using the same number of mineral fertilizers, lower pH values were found with F and B+F treatments. This can be a result of acidifying caused by the active release of metabolites by fungi (Fig. 1).

Influence of the microbial associations on the number of soil microorganisms

The dynamic of soil heterotrophic bacteria, HOB and microfungi in various soil treatments shown on the Fig. 2. The initial number of culturable soil microfungi was 3×10^3 CFU/g. During the first day after the soil contamination with treatment DF and DF+B, their number reduced by 2.4 and 2.8 times, respectively, that can be explained by toxic effect of diesel fuel on indigenous microfungi. At the same time, the number of the microfungi with treatment DF+F and DF+BF were 154×10^3 and 56×10^3 CFU/g respectively after 1 day. To the next year, the number of the soil fungi increased by 3-4 times, that is to 193-226×10³ CFU/g. These values were about one order of magnitude higher than in the treatment DF and 3-4 times higher than in the treatment B (Fig. 2A).

In contrast to microfungi, the soil contamination with diesel fuel did not inhibit growth either HOB or heterotrophic bacteria. The number of heterotrophic bacteria in the pure soil was $(16.8\pm1.7)\times10^6$ cells/g. During the first day after contamination and fertilizing, their number with

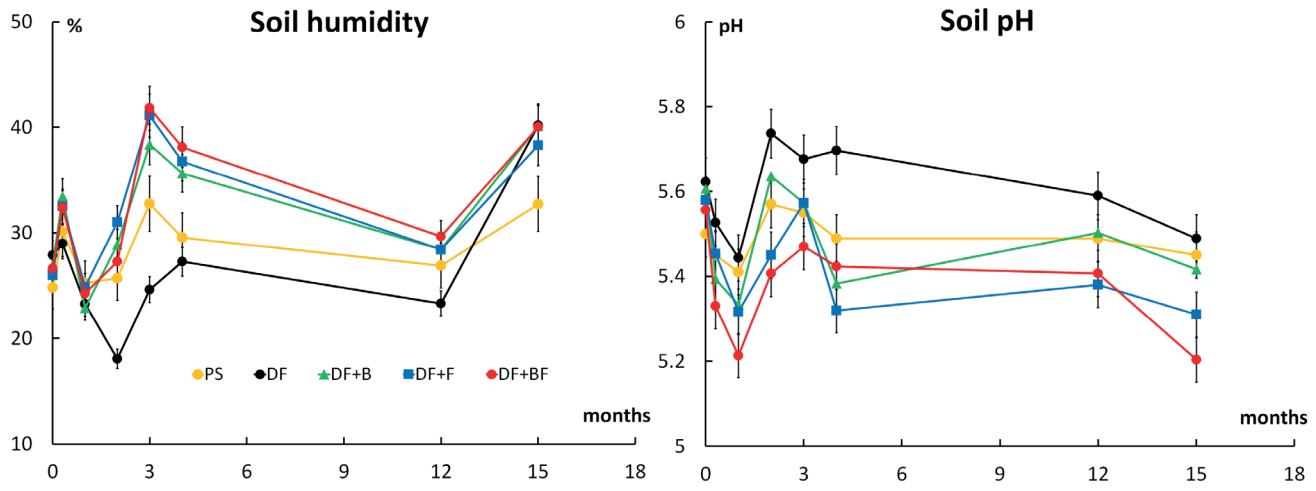

Fig. 1. Dynamics of soil humidity (A) and pH (B) in the background (pure) soil (PS), in diesel fuel contaminated soil (DF) and with association of bacteria (DF+B), fungi (DF+F), mix with bacteria-fungi (DF+BF)

treatment DF and DF+F were increased to (20.5±0.6)×10⁶ cells/g. In the next year those values remained increase – (37.5±3.5)×10⁶ CFU/g compared to the pure soil. After one day of inoculation the number heterotrophic bacteria in the soil with treatments DF+B and DF+BF were 58.2×10⁶ and 31.4×10⁶ CFU/g, respectively. Already in 3 months of the soil treatment, their number increased by 6-7 times and remained practically the same till the next year (Fig. 2B).

The dynamic of indigenous HOB in those soils was almost similar. Their number increased from 3.2×10⁶ CFU/g in the pure soil to 6.3×10⁶ and 11.9×10⁶ with treatment DF and DF+F, respectively. The number of culturable heterotrophic bacteria in the soil with treatment DF+B and DF+BF were 30.2 ×10⁶ and 12.0×10⁶ CFU/g after 1 day, and their number increased by 2-9 times respectively after 12 months treatment (Fig. 2C).

In accordance with the received data, the bacterial preparations supported a high number of culturable heterotrophic and HOB during all term of the observation. The microfungi, which are higher-level organisms than bacteria, are likely to be more sensitive to environmental changes but better able to adapt to a changing environment due to a powerful enzymatic system and abundant sporogenesis (Kireeva et al. 2009).

Influence of the microbial associations on the species diversity of soil microfungi

Soil contamination by diesel fuel reduced fungal species diversity in the soils (from 16 species initial to 11 species at the end of experiment) and changed the structure of the soil culturable microfungal community (Table 2). The fungi of genus *Penicillium* dominated in all soil samples. The increase amount of the fungi *Penicillium* with treatment DF+F and DF+BF from 50% initial to 75% after 3 months of experiment were noted.

The microbial preparations lead to changes in the structure of microscopic fungi complexes both at the beginning of the experiment and after 3 months. Initial the *P. simplicissimum* was the absolute dominant in the fungi community with treatment DF+F and DF+B+F. After 3 months *P. canescens* dominated in the soil with treatment DF+F and DF+B+F. According to previous our research this species is an active decomposer of petroleum products.

Toxicity of microfungi for plants

The biological preparations can influence both microorganisms and higher plants. Because of rearrangement in the species structure of the fungal communities, the toxin-forming microfungi can be dominant in petroleum contaminated soils. This can be considered as the supplementing factor conditioning the high toxicity of petroleum-contaminated soil toward the plants. Therefore, it is necessary to choose groups of microorganisms that have a low hazard effect on the environment.

In our experiment the culture liquid of microfungi (*Penicillium canescens, P. commune, P. simplicissimum*) suppressed the germination of test plant seeds to one degree or another. The maximum degree of phytotoxicity was observed with P. *simplicissimum* St. 1, which inhibits the germination of *Lepidium sativum* by 92% and *Triticum aestivum* by 30%. At the same time, Czapek's sterile medium also reduced the germination of *Lepidium sativum* seeds by 17% and Triticum aestivum seeds by 5% respectively. The lowest degree of phytotoxicity was shown by the species *P. commune,* which inhibited seed germination of plants by 29% for *Lepidium sativum* and 24% for *Triticum aestivum.* According Bilaj and Koval (1980), these fungi species are not toxic for plants and can be used in the biological preparations.

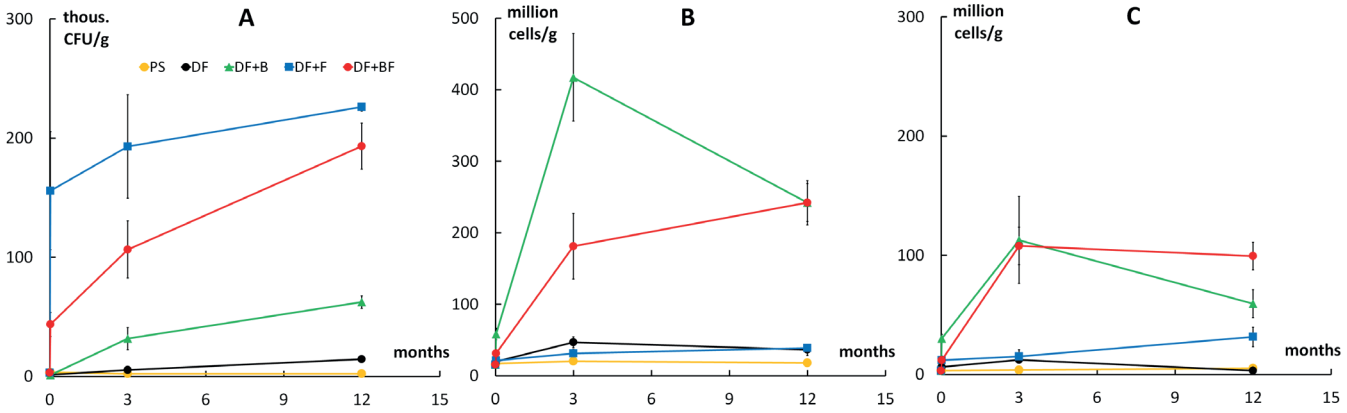

Fig. 2. The dynamics of the number of soils microfungi (A), heterotrophic bacteria (B) and HOB (C) in the soil. The legend is similar to Fig. 1.

Table 2. Dominating species of fungi in initial and after 3 months of experiment

Treatment	Initial	After 3 months
PS	*Sterilia mycelia* (24%), *P. ochrochloron* (24%), *P. janczewskii* (20%)	*P. simplicissimum* 25.3%
DF	*P. ochrochloron* (30%)	*Cephalosporium asperum* 27.5%
DF+B	*Pseudogymnoascus pannorum* (26%) *P. ochrochloron* (26%)	*P. simplicissimum* (30.2%)
DF+F	*P. simplicissimum* (98%)	*P. canescens* (46%)
DF+BF	*P. simplicissimum* (94%)	*P. canescens* (48%)

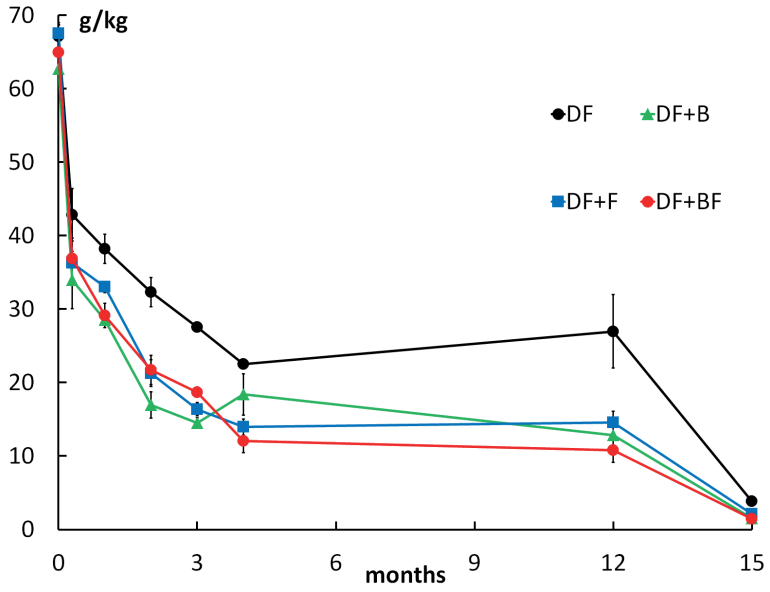

Fig. 3. The dynamics of TPH content in the contaminated soil. The legend is similar to Fig. 1.

Table 3. The rate of petroleum product destruction in soil (g/day)

Variant	Time period, days				
	1-10	10-30	30-60	60-90	90-120
DF	2.43±0.22	0.23±0.02	0.20±0.02	0.16±0.01	0.17±0.02
DF+B	2.87±0.20	0.27±0.02	0.39±0.03	0.08±0.01	0.01±0.01
DF+F	3.12±0.25	0.16±0.01	0.39±0.02	0.16±0.02	0.08±0.01
DF+BF	2.81±0.19	0.39±0.02	0.25±0.02	0.10±0.01	0.20±0.02

Influence of microbial associations on degradation rate of TPH in soil

The results presented on Fig. 3 indicates that all the microbial associations significantly accelerated degradation rate of diesel fuel in the soil compared to uninoculated control.

After 1 month, the petroleum products content with treatment B+F decreased by 57% from the initial and amounted to 29 g/kg. After 120 days the decrease was 82%, that 15% more than with no microbial associations. After 12 months the decrease was 23% more than DF variant. With treatment DF+B and DF+F the content of petroleum products decreased slightly less.

During the first month of the experiment, the petroleum product content reduced quickly due to evaporation (Myazin and Evdokimova 2012). The microbial preparations accelerated oil product decomposition by 10–20%. The rate of petroleum product destruction in soil changed throughout the experiment (Table 3). During the first 10 days, the rate was maximal because of intensive evaporation. Thereafter, the rate of oil product decomposition decreased. The most destruction of petroleum product with treatment DF+B+F was observed between 10 and 30 days of the experiment and for separately treatment DF+B and DF+F was revealed between 30 and 60 days, however the bacterial-fungi community (DF+B+F) maintained its efficiency even after 120 days.

CONCLUSIONS

The microbial associations based on indigenous microorganisms accelerated the decomposition of oil products in the Hortic Arthrosol soil on the Kola Peninsula and can be advised for clearing the environment from oil hydrocarbons. In the field experiment, the bacterial–fungi associations demonstrated a significant effect: the content of oil products reduced by 82% after 120 days that 15% more than with treatment DF (with no microbial associations). After 1 year the decrease was 23% more with treatment DF. The decomposition rate of oil products reached the maximum after 30 and 120 days. Because the soil of the Kola Peninsula is acidic, the efficiency of microbial associations based on fungi will likely be higher than those of bacterial associations. However, the difference between the variants in this field experience is not significant.

At the first period after contamination, the number of microfungi decreased in variants DF and DF+B relative to the control and after 90 days increased in all variants of experiment. Soil contamination by oil products resulted in a reduce in fungal species diversity and change of structure of the soil culturable microfungal community.

The species *Penicillium commune*, *P. simplicissimum*, *P. canescens,* being an active decomposers of oil products, showed almost no phytotoxic effect. They suppressed *Triticum aestivum* seed growth by 24% and *Lepidium sativum* seed growth by 29%. These species may be prospective for the creation of biological preparations and can be advised for the bioremediation of soil contaminated by oil products. However, the use of microbial preparations and phytoremediation have to separated.

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