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EXPERIMENTAL ARTICLES

Effect of Activated Charcoal on Bioremediation of Diesel Fuel-Contaminated Soil

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Abstract—Elevated plant and microbial toxicity throughout the season was found in field experiments on bioremediation of diesel fuel (DF)-contaminated soil (4.5%). This was an indication of the presence of mobile toxic DF components and their metabolites. Introduction of granulated activated charcoal (GAC) was shown to decrease the bio- and phytotoxicity of petroleum-contaminated soil, resulting in a sharp increase in the abundance of petroleum degraders (both aboriginal and inoculated ones), as well as to create conditions for improved growth at the stage of post-treatment by phytoremediation. In spite of short-time deceleration of DF degradation, more complete decrease of hydrocarbon concentrations occurred in the presence of GAC, while simultaneous introduction of the sorbent and a biopreparation (and association of mesophilic petroleum-degrading strains) provided the best results. In these variants the concentration of petroleum hydrocarbons decreased to 0.19–0.21 and 0.13–0.14%, respectively, which was 1.5 and 2 times lower than the values for unsupplemented control. Thus, GAC introduction during bioremediation of DF-contaminated soils increases the efficiency of remediation and localizes the pollutants in the treated layer, which decreases the risk of their penetration into groundwater during in situ soil treatment.

Keywords: diesel fuel, in situ soil bioremediation, biopreparation, petroleum degraders, activated charcoal, biotoxicity, phytotoxicity

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Oil and petrochemical pollution of soil cover is a global ecological problem. Diesel fuel is one of the most widespread and toxic petrochemicals [1]. Its constituent hydrocarbons are able to migrate from soil into surface and ground waters [2, 3] and demonstrate phyto- and biotoxicity [4]. The leaks and spills of petrochemicals on the territories of gas stations (GS) and haulage contractors are a serious source of soil contamination with DF. Now there are more than 900 GSs only in the city of Moscow and in the Moscow region, with the soil contaminated within a radius of 25–50 m [5]. Thousands of tons of hydrocarbons get into soil and ground waters as a result of liquid fuel leaks from the subsurface storages of fuels and lubricants. Pipeline breaks may result in hundred- and thousand-ton spills of petroleum and petrochemicals. They are often accompanied by penetration of the pollutants into deep soil layers and groundwater. Groundwater contamination was revealed in approximately 3000 out of 10000 sites in Finland contaminated with petroleum hydrocarbons [3].

DF is a complex mixture of petroleum hydrocarbons (TPH), within a range of C8–C40, with 75–80% alkanes and 20–25% aromatic hydrocarbons. In addition to TPH, DF comprises 1–5% N-, S-, and O-containing compounds and up to 10% additives: surface-active substances, antiknock agents, and antifreezes [6]. Soil microorganisms can utilize the most of DF hydrocarbon components as the only carbon and energy source with different efficiency [1]; however, high concentrations of these compounds may be toxic for microbial cells, mainly due to their negative effect on cell membrane structure. Light TPH fractions (low-boiling *n*-alkanes, monoaromatic compounds, naphthalene derivatives) are more toxic than heavy fractions because of high water solubility; however, heavy fractions may have negative influence on the physical properties and structure of soil [7]. Aboriginal petroleum-degrading microorganisms have been found in almost all soils, and the maximum rate of TPH degradation is observed under aerobic conditions [1].

The most efficient method of soil cleanup from petrochemicals, including DF, is bioremediation by the method of activation of the natural aerobic petro-

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leum-oxidizing microflora; in some cases, complementary bioaugmentation is performed by introducing biopreparations on the basis of specially isolated microorganisms. The optimal conditions of vital activity for petroleum degraders are created by aeration (the soil is blown with the air, periodically mixed up, tilled or loosened depending on the technology), moistening as and when necessary, and addition of nutrients. The optimal intervals are 60–70% FWC for soil humidity and 20–35°C for temperature [1]; however, TPH biodegradation under the conditions of boreal climate is possible also at low positive temperatures [8]. Nitrogen as the most important nutrient, as well as less important phosphorus and potassium, are introduced as mineral and sometimes organic fertilizers. The recommended C : N ratio varies from 10 : 1 to 100 : 1 [9]; however, at a high level of hydrocarbon contamination it is recommended to add fertilizers intermittently to avoid strong pH deviations from the value of soil acidity optimal for microorganisms: pH 6.5–7.5 [10].

Landfarming is considered the simplest and cheapest method of soil cleanup. This bioremediation technology is based on periodic moistening and intensive tilling of contaminated soil. Depending on the depth of pollutant penetration and the degree of contamination, soil is either treated in situ, i.e., directly in the upper layer (<1–1.5 m), or, in case of deeper contamination (>1.5 m), excavated and treated on site at a ground with waterproof layer and barriers. Soil bioremediation is recommended for TPH contamination of no more than 5 mass % [10]. At the stage of final purification or in case of moderately polluted soils (0.5–2% TPH), it is convenient to clean up the soil by the method of phytoremediation based on the ability of plants to accelerate hydrocarbon degradation [11, 12].

Soil cleanup by in situ landfarming that requires neither soil excavation nor construction of special treatment facilities seems to be most attractive. However, its applications are limited by the hazard of carrying the pollutants out of the cleaned-up site. We have developed the method of sorption bioremediation to expand the possibilities of soil purification in situ. It is based on the application of granulated activated charcoal, which localizes pollutants within the limits of the treated site and simultaneously reduces the toxicity of contaminated soils for microorganisms and plants. In addition to the sorption of pollutants, GAC enhances the water retention capacity and degree of aeration of soils and serves as a reservoir for microorganisms. This method has been successfully used in the sorption bioremediation of soil for elimination of the accidental spill of 17 tons of propanide [13]; its efficiency has been also proved during bioremediation of soils contaminated with 2,4,6-trinitrotoluene and polychlorynated biphenyls [14, 15].

Activated charcoal is a highly porous sorbent obtained by thermal reprocessing of various natural carbonaceous materials (wood, coal, peat, lignin, organic wastes, etc.) followed by activation with vapor or chemical reagents. Owing to the large specific surface (up to 500–2000 m^2/g) and microporous structure (the average and minimal pore diameter is 1–2 nm and 0.35 nm, respectively), AC has higher absorption capacity compared to many organic and inorganic substances [16]. In the late 90s, AC was recommended for oil gathering in case of emergencies in water areas and elimination of minor spills of petrochemicals on a solid surface by simultaneous localization and gathering of spilled hydrocarbons from the surface [17], as well as for catching volatile BTEX hydrocarbons when composting petroleum-contaminated soils [18]. The recent article [19] has demonstrated the potential application of GAC as a carrier for biopreparation developed for bioremediation of petroleum-contaminated soils.

In the present work, we have posed for the first time the problem of studying the influence of GAC on the rate of DF biodegradation in soil, the dynamics of variation of the quantity of soil microorganisms, and soil phytotoxicity in order to expand the possibilities of bioremediation.

MATERIALS AND METHODS

Gray forest soil for the studies was taken from the upper 20-cm layer under broad-leaved forest in the region of Pushchino (Moscow oblast). This loam soil has the following characteristics: C_{org}, 1.9%; pH 6.9; total nitrogen content, 4.4 mg N/kg; accessible phosphorus, 46 mg P_2O_5/kg; exchangeable potassium, 94 mg K_2O/kg. Commercial winter diesel fuel DZr-25 (0.84 g/cm^3) was used as a pollutant.

Granular activated charcoal (GAC VSK, Nizhny Novgorod) added to the soil had the following physicochemical characteristics: grain size, 1–1.5 mm (91%); apparent density, 450 g/dm^3; rubbing fastness, 80%; total pore volume by water, 0.8 cm^3/g; effective micropore volume, 0.45 cm^3/g; adsorption capacity, 1000 mg/g by iodine and 300 mg/g by methylene blue.

The association of four microorganisms from the collection of the Laboratory of Plasmid Biology (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) was used as a biopreparation. These microorganisms (*Rhodococcus* sp. X5, *Rhodococcus* sp. S67, *Pseudomonas putida* BS3701, and *Pseudomonas fluorescens* 142NF) are highly efficient petroleum degraders and can utilize aliphatic and aromatic hydrocarbons as growth substrates. Microbial biomass was obtained in different cultures on a complete medium as described in [20]. The concentrated suspension of the biopreparation was obtained as follows: the biomass of each microorganism was mixed in equal proportion and diluted with saline to a concentration of about 10^9 CFU/mL.

The microfield experiment was performed on the vegetation plot of the Institute of Physicochemical and Biological Problems of Soil Science (Russian Acad-

Table 1. The scheme of microfield experiment

Variant no.	Code	Initial DF content, mg/kg	GAC dosage, mass %	BP	Mixing and moistening	Mineral fertilizers
1	UC		–	–	–	–
2	C		–	–	+	+
3	BP		–	+	+	+
4	2AC	45000	2.5	–	+	+
5	2AC BP		2.5	+	+	+
6	5AC		5.0	–	+	+
7	5AC BP		5.0	+	+	+
8	CC		0	–	+	+
9	CC 1AC		1	–	+	+
10	CC 2AC	0	2.5	–	+	+
11	CC 5AC		5.0	–	+	+
12	CC 10AC		10	–	+	+

The following abbreviations were used in the ciphers: CC, clean control; UC, untreated control; C, control without the additives; the addition of GAC and biopreparation (separately and together) was designated as AC, BP, and AC BP, respectively; the numbers 1, 2, 5, and 10 before AC correspond to sorbent dosage.

emy of Sciences, Pushchino) under the conditions of natural humidity and insolation from June to November. The soil cleaned from roots and sieved through a mesh with 5-mm holes was poured into the prepared vegetation PVC vessels (without the bottom), $30 \times 40 \times 40$ cm, dug in soil so that the top edge of the vessel would be 5 cm above the soil level. Polyethylene netting was put onto the initial soil in the vessels at a depth of 15 cm below the top edge to separate the experimental sample. Experimental soil (12 kg dry weight) was poured from above; after it was compressed to natural state, 650 mL of DF was evenly applied to soil surface, until its concentration in the upper 8- to 10-cm soil layer was about 45 g/kg (4.5 mass %).

The treatment of experimental vessels was started in 3 days (air temperature 10–23°C; without precipitation), with the exception of untreated control (UC). The experimental soil layer was mixed; GAC and BP were added, together or separately, to different variants according to Table 1. GAC was added first at a dose of 2.5 or 5 mass %, and the biopreparation was added in 24 h as a suspension of newly grown petroleum-degrading strains (300 mL/vessel) so that the initial cell density in the soil would be 3×10^7 cells/g. In addition to the variants with contaminated soil, some vessels contained clean soil (clean control, CC) with GAC (1, 2.5, 5, and 10%) or without it. The experiment was carried out in triplicate.

The optimal water–air regime was maintained in all vessels by periodic moistening with settled tap water and weekly soil mixing to create the optimal conditions for vital activity of petroleum degraders. The maximum precautionary measures were taken: first inoculated and then noninoculated samples were mixed. On day 7, urea and granulated superphosphate (26% P and 6% N, 5 g/vessel) and potassium chloride (4 g/vessel) were added into all vessels except for UC so that the final macroelement ratio would be C : N : P : K = 100 : 1 : 0.6 : 1.

In order to model the process of secondary treatment of soil by phytoremediation and to assess soil phytotoxicity, all vessels were seeded in 1.5 months with alfalfa (*Medicago sativa*): 40 seeds per vessel. In 2 weeks, the sprouts were thinned and 15 plants were left in each vessel. In the next 2.5 months (in the late September), the plants were taken out of the vessels, the soil was thoroughly shaken off the roots and put back into the vessel, the roots were washed with running water and the plants were dried at 105°C and weighed up.

Soil samples were regularly taken for analysis during the experiment for TPH measurement and for express determination of phytotoxicity, the number of microorganisms, and pH value of the soil.

TPH in soil was determined according to methodical recommendations [21]. Hydrocarbons were extracted from soil samples in the state of natural humidity after mixing a 1-g soil sample with an equal amount of dehydrated Na_2SO_4. The sample was extracted with carbon tetrachloride (for chromatography) by shaking. The resultant extract was released from polar compounds in a column with Al_2O_3 (3% H_2O). The total content of nonpolar hydrocarbons in purified extracts was measured with a KH-2 concentration meter (Sibecopribor Production and Ecological Enterprise, Novosibirsk). The device was calibrated by the solution of petrochemicals in carbon tetrachloride (State Reference Standard no. 7554-99). The measurement was performed in duplicate for each

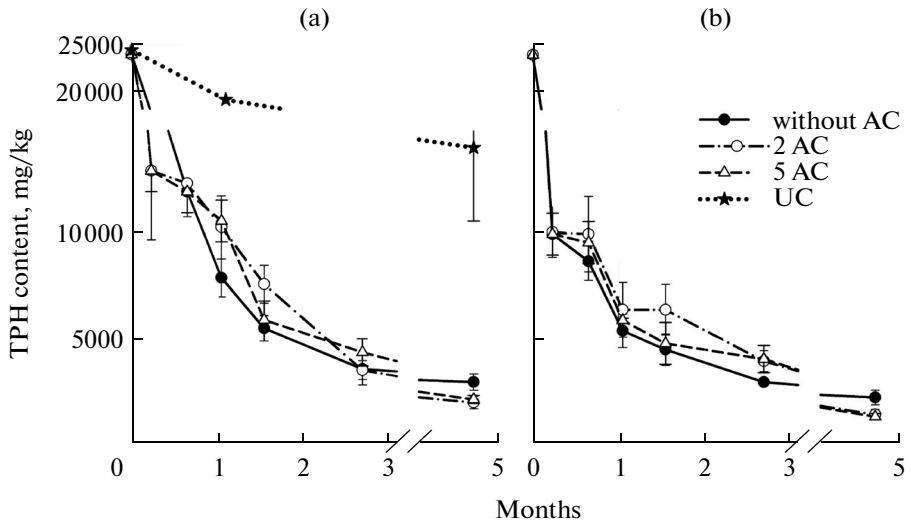

Fig. 1. The dynamics of TPH content in DF-contaminated soil during its bioremediation by the method of activation (a) and bioaugmentation (b) at different GAC doses (0, 2.5, and 5%: without AC, 2AC, and 5AC, respectively) compared to untreated control (UC).

sample and all results were recalculated per absolutely dry soil.

The number of the colony-forming units (CFU) of heterotrophic bacteria and petroleum degraders (PD) was determined by inoculating soil suspension onto solid media. Soil suspension was inoculated (1) on a standard meat–peptone agar (MPA) for counting heterotrophic microorganisms and (2) on a solid mineral medium of the following composition for counting petroleum degraders: KNO_3, 4.0 g; K_2HPO_4, 0.6 g; $NaH_2PO_4 \cdot 12H_2O$, 1.4 g; $MgSO_4$, 0.8 g; and agar, 20 g (per 1 L of distilled water), with DF as the sole carbon and energy source. DF (0.2 mL) was poured onto the bottom of the petri dish before pouring the prepared mineral medium. The measurements were performed in triplicate for each sample.

Soil phytotoxicity was determined by our express method. Integral soil toxicity was assessed by the germination ability of white clover (*Trifolium repens*), which is highly sensitive to hydrocarbon contamination. The soil was put into petri dishes by 40 g and moistened to 75% FWC; then clover seeds were sown onto the surface (35 seeds per dish) and covered. Germinating seeds were counted (G) after 7-day incubation at room temperature. Simultaneously, the germination ability of seeds (G_s) was determined in petri dishes with moistened filter paper incubated under the same conditions. Soil phytotoxicity was assessed by the death of seeds (%) by the equation $P = 100 \times (1 - G/G_s)$. The assessment was performed in triplicate for each sample.

The results were statistically processed in Excel.

RESULTS AND DISCUSSION

Figure 1 shows the dynamics of reduction of TPH content in soil. In the first 3 days, there was a rapid decrease in TPH concentration from the predicted level (45 g/kg) to 24.5 g/kg (2.45 mass %), which we will hereinafter consider as the initial TPH concentration in soil before starting the treatment. TPH content in soil decreased mainly due to the evaporation of its light fraction from soil surface: 46% of the calculated TPH value. The modeling of accidental DF spill (1 L/m^2) under the natural conditions of Spain also showed the evaporation of about 50% TPH from soil surface, which is accounted for by the high percentage of volatile petroleum hydrocarbons [22]. Approximately the same amount of hydrocarbons evaporated from the dry soil mixed with DF (from 5 to 50 g/kg) after 2-week storage at room temperature [23].

Henceforth, DF content in the untreated control decreased comparatively slowly, and TPH concentration in the soil at the end of the season was still 14.1 g/kg (58% of the initial value). The activation of aboriginal microorganisms by loosening, moistening, and introduction of mineral fertilizers (Fig. 1a) substantially accelerated DF degradation, providing a decrease in TPH concentration to 3.5 g/kg in 3 months and to 2.9 g/kg at the end of the season (by 86 and 88%, respectively). Additional introduction of the biopreparation as petroleum degraders slightly accelerated DF degradation (Fig. 1b) but only in the first 1–2 months; within 3–5 months the difference between variants (a) and (b) became unreliable. The addition of GAC somewhat slowed DF degradation in the first 1–2 months; however, TPH content in the variants with 2.5 and 5% GAC was reliably lower at the end of the season (in 5 months) compared to the con-

Table 2. Residual content of TPH in DF-contaminated soil in 5 months after the treatment. The average values are presented; standard deviations are given in brackets

Variant	g/kg		% of the initial value		without BP / with BP
	without BP	with BP	without BP	with BP	
UC	14.1 (3.1)	–	57.5 (12.8)	–	
C	2.93 (0.39)	2.21 (0.35)	12.0 (1.6)	9.0 (12.4)	1.3
2AC	1.95 (0.30)	1.40 (0.25)	8.0 (1.2)	5.7 (1.0)	1.4
5AC	2.05 (0.20)	1.30 (0.15)	8.6 (0.8)	5.3 (0.5)	1.6

trol without GAC: 2.0 and 2.1 g/kg in inoculated and 1.4 and 1.3 g/kg in uninoculated samples, respectively (Table 2).

Analogous data on the rate of bioremediation of DF-contaminated soil were obtained in the microfield experiment performed in Taiwan. After 3.3-month landfarming with the introduction of mineral fertilizers, TPH concentration decreased from 25 to 3.6 g/kg and even to 3.2 g/kg in the variant with complementary bioaugmentation (i.e., by 86 and 87%, respectively) [24]. In the laboratory experiment with moderately DF-contaminated soil (6 g/kg), with the activation of aboriginal microorganisms by the introduction of mineral fertilizers, daily mixing and maintaining the optimal humidity, TPH was degraded faster: up to 90% in 1.5 months [25].

In the book of M. Alexander [1] concerned with soil bioremediation, it was shown that the effects of biopreparations based on petroleum degraders were temporal or absent at all in many studies. However, some works demonstrated quite an appreciable positive influence of inoculated petroleum degraders on the rate of DF degradation. The review [26] considers various causes of ambiguous effects of biopreparations. For example, introduction of a bacterial consortium consisting of *Pseudomonas putida* (K3) and *Acinetobacter lwoffi* (K29) into DF-contaminated soil accelerated decomposition of only easily degradable TPH at the 1st stage; then hydrocarbon degradation slowed down, and the effect of the introduced biopreparation became barely noticeable. However, inoculation of the strain *Rhodococcus erythropolis* (K45) that was able to degrade stable DF components (alicyclic alkanes and PAH) considerably accelerated TPH decomposition at the 2nd stage [27]. Substantial acceleration of TPH degradation was observed in the soil weakly contaminated with DF with multiple introduction of the biopreparation every 3 days [28].

Thus, the low efficiency of biopreparations may be accounted for by several facts: (1) the presence of aboriginal adapted petroleum degraders in the initial soil, especially with old contamination; (2) the high heterogenicity of DF components needing different microorganisms for their degradation; and (3) the absence of conditions for the survival of inoculated strains. In our case, with the first two conditions optimized, the main factor that weakens the effectiveness of biopreparation seems to be the toxicity of contaminated soils.

The results of soil biotesting obtained during bioremediation support these assumptions. Figure 2 shows the dynamics of the number of heterotrophic bacteria and petroleum degraders in the clean and contaminated soil (treated and not treated with the biopreparation) against different doses of GAC (0, 2.5, and 5%). The initial content of heterotrophic bacteria and petroleum degraders in the clean GF soil is 6×10^6 and 2×10^6 CFU/g, respectively. The latter value is higher by 3 orders of magnitude than the minimal level of petroleum degraders (10^3 CFU/g) when bioremediation of petroleum-contaminated soils by the method of activation is considered possible according to the US EPA Manual [29].

During our experiments, the quantity of the first and second groups of microorganisms in clean soil varied in the range of $(6–11) \times 10^6$ and $(2–6) \times 10^6$ CFU/g, respectively; in the presence of 2.5 and 5% GAC, their quantity changed insignificantly (Figs. 2a and 2c). In contaminated soil that was cleaned up without the additives (variant K), the quantity of heterotrophs was little different from the clean control (CC) throughout the period of observation, while the quantity of petroleum degraders exceeded the level of CC due to the additional carbon and energy source (DF) only slightly: no more than twofold. The level of petroleum degraders in soils without the sorbent in the first 2 months was higher in the inoculated variants (Figs. 2b and 2d) compared to uninoculated soils. However, it was on more than 12×10^6 CFU/g, i.e., nearly 3 times lower than the calculated quantity of introduced cells (30×10^6 CFU/g).

Contrariwise, the number of petroleum degraders in contaminated soil showed a positively response to GAC introduction. In the soil cleaned up by the method of activation, the quantity of aboriginal microorganisms (both petroleum degraders and all heterotrophs) drastically increased after a short lag period (Figs. 2a and 2c). It especially increased in the presence of the maximum dose of GAC: approximately tenfold (up to 45×10^6 CFU/g); the maximum quantity of microorganisms coincided in time with the period of DF semi-degradation (1 month). In

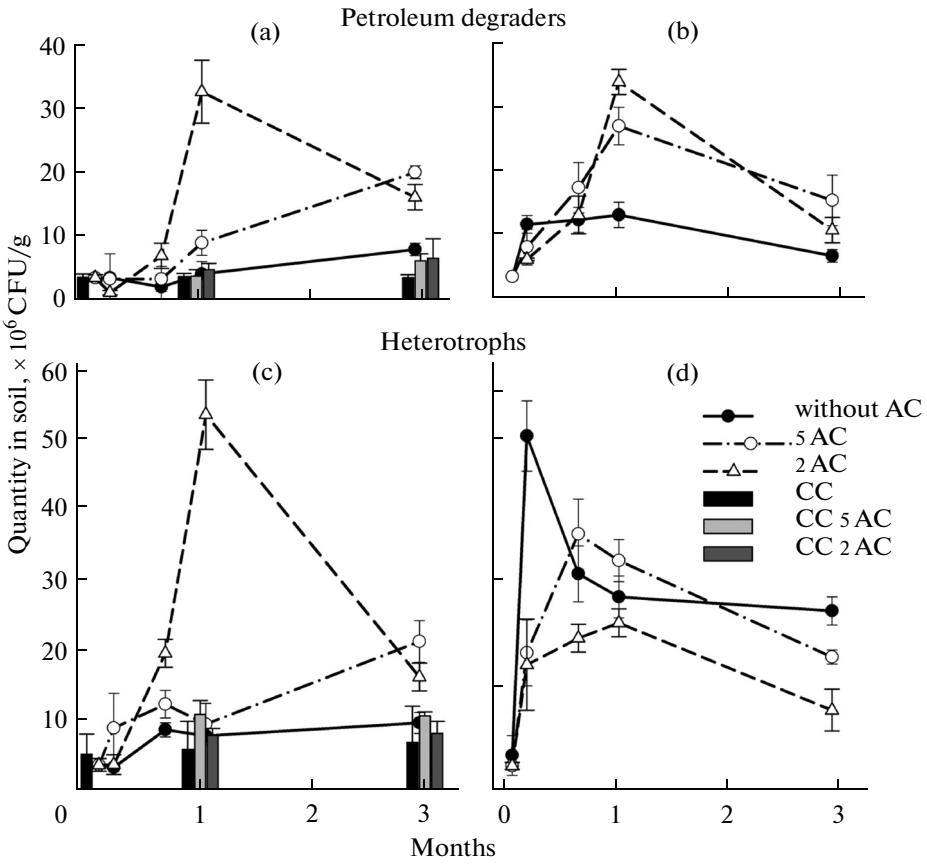

Fig. 2. The dynamics of quantity of petroleum degraders and heterotrophic microorganisms in DF-contaminated soil during its bioremediation by the method of activation (a) and bioaugmentation (b) at different GAC doses (0, 2.5, and 5%) compared to clean soil and the same doses of GAC: CC, CC 2AC, and CC 5AC.

3 months, the quantity of soil microflora in all soils gradually returned to the initial level as DF was degraded, but in the presence of GAC remained two- to threefold higher than in its absence.

It is important to note that the quantity of aboriginal petroleum degraders in the uninoculated soil with 5% GAC reached the same maximum as in the biopreparation-containing samples with the same dosage of the sorbent and was threefold higher in the soil containing the biopreparation only. This fact indicates that aboriginal petroleum degraders can actively propagate in the soil heavily contaminated with petroleum hydrocarbons in the presence of sorbent. In our case, such a drastic increase in the number of aboriginal and inoculated petroleum degraders is obviously associated with the reduced toxic effect of DF components and their metabolites as a result of reversible sorption by activated charcoal.

The conclusions about the toxicity of DF-contaminated soil are in good agreement with the data of the work [29] showing that the resistance of soil microorganisms in petroleum-contaminated soils beings to decrease over the range of TPH concentrations of 4–8 g/kg, i.e., the total quantity of microorganisms and the number of their species are reduced. Simultaneously, the bioremediation of petroleum-contaminated soils is accompanied by the propagation of the most resistant species of petroleum degraders due to utilization of hydrocarbon substrates; as a result, their quantity may increase.

GAC has a strong influence also on soil phytotoxicity as follows from express assessment by white clover germination (Fig. 3). Initially, the high value of soil phytotoxicity (85%) decreased to 20% after 1.5-month treatment by the method of stimulation without sorbent introduction. However, clover germination in this soil remained reliably higher (by 10–15%) than in the clean control (Fig. 3a). At the same time, the rapid decrease in phytotoxicity was observed in the presence of GAC (especially the maximum dose), and as early as in 10–20 days this value dropped to the minimum and henceforth did not exceed the standard deviation of this method (±5%).

In the soil with the biopreparation (Fig. 3b), the positive effect of GAC on phytotoxicity reduction was also observed throughout the season. However, in the first months, this index was almost reliably higher in all inoculated samples compared to uninoculated sam-

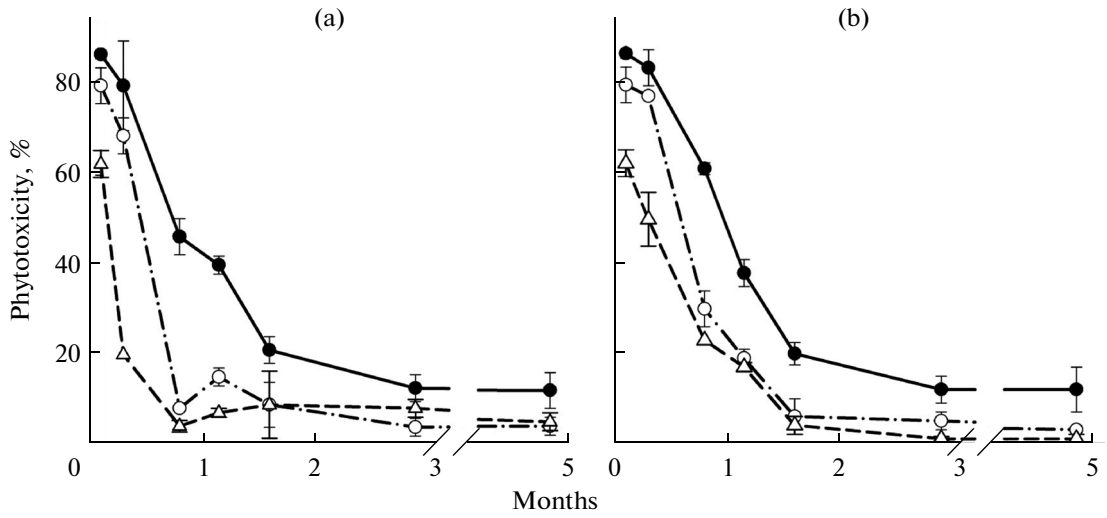

Fig. 3. The dynamics of phytotoxicity of DF-contaminated soil during its bioremediation by the method of activation (a) and bioaugmentation (b) at different GAC doses: 0, 2.5, and 5% (the legend is the same as in Fig. 1).

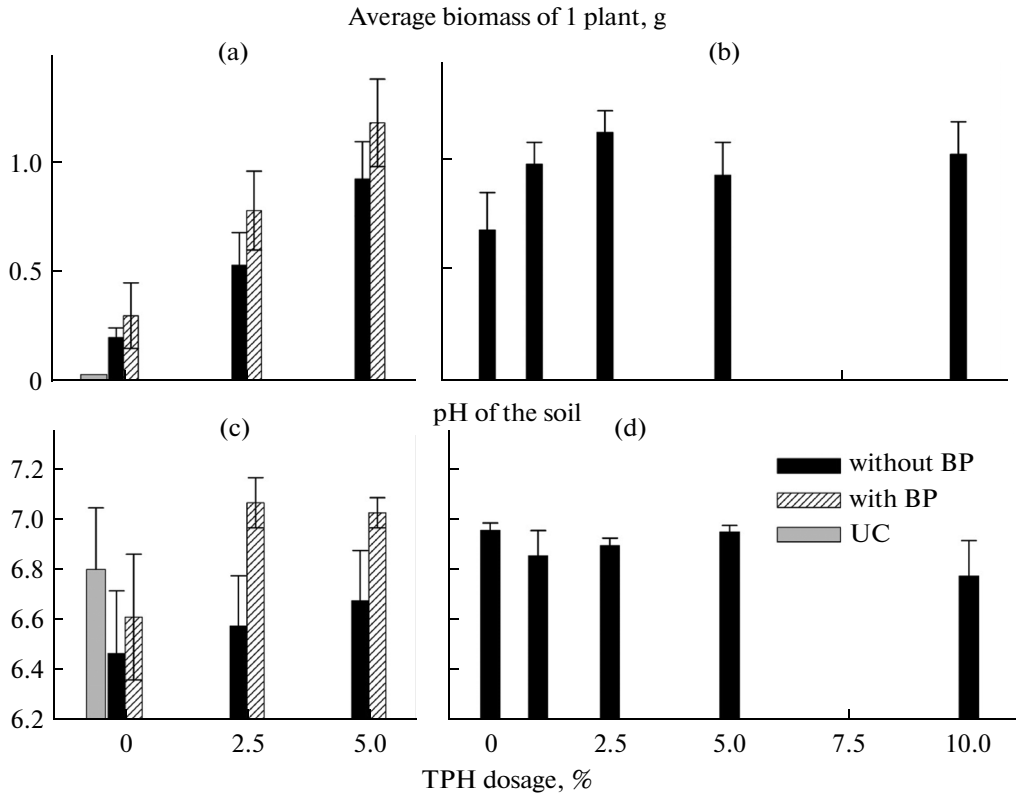

Fig. 4. The effect of GAC dosage on dry alfalfa biomass (per 1 plant) grown in clean (b) and DF-contaminated soil (a) cleaned up without and with BP, compared to untreated control (UC), as well as on pH values of the same samples of contaminated (c) and clean (d) soils determined before seeding the plants.

ples, though at the end of the season this effect disappeared and phytotoxicity of the variants approached that in uninoculated soils. This temporary effect is probably associated with low phytotoxicity of the biopreparation, as it has been demonstrated on petri dishes with the suspension of freshly grown strains in which the clover seeds were soaked. The cause of this phenomenon has not yet been elucidated.

The measurement of alfalfa plant biomass harvested at the end of the season (Figs. 4a, 4b) showed

that the introduction of GAC into clean soil slightly stimulated plant growth (by 20–25%). The death of nearly all plants was observed in contaminated soil (untreated control). In the soil samples cleaned up without sorbent (with and without the biopreparation), the growth of alfalfa was also substantially inhibited and its biomass at the end of the season was 3–4 times lower than in CC. At the same time, in the presence of a half dose and especially the maximum dose of GAC, alfalfa growth in the cleaned-up soil noticeably intensified, while the average dry biomass per 1 plant was as follows: 64 and 109% in the soils without BP, 85 and 120% of CC in the soil with the biopreparation, respectively.

The comparison of plant biomass with pH value of the soil measured before seeding the plants demonstrates a close relationship between these indices (Figs. 4c, 4d). The drastic decrease in pH of the soil in the variant K (from 6.8 to 6.4) may be accounted for by accumulation of acidic metabolites (organic acids) and/or acidification of the medium in the presence of enhanced doses of nitrogen and phosphorus fertilizers [30]. The lesser pH fluctuation in the presence of GAC seems to be determined by the sorption of acidifying DF metabolites and by the buffer effect of charcoal on excessive protons accumulated during urea biotransformation and potassium dihydrophosphate hydrolysis. Introduction of the biopreparation had an additional positive effect on pH of the soil and alfalfa biomass, due to either the accelerated metabolism of acidic DT metabolites or the neutralizing effect of buffer salts in combination with the culture medium in which petroleum degraders were introduced.

The work [31] has shown that enhanced phytotoxicity of DF-contaminated soils is a result of the toxic effect of the most water-soluble components, in particular, monoaromatic compounds of the BTEX group (benzene, toluene, ethylbenzene, and xylenes), as well as binuclear PAHs (naphthalene and its methyl derivatives). In addition to these compounds, various oxidized TPH derivatives were found in lysimetric waters of the soil freshly contaminated with DF: alcohols, ketones, lactones, phenols, dions, and dion esters, which apparently were originally contained in DF. In 2 months of incubation, these compounds were replaced by other ketones, as well as alcohol esters, carboxylic acids, and heteroatomic compounds with complex structure. The longer incubation results in the accumulation of usually polyoxidized hydrocarbons in petroleum-contaminated soils, in particular, oxy-carboxylic acids that are rather well soluble in water. All of these compounds may have toxic effects on microorganisms and plants and, consequently, inhibit the process of bioremediation.

Hence it follows that the enhanced soil phytotoxicity observed in our experiments in the variants without the sorbent indicates the presence of mobile toxic compounds that may migrate to the underlying soil layers until penetrating the groundwater. At the same time, soil phytotoxicity in the presence of GAC is minimal and, consequently, the probability of groundwater contamination is minimized.

Thus, soil self-purification after contamination with 4.5% DF proceeds slowly because of enhanced phyto- and biotoxicity of DF components and their metabolites. GF soil under study was shown to have a comparatively high initial density of aboriginal petroleum degraders (about 2×10^6 CFU/g). Therefore, the activation of soil microflora by means of agricultural techniques noticeably accelerated hydrocarbon degradation. Soil bioaugmentation by the biopreparation on the basis of isolated mesotrophic strains resulted in a slight temporary acceleration of pollutant degradation. However, the activity of both aboriginal and inoculated petroleum degraders in DF-contaminated soil is strongly inhibited, impeding the process of biodegradation, and the final TPH concentrations in soil are 0.29 and 0.22%, respectively. It has been shown for the first time that the number of petroleum degraders can increase many times and the conditions of phytoremediation plant growth can be improved by means of GAC introduction. It is a result of reduction of toxic effect due to the reversible sorption of toxic DF components and their metabolites. As a result, TPH concentration decreases to 0.19–0.21% in the presence of GAC only and to 0.13–0.14% in case of GAC introduction in complex with the biopreparation, which is close to the approximately permissible TPH level in soil. Though MPC values for petroleum hydrocarbons in soil have not yet been established in Russia, the quality of soil cleanup can be assessed by the standards accepted in some western countries, where the permissible level of TPH (or only light TPH fractions) is no more than 0.1% [3, 32]. We may consider that in our studies the soil has not been cleaned up completely and requires further purification for one more season.

The dynamics of reduction of TPH concentration in soil showed a certain temporary slowdown of TPH degradation in the first two months instead of expected acceleration of the process, in spite of the increasing quantity of petroleum degraders in the presence of GAC. However, the final TPH concentrations in sorbent-enriched soils were reliably higher at the end of the season compared to the soil without the sorbent. It is probably associated with temporary reduction in accessibility of pollutants adsorbed by charcoal, followed by their deeper utilization by microbial degraders colonizing the surface of sorbent granules.

The analogous mechanism of GAC action was demonstrated in our studies with 3,4-dichloroaniline, where is was shown that pollutant degradation in strongly contaminated soil is accelerated due to reduction of its toxicity as a result of reversible sorption [33]. The accessibility of BTEX compounds adsorbed on GAC for degraders was proved in the experiment with liquid culture [34]. The addition of GAC to the compost with contaminated soil considerably stabilized the purification of filtered gases from BTEX hydrocar-

bons, while the efficiency of contaminant removal increased to 90% and even more [18].

The bioreactor with GAC as a solid carrier was constructed for isolation of petroleum-degrading microorganisms from the soil historically contaminated with hydrocarbons. In such a bioreactor, favorable conditions for the build-up of petroleum degrader biomass were created in the liquid medium flowing out of the bioreactor, where cell density was up to 4×10^{11} cells/mL [35]. Liang et al. [19] developed a biopreparation on the basis of petroleum degraders immobilized on GAC and zeolite. Colonization of the surface of sorbents by spherical and rod-like microorganisms and formation of a microbial biofilm (5–20 microns thick) was demonstrated in an electron microscope. GAC provided the best conditions for the growth of petroleum degraders, and their number reached 10^{10} CFU per 1 g of the sorbent. As a result of inoculation of this biopreparation into petroleum-contaminated soil, the quantity of petroleum degraders increased to 10^8 CFU/g, i.e., was higher by 2 orders of magnitude than the value in the control without the sorbents, while the total microbial and dehydrogenase soil activities were 12-fold higher than the control values. After the introduction of petroleum degraders immobilized on GAC, petroleum biodegradation in the soil reached 49%, compared to 37% with free-living cells, 26% with fertilizers only, and 13% without the additives. Biocarrier increased the quantity of petroleum degraders in soil up to 10^8 CFU/g, which is in good agreement with the results of our studies. The authors attribute the positive effect of GAC on the rate of petroleum biodegradation to enhanced soil water capacity, oxygen-enriched soil microflora, and improved mass transfer of nutrients [19]. Though these factors can also have positive effects on the rate of bioremediation of petroleum-contaminated soils, the key role of introduced GAC, from our point of view, is to reduce the phyto- and biotoxicity of individual petroleum components and particularly their more mobile metabolites due to reversible sorption.

This study has shown for the first time that the addition of only granulated activated charcoal considerably accelerates bioremediation of gray forest soil strongly contaminated with medium-volatile petroleum products such as DF (4.5%). It occurs as a result of reduction of phyto- and biotoxicity of soil due to the reversible sorption of DF components and their metabolites, providing a substantial increase in the quantity of aboriginal petroleum degraders and creating conditions for the better growth of phytoremediation plants (alfalfa) at the stage of post-treatment. As a result, in spite of the short-term slowdown of DF degradation in the presence of GAC, the total content of TPH in soil at the end of the season is up to 0.19–0.20%, while the addition of GAC in complex with the biopreparation provides still more complete reduction of TPH concentration: to 0.13–0.14%. This value is 1.5–2 times lower compared to the control or to the variant with only the biopreparation. Thus, the addition of GAC during bioremediation of the soil heavily contaminated with DF enhances cleanup efficiency and simultaneously localizes the pollutants in the cleaned layer, reducing the risk of their penetration into groundwater during soil treatment in situ.

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