

## Article

# The Influence of Remediation with *Bacillus* and *Paenibacillus* Strains and Biochar on the Biological Activity of Petroleum-Hydrocarbon-Contaminated Haplic Chernozem

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**Abstract:** The effect of bacterial strains on certain genera, both independently and in combination with biochar in various options, on petroleum hydrocarbon decomposition in chernozem and the restoration of the ecological state of the soil were studied. To simulate petroleum hydrocarbon contamination, petroleum hydrocarbons were introduced into soil in the amount of 5% of soil weight. Strains of *Bacillus* and *Paenibacillus* bacteria (in recommended and increased doses  $\times 100$ ) and biochar (1% of soil weight) were introduced into contaminated soil separately and together. It was found that after 30 days, the oil content decreased with the joint introduction of an increased dose of *Bacillus*, *Paenibacillus* and biochar by 64%, as well as with the inoculation of biochar with *Bacillus* and *Paenibacillus* bacteria at the recommended dose by 67%. The introduction of biochar, inoculated with BP and BP  $\times 100$ , contributed to an increase in the intensity of CO<sub>2</sub> emission compared to the background by 5–10%. With the joint introduction of BP + B, stimulation was 70%, with an increase in the concentration of BP  $\times 100$ –115%. The preparation BP and BP  $\times 100$  introduced with biochar stimulated the activity of the enzyme by 49 and 61%; with the preinoculation of BP in biochar, stimulation was 27% relative to the background value. The most informative biological indicators when introducing ameliorants of biochar, *Bacillus* and *Paenibacillus* were the total number of bacteria, the length of the barley roots and the catalase activity, demonstrating the greatest sensitivity. The results of the study should be used for the remediation and biomonitoring of the state of oil-contaminated soils.

**Keywords:** pollution; soil; bioremediation; immobilization; enzymatic activity; barley; seed germination; restoration of the ecological state

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## 1. Introduction

Methods of soil purification in the case of contamination with petroleum hydrocarbons in various emergency situations have been studied everywhere [1–3]. Petroleum hydrocarbon and petroleum hydrocarbon product spills put a critical load on the communities of soil organisms, resulting in the soil losing its ability to perform its ecosystem functions. Petroleum-hydrocarbon-contaminated soils have been excluded from agricultural use for many years due to causing adjacent ecosystems to suffer and, therefore, inflicting significant economic damage on the population of adjacent territories. The ecological state of oil-contaminated soil after remediation is generally assessed by looking at the indicators of soil biological activity, including the number of soil bacteria, enzymatic activity and soil phytotoxicity [4–9]. To decontaminate soils, various remediation technologies have been used to return them to their original state, such as bioaugmentation and biostimulation.

Bioaugmentation and biostimulation have been distinguished as being among the most effective technologies for the bioremediation of oil-contaminated soils [10]. Bioaugmentation is associated with the introduction of microbiological preparations into contaminated soil, including one or more strains of bacteria, fungi, algae and other microorganisms, such as *Achromobacter*, *Alcaligenes*, *Xanthobacter*, *Arthrobacter*, *Pseudomonas*, *Bacillus*, *Mycobacterium*, *Corynebacterium*, *Flavobacterium*, *Nitrosomonas* and others [11–13]. Aerobic or anaerobic microorganisms can be effective in the decomposition of various types of organic pollutants [14–16]. Aerobic bacteria were previously effectively used for the decomposition of polycyclic aromatic hydrocarbons [17,18]. Anaerobic bacteria are increasingly being used to remediate soils contaminated with polychlorinated biphenyls, chlorine-containing compounds and chlorinated solvents, such as trichloroethylene and chloroform [19–22]. The decomposition of petroleum hydrocarbons and products can also occur under aerobic and anaerobic conditions; however, the use of bacteria with an aerobic metabolism is a more common method of biodegrading petroleum hydrocarbons. The most easily biodegradable fraction of petroleum hydrocarbons is n-alkanes, and the least biodegradable are PAHs and asphaltenes [23]. In turn, the mechanisms of the microbial degradation of petroleum hydrocarbons under aerobic conditions include enzymatic activity (using monooxygenases and dioxygenases), the synthesis of biosurfactants (lipopeptides, glycolipids, etc.) and the synthesis of bioemulsifiers (lipopolysaccharides, polysaccharides, etc.) [24]. It is important to note that bacterial strains of the genus *Bacillus* have been researched in a number of studies aimed at searching for strains of petroleum hydrocarbon destructors [25] or bacteria actively producing biosurfactants [26]. Thus, in the paper by Fan et al. (2020), the strain of *B. licheniformis* DM-1 could decompose a wide range of n-alkanes with a chain length of C<sub>12</sub>–C<sub>36</sub>, contributing to reducing petroleum hydrocarbon viscosity due to the production of exopolysaccharides [25]. Bacteria of the genus *Paenibacillus* can also synthesize various biosurfactants and bioemulsifiers, and the possibility of using cultures of bacteria of the genus *Paenibacillus* as bioremediants has also been demonstrated [27].

The use of biochar as a biostimulator and adsorbent for the remediation of contaminated soil is due to the physicochemical properties of this substance, such as its high porosity, specific surface area and sorption capacity [28–32]. Almost any organic material can be used as a raw material for biochar production, which is then used for high-temperature pyrolysis under anaerobic conditions for food, forestry and agricultural waste [33–35]. The use of biochar as a product in high-temperature pyrolysis reduces greenhouse gas emissions and is a more environmentally friendly technology for the production of biological adsorbents compared to open combustion [36–38]. The immobilization or inoculation of bacteria on a biochar is a fairly common, although still relatively poorly studied, direction for restoring the fertility and remediation of soils [39–42]. Biochar is a stimulant and carrier for the bacteria inoculated onto it. Thus, the research by Medeiros et al. (2020) allowed for establishing the use of biochar produced from coffee grounds together with *Trichoderma* spp. Fungi, which was shown to be more effective in growing melons than biochar from husks of beans and coffee [40]. In turn, recent research has shown the possibility of using the aerobic spore-forming bacteria *Bacillus* sp. MN54 together with biochar. Thus, the combined use of bacteria and biochar on soil contaminated with diesel fuel contributed to an increase in the efficiency of phytoremediation using corn (*Zea mays* L.) [43]. Biochar affects the ecological state of soils distinctly, differing in their physicochemical properties [8,44]. In addition, the enzymatic activity of soils and the number of soil bacteria are affected by the concentration of biochar in oil-contaminated chernozem [7,45]. Along with the ecological efficiency of biochar, the economic profitability of its use in restoring the ecological state of oil-contaminated soils has also been established [6]. In this regard, it is advisable to assess the ecological state of oil-contaminated soils during remediation with biochar and microbiological preparations containing strains of aerobic spore-forming bacteria [46].

In this regard, the work objective of this paper was to evaluate the effect of strains of aerobic spore-forming bacteria of the genera *Bacillus* and *Paenibacillus* and biochar on petroleum hydrocarbon decomposition and the biological activity of haplic chernozem.

## 2. Materials and Methods

### 2.1. Soil Site

The object of the study was ordinary carbonate chernozem (A, 0–25) or Haplic Chernozem Calcic [47]. The place of selection was the botanical garden of the Southern Federal University, Rostov-on-Don (47° 14′ 17. 54 N, 39° 38′ 33. 22 E). The Botanical garden is in the center of Rostov-on-Don (Rostov Region, Russia), and is characterized by its minimal anthropogenic impact on soil cover. Haplic Chernozem are among the most fertile soils in Russia and the world [48]. The physicochemical properties of the Haplic Chernozem were: pH = 7.5–7.8; organic matter content—5.5–6.0%; carbonate content—0.3–0.5%; sum of absorbed bases—34.0–36.0 mg-eq/100 g of soil [49].

### 2.2. Biological Preparation Based on Bacteria of the Genera *Bacillus* and *Paenibacillus*

A microbiological preparation with biofungicidal action against *Fusarium* fungi based on a consortium of strains of aerobic spore-forming bacteria *B. amyloliquefaciens* V3.14 and R4.6, *P. polymyxa* R5.31, as well as *P. peoriae* O1.27, O2.11, R3.13, R4.5 and R6.14, and *P. jamilae* K1.14, R4.24, was developed in the laboratory of new biological products of the Academy of Biology and Biotechnologies of the Southern Federal University. The biopreparation was based on liquid-phase fermentation technology through the use of bacterial cultures of a liquid broth based on 2.5% beet molasses by volume and a complex of salts (nitrogen–phosphorus–potassium fertilizer) at a concentration of 1.95 g/l, which corresponded to 1:25 in the ratio of N (nitrogen):C (carbon) [50]. The concentration of viable bacterial spores in the resulting biological product was no less than  $1 \times 10^9$  CFU/ml. Preparations of this kind have been investigated in various studies and used to protect different crops from phytopathogenic fungi [51–54].

In this study, the bacterial preparation (BP) was introduced at the recommended concentration of 20 mL/ha or 7,500 CFU/kg of soil. This dose is used in agricultural fields to suppress *Fusarium* fungi when processing plants by leaf. In this regard, an additional 100 times higher concentration of the drug (BP  $\times$  100) was investigated. The solution was introduced into the soil contaminated with petroleum hydrocarbons and thoroughly mixed.

### 2.3. Biochar

The biochar was pure birch charcoal (*Betula alba* L.) grade A GOST 7657-84 with a carbon content of at least 85%. The product was produced through the pyrolysis of wood (800 °C) in retort installations without oxygen. The product had a high carbon content (93–98%) and did not contain harmful or toxic impurities. The amount of carbon in 1 ton of biochar is equivalent to the content of 3 tons of carbon (C) in carbon dioxide (CO<sub>2</sub>). When using biochar, plants capture carbon from the air and bind it in the soil [53,54]. In relation to the soil contaminated with petroleum hydrocarbons, the biochar served as a sorbent and a stimulator of the biota of native soil. In the prepared soil samples with petroleum hydrocarbons, 1% biochar was applied to the soil weight.

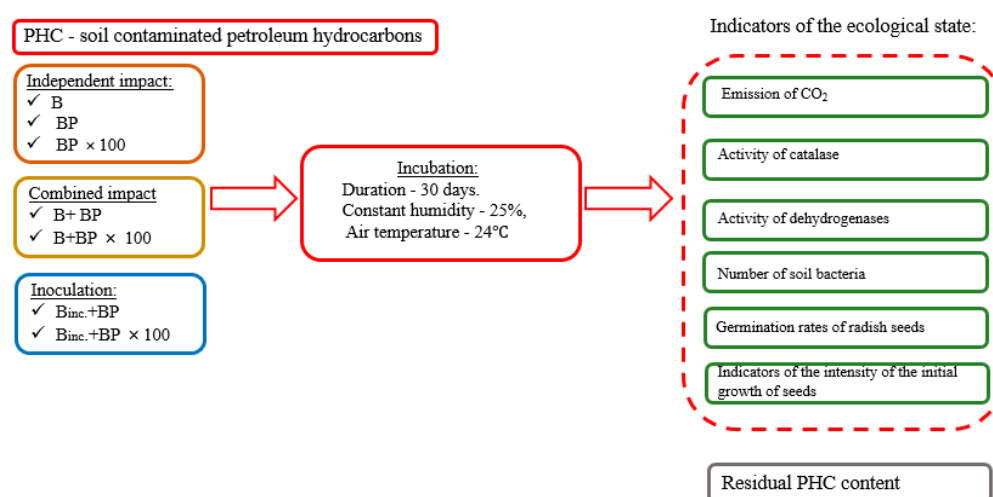
### 2.4. Petroleum Hydrocarbons

To model contamination, petroleum hydrocarbons from the Novoshakhtinsky refinery were used (Novoshakhtinsk, Rostov Region). Petroleum hydrocarbons are a mixture of hydrocarbons with a density of 0.818 g/m<sup>3</sup>, a mass fraction of sulfur of 0.43%, a mass fraction of mechanical impurities of 0.0028%, a mass fraction of water of 0.03% and a concentration of chloride salts of 40.1 mg/dm<sup>3</sup>. Petroleum hydrocarbons were added to soil samples at a concentration of 5% of soil weight. This level of soil contamination with oil is most often encountered during accidental oil spills [55].

## 2.5. Model Experiment

The prepared soil was premoistened and the petroleum hydrocarbons were applied to 5% of soil weight. A biological preparation based on aerobic spore-forming bacteria was introduced into the soil at the recommended dose (BP) and 100 times more than the recommended one ( $BP \times 100$ ). According to the experimental scheme, a biochar of 1% (B) of soil weight was introduced into soil samples with petroleum hydrocarbons. This dose of biochar was chosen based on previous studies on the remediation of organic compounds [56, 57]. At a dose of biochar above 2–5%, there is a high probability of disturbing the soil microbiota, increasing hydrophobicity and changing the response of the soil environment [58].

In the treatment option with the preliminary inoculation of the biochar with bacteria ( $B_{inc.}+BP$ ), the prepared biochar was soaked in the finished biological product for 48 hours. The general scheme of a model experiment on the remediation of petroleum-hydrocarbon-contaminated chernozem using biochar and aerobic spore-forming bacteria (independently and in combination, including inoculation) is shown in Figure 1. To compare the remediation effect of the biochar and the tank of the preparation in oil-contaminated soil, the independent effect of each substance separately (B, BP and  $BP \times 100$ ), the combined effect ( $B+BP$ ,  $B+BP \times 100$ ) and the inoculation of bacteria on the biochar ( $B_{inc.}+BP$ ,  $B_{inc.}+BP \times 100$ ) were evaluated.



**Figure 1.** Scheme of a model experiment. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation;  $B_{inc.}$ —biochar inoculated with a bacterial preparation.

The scientific hypothesis of the study: the addition of bacteria of the genera *Bacillus* and *Paenibacillus* and biochar (both independently and separately) should lead to an increase in the rate of petroleum hydrocarbon decomposition in the chernozem to simple decomposition products (water vapor and carbon dioxide) and, as a result, stimulate the emission of CO<sub>2</sub>, enzymatic activity and an increase in the number of soil bacteria, eventually providing a decrease in phytotoxicity.

## 2.6. Residual Petroleum Hydrocarbon Content

The petroleum hydrocarbon content after various treatment options was analyzed through the extraction of soil samples with carbon tetrachloride, followed by the determination of the content using an IKN-025 infrared analyzer. The petroleum hydrocarbon content was estimated in mg/kg of soil.

## 2.7. Methods for Assessing Soil Biological Activity

To assess the soil's biological activity, the authors determined the CO<sub>2</sub> emission, catalase and dehydrogenases activity, the total number of bacteria, indicators of germination of barley seeds (*Hordeum vulgare* L.) (germination, energy of germination, germination vigor and germination rate) and indicators of the intensity of the initial seed growth (length of shoots and roots, air-dried mass of shoots and roots) (Table 1). Carbon dioxide emissions were measured during the 30 days of incubation (every 3–4 days) using an EGM PP Systems (USA) gas analyzer.

**Table 1.** Methods for determining the biological activity of the soil.

Indicator	Measurement	Units	References
CO <sub>2</sub> emission	Measurement using gas analyzer EGM-5 PP Systems (USA) every 3–5 days during the entire period of incubation	ppm	No references
Catalase activity (H <sub>2</sub> O <sub>2</sub> : H <sub>2</sub> O <sub>2</sub> —oxidoreductase; EC 1.11.1.6)	Volumetric method according to the rate of decomposition of a 3% solution of hydrogen peroxide in contact with the soil	ml O <sub>2</sub> /1 grams of soil for 1 min	[59]
Activity of dehydrogenases (substrate: NAD (P)—oxidoreductase, EC 1.1.1.1)	Colorimetric method for the conversion of triphenyltetrazolium chloride to triphenylformazan (TPF)	mr TPF/10 g of soil/24 hours	[60]
Total number of bacteria	The total number of soil bacteria was determined with fluorescence microscopy with preparations stained with fluorescent dye acridine orange (1:10,000) and viewed on a Carl Zeiss Axio Lab microscope at 40x magnification	10 <sup>-9</sup> /1 g of soil	[61]
Germination indicators of seeds ( <i>Hordeum vulgare</i> L): germination, energy, vigor, germination rate	Seed germination calculated daily for 7 days Energy, friendliness and speed of germination were calculated from the daily values of germination	%	[62]
Indicators of the intensity of the initial growth of barley seeds ( <i>Hordeum vulgare</i> L.): length of shoots and roots, mass of shoots and roots	Measurement of the length of shoots and roots at the end of 7 days of the vegetation experiment	mm	
	Measurement of the air-dried mass of shoots and roots at the end of 7 days of the vegetation experiment	grams	

It was efficient to use sensitive biological indicators to diagnose soil conditions after chemical pollution [63,64]. These biological indicators allowed us to assess the state of the soil at various levels of organization, from bacteria and enzymes to plants [65]. The total number of bacteria in the soil reflected the state of reducers in the ecosystem [66]. Oxidoreductases (catalase and dehydrogenases) were more sensitive to chemical pollution than other enzymes [67]. The germination rate and root length of the radishes were the most informative of the many indicators of soil phytotoxicity [68,69].

To assess soil phytotoxicity before and after the introduction of bacteria and biochar, two groups of indicators were studied: the germination of barley seeds (germination, energy, seedling vigor and germination rate) and indicators of the intensity of the initial growth (length of shoots and roots, air-dried mass of shoots and roots). The germination of barley seeds was assessment with Equation 1:

$$G = \frac{G_7}{G_n} \quad (1)$$

where  $G_7$ —the number of germinated barley seeds on the 7<sup>th</sup> day;  $G_n$ —the total number of seeds planted at the beginning of the phytotoxic experiment.

The method for calculating the germination rate of barley seeds was represented by Equation 2:

$$GV = G/n \quad (2)$$

where  $GV$ —germination vigor;  $G$ —germination rate;  $n$ —number of seeds.

Based on the above informative and sensitive biological indicators the integral indicator of the biological state (IIBS) of haplic chernozem was calculated [63].

According to microbiological, biochemical and phytotoxic indicators, an integral indicator of the biological state of the soil (IIBS) was calculated [59]. The IIBS made it possible to assess the ecological state of soils, since it includes a set of microbiological, biochemical and phytotoxic soil parameters. To calculate this indicator, the control (noncontaminated) values of each determined biological parameter were taken as 100%, and in relation to it, the percentage value of the indicator was found already in the soil with the introduced pollutant. Next, the IIBS was calculated using Equation 3:

$$IIBS = (A_{cat} + A_{deh} + B + L_r)/N \quad (3)$$

where  $A_{kat}$ —catalase activity;  $A_{deh}$ —dehydrogenases activity;  $B$ —the total number of soil bacteria;  $L_r$ —length of roots, mm;  $N$ —the number of biological indicators.

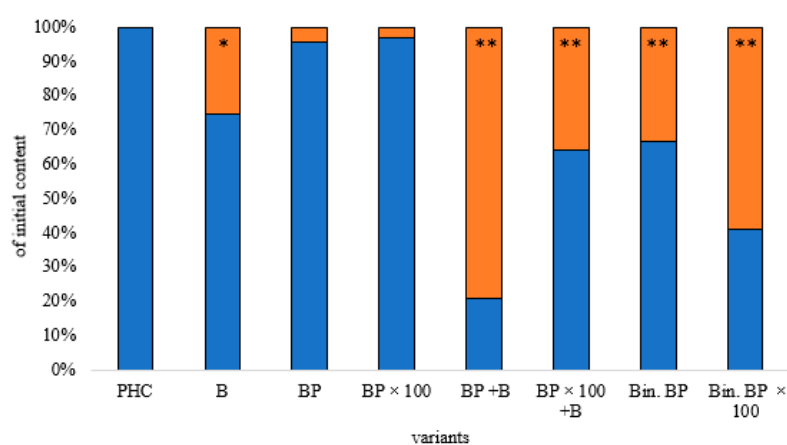
## 2.8. Statistical Processing

The statistical processing of the obtained data was carried out using the STATISTICA 12.0 (Russia) and Python 3.6.5 Matplotlib (Russia) software packages [70,71]. The significance was tested at \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ . Statistical data (average values and variance) were determined, and the reliability of various samples was established using a variance analysis (Student's t-test).

## 3. Results

### 3.1. Residual Petroleum Hydrocarbon Content

The use of biochar made it possible to reduce the petroleum hydrocarbon content by 25% compared to the background (Figure 2). At the same time, the independent introduction of bacteria, both in the recommended and 100 times higher concentration ( $BP \times 100$ ), did not significantly affect the petroleum hydrocarbon content.



**Figure 2.** Residual petroleum hydrocarbon content in haplic chernozem after the introduction of a biological product and biochar, %. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation;  $BP \times 100$ —bacterial preparation concentration 100 times greater; B + BP—biochar combined with bacterial preparation; B +  $BP \times 100$ —biochar combined with bacterial preparation 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP

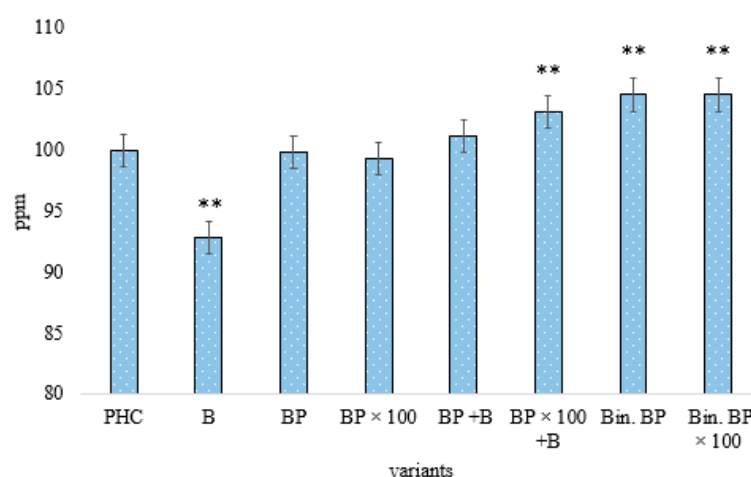
$\times 100$ —biochar-inoculated bacterial preparation 100 times greater; statistical confidence: \*— $p < 0.05$ ; \*\*— $p < 0.001$ .

The introduction of biochar together with the bacteria contributed to petroleum hydrocarbon decomposition by 79%, compared to the background. With an increase in the concentration of bacteria ( $BP \times 100$ ), the decomposition efficiency decreased by 43% compared to B+BP; however, a decrease in the petroleum hydrocarbon content by 36% compared to the control was noted. At the same time, in the treatment option, in which the bacteria were previously inoculated into biochar, the petroleum hydrocarbon decomposition was at a level comparable to the  $BP \times 100$  treatment option—34–36% of the initial petroleum hydrocarbon content. With an increase in the concentration of bacteria ( $BP \times 100$ ) with preliminary inoculation in the biochar, a decrease in the petroleum hydrocarbon content of 59% was noted, compared to the oil-contaminated background.

According to the obtained results, it was most effective to use the following combinations of biochar with BP for petroleum hydrocarbon decomposition in the soil: biochar + BP and biochar-inoculated  $BP \times 100$ . The least effective application to oil-contaminated soil was BP only.

### 3.2. CO<sub>2</sub> Emission

Oil decomposition contributed to an increase in CO<sub>2</sub> concentration (Figure 3). At the initial stage of measuring the CO<sub>2</sub> concentration in the soil air, a low decomposition rate was determined in the option with biochar and the option of joint treatment with biochar + BP. However, toward the end of the experiment, it was found that the introduction of biochar inhibited the release of CO<sub>2</sub> by 8%, and BP and  $BP \times 100$ , biochar with BP and  $BP \times 100$  did not significantly differ from the oil-contaminated background, i.e., they did not affect the emission process.



**Figure 3.** Emission of CO<sub>2</sub> of haplic chernozem (averaged values for 30 days) contaminated with petroleum hydrocarbons, after the introduction of BP and biochar, ppm. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation;  $BP \times 100$ —bacterial preparation concentration is 100 times greater; B + BP—biochar combined with bacterial preparation; B +  $BP \times 100$ —biochar combined with bacterial preparation is 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin.  $BP \times 100$ —biochar-inoculated bacterial preparation is 100 times greater; statistical confidence: \*\*— $p < 0.001$ .

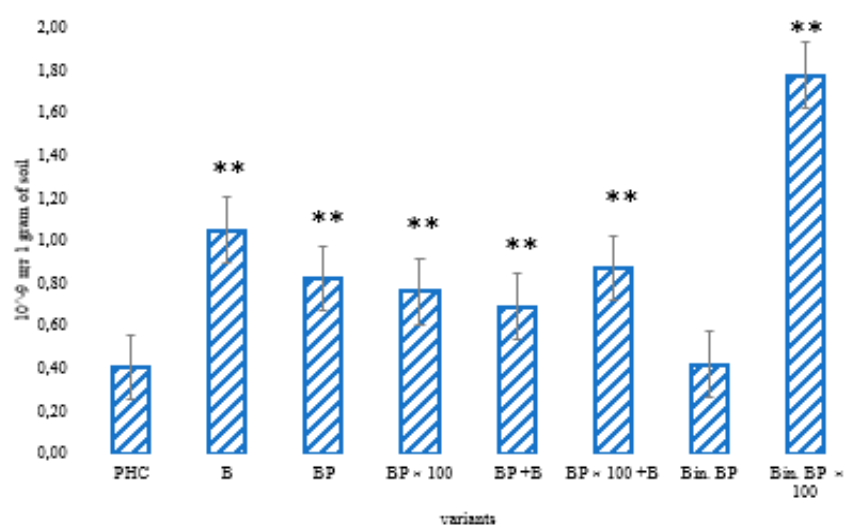
The introduction of biochar-inoculated BP and  $BP \times 100$  contributed to an increase in the intensity of CO<sub>2</sub> emission compared to the background by 5–10%. Considering that the process of petroleum hydrocarbon decomposition in options with biochar and BP, as

well as biochar inoculated with bacteria, was the most intense, the emission was higher than in other options.

The highest content of carbon dioxide in the soil air was observed in such treatment options as biochar-inoculated BP and BP  $\times$  100, and biochar + BP and biochar + BP  $\times$  100.

### 3.3. Total Number of Soil Bacteria

The total number of bacteria after the introduction of biochar was stimulated by 128% compared to the background (Figure 4). The use of BP and BP  $\times$  100 stimulated the number of bacteria by 103 and 70% compared to the background.



**Figure 4.** The total number of bacteria in oil-contaminated haplic chernozem after the introduction of BP and biochar, 10<sup>9</sup>/g of soil. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation; BP  $\times$  100—bacterial preparation concentration is 100 times greater; B + BP—biochar combined with bacterial preparation; B + BP  $\times$  100—biochar combined with bacterial preparation is 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP  $\times$  100—biochar-inoculated bacterial preparation is 100 times greater; statistical confidence: \*\*— $p < 0.001$ .

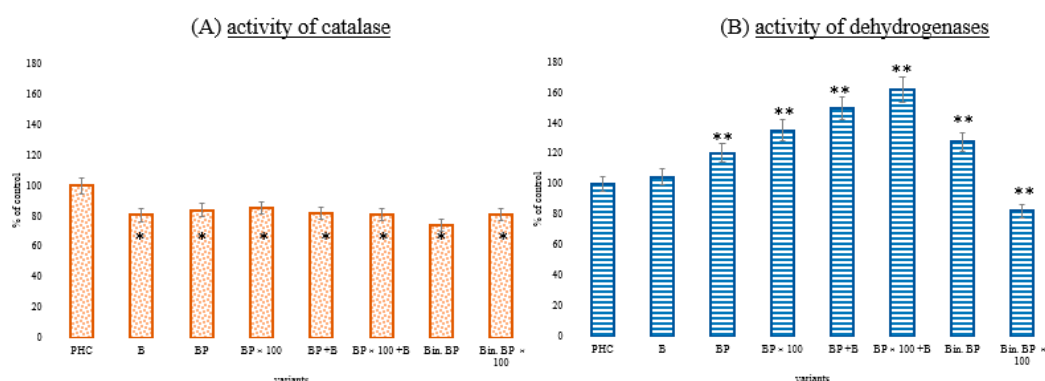
With the joint introduction of BP+B, the stimulation was 70%, with an increase in the concentration of BP  $\times$  100—115%. Biochar-inoculated BP had no effect on the number of bacteria, and at a dose of BP  $\times$  100 + biochar, stimulation was 34 times higher compared to the control.

The total number of soil bacteria was highest when introducing biochar without bacteria, biochar + BP  $\times$  100, BP  $\times$  100 inoculated into biochar.

### 3.4. Enzymatic Activity

The activity of oxidoreductases (catalases and dehydrogenases) during the introduction of biochar and BP differed depending on the type of enzyme (Figure 5). Catalase activity during the introduction of BP and BP  $\times$  100 and biochar was inhibited by 14–26% regardless of BP concentration. The greatest inhibition of catalase was found with the introduction of biochar-inoculated BP.





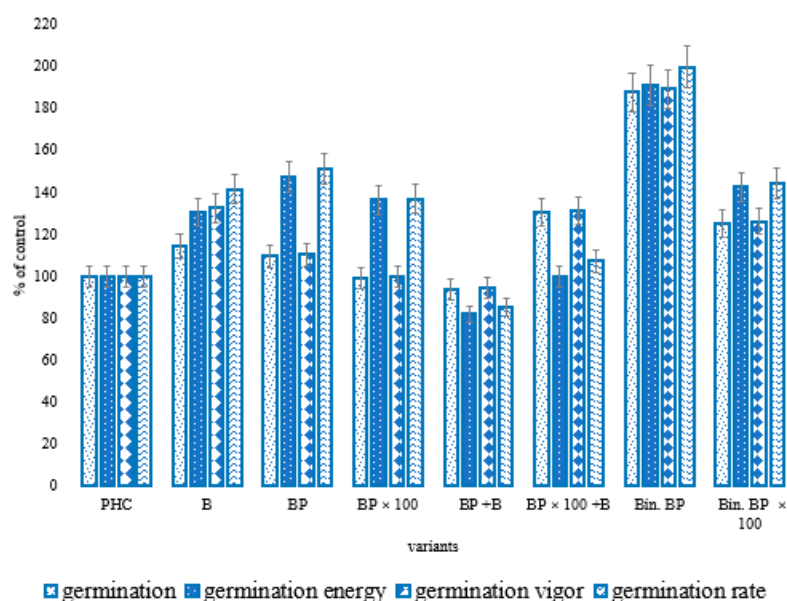
**Figure 5.** Enzymatic activity of haplic chernozem contaminated with petroleum hydrocarbons after the introduction of BP and biochar, % of petroleum hydrocarbon pollution. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation; BP × 100—bacterial preparation concentration 100 times greater; B + BP—biochar combined with bacterial preparation; B + BP × 100—biochar combined with bacterial preparation 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP × 100—biochar-inoculated bacterial preparation 100 times greater; statistical confidence: \*— $p < 0.05$ ; \*\*— $p < 0.001$ .

Dehydrogenase activity after the introduction of biochar did not significantly differ from the control. The introduction of BP and BP × 100 stimulated the activity of dehydrogenases by 20 and 49%, respectively, relative to the background content. The preparation BP and BP × 100 introduced with the biochar stimulated enzymatic activity by 49 and 61%. With the preliminary inoculation of BP in biochar, the stimulation was 27% relative to the background; with an increase in the concentration of BP × 100, the inhibition of dehydrogenase activity was established to be 18%, compared to the background.

Thus, the catalase activity was inhibited by the introduction of biochar and BP. At the same time, the activity of another representative of the oxidoreductase class—dehydrogenases—was stimulated in the options of BP × 100, biochar with BP, biochar with BP × 100 and BP inoculated in biochar.

### 3.5. Indicators of Initial Seed Germination and Intensity of Initial Growth of *Hordeum vulgare* L.

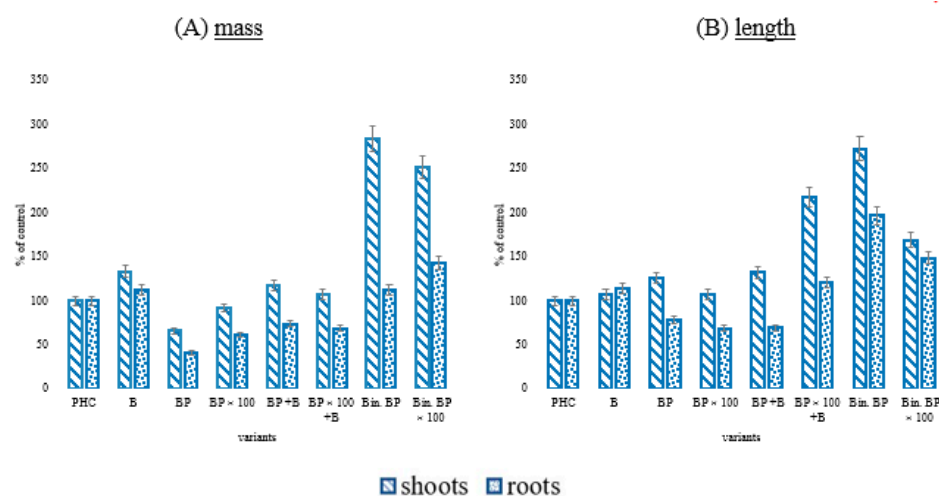
Indicators of the intensity of the initial growth of barley (*Hordeum vulgare* L.) when applying biochar were stimulated by 8–21%, relative to the background (Figure 6). The introduction of BP resulted in the significant stimulation of energy and germination rate by 22 and 29%, respectively. With an increase in the dose of BP × 100 times, the inhibition of germination and the germination of seeds of *Hordeum vulgare* L. was established; the energy and speed of germination were stimulated by 36 and 37%, respectively.



**Figure 6.** Changes in germination vigor, germination energy and germination rate of barley on haplic chernozem contaminated with petroleum hydrocarbons after the introduction of BP and biochar, % of petroleum hydrocarbon pollution. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation; BP × 100—bacterial preparation concentration 100 times greater; B + BP—biochar combined with bacterial preparation; B + BP × 100—biochar combined with bacterial preparation 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP × 100—biochar-inoculated bacterial preparation 100 times greater.

The joint application of BP and biochar inhibited the energy and seedling vigor of *Hordéum vulgäre* L. by 18 and 15%, respectively, relative to the oil-contaminated background. When BP × 100 was added with biochar, it was found that the stimulation of germination and the germination vigor of *Hordéum vulgäre* L. compared to the background were, respectively, 30 and 31% higher than the background, and the energy and rate did not differ from the background.

The morphometric indicators of *Hordéum vulgäre* L., characterizing the length of shoots and roots and the mass of shoots and roots, are shown in Figure 7.



**Figure 7.** Morphological parameters of haplic chernozem barley on soil contaminated with petroleum hydrocarbons after the introduction of BP and biochar and the % of petroleum hydrocarbon pollution. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial

preparation; BP  $\times$  100—bacterial preparation concentration 100 times greater; B + BP—biochar combined with bacterial preparation; B + BP  $\times$  100—biochar combined with bacterial preparation 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP  $\times$  100—biochar-inoculated bacterial preparation 100 times greater.

The introduction of biochar stimulated the mass of shoots by 21% more than the roots, but the length of the shoots and roots did not significantly differ from the background. The introduction of BP and BP  $\times$  100 caused an inhibition of the root system length and root mass by 21–59%, relative to the background. At the same time, the mass of shoots was inhibited by 33% when BP was applied. The stimulation was established for the length of shoots when the BP was applied at 26%, and at BP  $\times$  100, it did not significantly differ from the control. The combination of BP with biochar led to the stimulation of shoot growth by 33–117% and an increase in mass by 10–18%. Biochar inoculated with BP and BP  $\times$  100 stimulated the growth of the mass of shoots by 225 and 151% and roots by 12 and 43%, relative to the background, respectively. The length of barley shoots when applying biochar with BP and BP  $\times$  100 was stimulated by 173 and 69%, and the roots by 96 and 49%, relative to the oil-contaminated background.

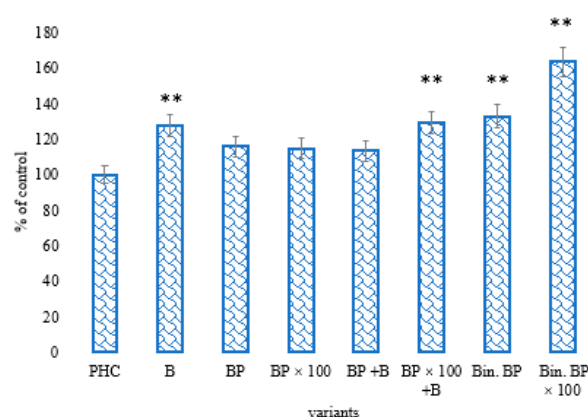
Thus, the intensity of the initial growth of *Hordéum vulgare* L., according to all indicators, was maximally stimulated when the inoculated BP was introduced into the biochar. The inhibition of the germination rate and energy by 18 and 15%, respectively, was established in the option BP with biochar.

The mass of shoots and roots of barley differed with the introduction of ameliorants: the mass of shoots in all options, except for BP and BP  $\times$  100, was stimulated by 33–184% (maximum stimulation corresponded to the option of biochar inoculation with BP). The length of barley shoots, as well as the mass of shoots, was stimulated by 7–173%, relative to the background. The maximum stimulation of the mass and length of shoots was established in the options biochar + BP  $\times$  100 and biochar-inoculated BP.

### 3.6. Integral Indicator of the Biological State of the Soil

Based on the obtained values of biological indicators, the IIBS was calculated (Figure 8).

The introduction of biochar and BP, both independently and in combination with each other, stimulated the IIBS. The introduction of biochar with BP  $\times$  100 increased the IIBS at a dose of 29%; for biochar with BP  $\times$  100 and biochar-inoculated BP  $\times$  100, it was 64% compared to the background.



**Figure 8.** The integral indicator of the biological state (IIBS) of haplic chernozem, contaminated with petroleum hydrocarbons, after the introduction of BP and B, % of petroleum hydrocarbon pollution. Note: PHC—soil contaminated with petroleum hydrocarbons; B—biochar; BP—bacterial preparation; BP  $\times$  100—bacterial preparation concentration 100 times greater; B + BP—biochar combined with bacterial preparation; B + BP  $\times$  100—biochar combined with bacterial preparation 100 times greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP  $\times$  100—biochar-inoculated bacterial preparation 100 times greater.

greater; Bin. BP—biochar-inoculated bacterial preparation; Bin. BP × 100—biochar-inoculated bacterial preparation; statistical confidence: \*\*— $p < 0.001$ .

The independent application of biochar and BP did not significantly affect the biological parameters of soil during petroleum hydrocarbon contamination. With the combined use of biochar with BP × 100, the stimulation compared with the independent introduction of substances was 15%, and with the inoculation of biochar BP and BP × 100, it was 17 and 49%, respectively.

The remediation of haplic chernozem using BP and biochar during petroleum hydrocarbon contamination had a significant impact on the biological parameters of the soil. The information value of each biological indicator was assessed through the closeness of the correlation coefficients with the residual petroleum hydrocarbon content in the soil after remediation with *Bacillus* and biochar. The following series was compiled:

The total number of bacteria (−0.61) = length of roots (−0.61) < length of shoot (−0.53) = germination (−0.52) < mass of shoots (−0.50) < germination vigor (−0.47) < germination rate (−0.43) = germination energy (−0.42) < emission (−0.38) < root mass (−0.35) < activity dehydrogenases (−0.19) < catalase activity (0.49).

The sensitivity of the biological indicators was assessed using the relative values of the biological indicators in relation to the background (Table 2). The more the value decreased relative to the background, the more sensitive the indicator was.

**Table 2.** Assessment of the sensitivity of biological objects to oil-contaminated areas of biochar and BP ingress, in % relative to the oil-contaminated background.

Biological indicators	B	BP	BP × 100	B+BP	B+BP × 100	Bin. BP	Bin. BP × 100
emission CO <sub>2</sub>	93	100	99	101	103	105	105
number of soil bacteria	259*	203*	188*	171*	216*	104	439*
activity of catalase	80*	84*	82*	79**	86*	74**	81*
activity of dehydrogenases	105	120*	149*	165*	120*	128*	82**
germination	116*	111*	95*	147*	179*	189*	126*
energy of germination	109	123*	68**	122*	144*	159*	119*
germination vigor	115*	95*	82*	127*	155*	164*	109
germination rate	121*	130*	73**	129*	152*	171*	124*
length of shoots	108	126*	133*	249*	358*	274*	170*
length of roots	114*	79**	70**	167*	235*	197*	149*
mass of shoots	133*	67**	119*	163*	307*	284*	252*
mass of roots	113	41**	74**	79**	174*	113	144*

Note: B—biochar; BP—bacterial preparation; Bin. BP—biochar-inoculated bacterial preparation. Note: statistical significance: \*— $p < 0.05$ ; \*\*— $p < 0.001$ .

Even though the catalase activity was the most noninformative indicator, it was the most sensitive (when introducing all options of biochar with BP), followed by the mass of the barley roots (BP, BP × 100 and biochar + BP) and the length of the barley roots (BP and BP × 100). When applying BP × 100, the indicators of the initial germination of barley seeds were also informative, including energy, seedling vigor and germination rate.

#### 4. Discussion

The most informative indicators for petroleum hydrocarbon pollution and remediation were the total number of bacteria and the length of the barley roots, and the least informative were the activity of the catalase and dehydrogenases. Previously, similar results were obtained when assessing the state of soils contaminated with petroleum hydrocarbons and heavy metals, as well as the remediation of petroleum-hydrocarbon-contam-

inated soils [4,6–8,63,72,73,74]. The correlation coefficient of the IIBS with the residual petroleum hydrocarbon content was equal to  $r = -0.88$ . This value represented a close relationship between the response of the integral value of all biological indicators of the soil and the petroleum hydrocarbon content; the lower the petroleum hydrocarbon content in the soil, the higher the biological activity of the soil and the more effective the remediation process. Catalase sensitivity was confirmed in a number of long-term experiments on the remediation of soil contaminated with petroleum hydrocarbons and petroleum products [75,76]. It was also confirmed that the activity of dehydrogenases correlated more closely with the residual petroleum hydrocarbon content ( $r = -0.90$ ) than the activity of catalase ( $r = -0.10$ ) and urease ( $r = -0.60$ ), and, as a result, was a more informative indicator.

The type of soil and the physicochemical properties of the soil mattered. The ordinary chernozem of the South of the European part of Russia is characterized by a slightly alkaline reaction of the medium and an organic matter content of 5.5–6.0% [49]. In this regard, chernozem has a slightly alkaline reaction ( $\text{pH} > 7$ ) in the presence of mineral fertilizers, which is sufficient for the intensive reproduction of bacteria of the genus *Bacillus* and *Paenibacillus* [77,78]. Thus, the intensive reproduction of bacteria occurs by consuming not only soil organic matter, but also the introduced petroleum hydrocarbon carbon as the carbon source [75].

According to the IIBS, the greatest stimulation of biological indicators was found on par with the decrease in the oil content in the soil after the application of biochar, biochar + BP  $\times 100$ , biochar inoculated with BP and B+BP  $\times 100$ . However, the highest petroleum hydrocarbon decomposition was achieved in the B+BP variant, while the biological indicators, according to the IIBS, were at the background level. However, of all biological indicators, only the number of bacteria and the activity of the dehydrogenases were stimulated by 70 and 65%, respectively. Such a high sensitivity of these indicators was due to the close relationship with the decomposition of petroleum hydrocarbons [79,80]. Previously, it was proved that the use of the microbiological preparation "Baikal EM-1", the fertilizer–biostimulant sodium humate and biochar contributed to the restoration of the ecological state of chernozem [6].

The results of the study made it possible to not only use biochar as an ameliorant, but also in combination with BP, stimulating not only the decomposition of petroleum hydrocarbons, but also the restoration of the ecological state through a change in biological indicators.

## 5. Conclusions

The introduction of biochar with bacteria of the genera *Bacillus* and *Paenibacillus* (BP) into the petroleum-hydrocarbon-contaminated soil had a greater remediation effect than the independent introduction of each ameliorant. The greatest reduction in the petroleum hydrocarbon content and the restoration of the ecological state of the soil according to biological indicators were achieved after the introduction of biochar and BP  $\times 100$  into the soil, as well as biochar-inoculated BP. The most informative biological indicators when applying ameliorants (biochar and BP) were the number of soil bacteria and the length of the barley roots, and the most sensitive indicator was the catalase activity. For the first time, the effect of biochar with bacteria of the genus *Bacillus* and *Paenibacillus* and biochar inoculated with bacteria of the genus *Bacillus* and *Paenibacillus* on the petroleum hydrocarbon content and ecological properties of the haplic chernozem was studied. The results of the study are recommended for use in the biomonitoring of the state of soils contaminated with petroleum hydrocarbons and petroleum hydrocarbon products after the introduction of ameliorants.

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