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Assessment of the Introduction of Microorganisms Capable of Destroying Toxic Compounds during Seed Germination †

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Abstract: This study evaluated the use of microorganisms to enhance seed germination in contaminated soil. Experiments introduced soil bacteria capable of growing on diesel fuel to clean the soil. Five experimental conditions included controls, soil with diesel fuel (DF), and soil with DF and bacterial suspension (BS) in both sterile and non-sterile conditions. Of 45 isolated microbial cultures, 13 used DF as a carbon source. The soil with 5% DF was slightly polluted. Wheat growth rates were 32% and 34% in DF-treated soil, and 86% and 88% in DF- and BS-treated soil, compared to 82% in the control. Thus, BS significantly boosted wheat seed germination.

Keywords: soil; diesel fuel; bioremediation; toxicity

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1. Introduction

For two centuries, oil has been one of the most important minerals, the use of which has been found in various fields of industry. The resulting petroleum products have found their use as fuel, various polymer components, and in the pharmaceutical and medical fields. At the same time, global demand for oil is growing every year. But the share of anthropogenic pollution with oil and oil products in the environment, including soils, is also growing [1].

The main sources of anthropogenic environmental pollution with oil and oil products include [2,3] oil production, which covers leaks and accidental spills in fields, both onshore and offshore; oil transportation, including leaks and accidents during transportation by pipelines, tankers, and rail tank cars; oil refining, namely possible emissions into the atmosphere and leaks into water bodies during oil refining at refineries (oil refineries); combustion of petroleum products (gasoline, diesel fuel), which results in the release of hydrocarbons and soot; and industrial waste of residual petroleum products that production enterprises discharge into the environment.

According to the European Environment Agency (EEA), about 1.3 million tons of oil and petroleum products end up in the world's oceans every year as a result of various anthropogenic processes. The percentages of pollution with oil and petroleum products are presented in Figure 1. Approximately 50% of all oil spills occur during oil production and transportation. Industrial activities account for about 25% of total pollution. About 20% of pollution is associated with accidents on oil tankers and oil pipelines. Approximately 5% of total pollution comes from leaks and spills from consumer use of petroleum products.

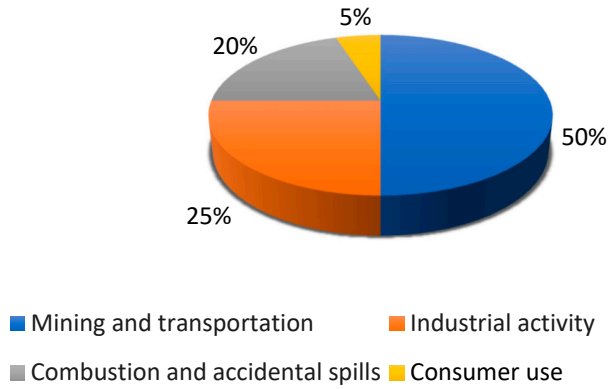

Figure 1. Percentages of oil and oil products pollution.

Pollution of aquatic ecosystems with oil accounts for the death of aquatic organisms, the disruption of ecosystems, and contamination of drinking water [4]. The main consequences of oil contamination of the aquatic environment are the formation of a film on water, which impairs gas exchange in the surface layers, prevents the penetration of light, and, as a consequence, photosynthesis, as well as the settling of heavy fractions to the bottom. Pollution with hydrocarbons leads to a deterioration in the physical and organoleptic properties of water and causes disturbances in the species and trophic structures of aquatic ecosystems. Once in the aquatic environment, oil is distributed along its profile and affects all groups of organisms living both in the surface layer and in the thickness and bottom sediments [3,5]. The negative impact of spills is especially strong in the coastal zone and on the shore [6]. Oil pollution results in soil degradation and deterioration of their fertility and chemical composition. Petroleum products lead to a sharp, catastrophic deterioration of soil properties, which are necessary to maintain ecological functions and create optimal living conditions for soil-dwelling organisms and plants that have a direct impact on soil fertility [7,8].

One of the most common petroleum product pollutants is diesel fuel, used in all types of transport, including agricultural machinery; in power generation; and in metallurgy and leather production. Being a mixture of heavy hydrocarbons, diesel fuel does not evaporate well from the surface, being absorbed into the soil and forming a synthetic film that interferes with gas exchange processes in the soil [9,10]. Also, diesel, even in minimal doses, has a significant phytotoxic effect on the generative and vegetative organs of plants. For example, it is characterized by increased phytotoxicity compared to fuel oil and has a stronger toxic effect on the soil and plant development [3,7,8].

The approximate composition of diesel fuel [10] is as follows: paraffinic hydrocarbons: 20–30%; naphthenic hydrocarbons: 30–40%; aromatic hydrocarbons: 10–20%; polycyclic aromatic hydrocarbons: <1%; sulfur: <0.05% (reduced to meet environmental standards); and additives and modifiers: <1%. All these components of diesel fuel, when released into the soil, can change its physical and chemical properties, which worsens the conditions for plant growth and can negatively affect biodiversity. Research has shown that soil contamination from diesel fuel reduces soil porosity, which limits oxygen availability and impairs water conditions [11].

To reduce the harmful effects of diesel fuel on soil, various remediation methods are used. Bioremediation, which involves the use of microorganisms capable of degrading hydrocarbons, has been shown to be effective in restoring contaminated soils [11]. However, the success of these methods depends on many factors, including soil type, climatic conditions, and pollutant concentrations.

Microorganisms, due to their enormous diversity and catabolic potential, are capable of degrading many toxic substances. In addition, they are able to adapt to various conditions; in particular, they change the properties of cell membranes to maintain the necessary biological functions and release surfactants [8,10,12]. The use of microorganisms for the remediation of oil-contaminated soils is based on these properties. Moreover, by

determining the microbial composition of soils, it is possible to use indigenous strains for bioremediation that do not disturb the balance of the local ecosystem [13]. During biodegradation, microorganisms are able to break down organic pollutants into less toxic or harmless substances. For example, *Pseudomonas putida* and *Rhodococcus erythropolis* degrade petroleum hydrocarbons by using them as a source of carbon and energy [14].

Factors influencing the effectiveness of bioremediation are as follows: pollutant type and concentration: some microorganisms may be highly effective at low pollutant concentrations but lose their activity at high toxicity levels [15]; soil physicochemical properties: pH, moisture content, nutrient availability, and soil temperature influence the viability of microorganisms and their ability to degrade pollutants [3,12]; bioavailability: long chain hydrocarbons and complex organic molecules may be less accessible to microorganisms, reducing their rate of degradation [16]; and microbial community: the composition and interactions of microorganisms in the soil influence the effectiveness of bioremediation. The combined action of different species can accelerate the degradation of complex pollutants [12,15].

Increasing the efficiency of the microbiological remediation of contaminated soils is achieved by introducing into the soil microbial biological products, where microorganisms have biodestructive activity, as well as nutrients (nitrogen, phosphorus, etc.) separately or as part of a complex mineral fertilizer. Thus, the soil is not only restored, but also improves its physical-mechanical and physical-chemical properties.

Thus, it is known to use various drugs containing, in addition to a number of excipients, microorganisms-pollutants and biodestructors of complex hydrocarbon compounds, among which “Putidoil”, “Ekoil”, “AVALON”, etc. have proven activity [12]. Examples of successful applications of bacteria for soil bioremediation are the following: 1. Cleaning up oil-contaminated soils: microorganisms such as *Alcanivorax borkumensis* are effective in breaking down oil and its derivatives, which are used in cleaning up soils and water bodies after oil spills [17]. 2. Pesticide removal: *Pseudomonas* bacteria are capable of degrading organophosphate pesticides, which are used for agricultural land restoration [18]. 3. Bioremediation of heavy metals: *Bacillus megaterium* is actively used for bioremediation of soils contaminated with lead and cadmium through sorption and precipitation of metals [14].

Thus, to reduce pollution, it is necessary to tighten control over oil transportation and production, introduce modern monitoring technologies, build and modernize treatment facilities at enterprises, and also use biodegradable sorbents, barriers, and technologies to quickly eliminate spills.

Microorganisms play an important role in soil bioremediation due to their abilities to degrade and transform various pollutants. Their use in environmental technologies makes it possible to restore contaminated soils, improving their quality and reducing toxicity. Further research in this area will help develop more effective methods for cleaning up and restoring ecosystems.

Microbiological bioremediation technologies are being improved with every detailed study of this issue, including the search for effective natural strains, which are characterized by greater efficiency and less demanding environmental conditions.

2. Materials and Methods

Soil samples were taken from next coordinates 50.534106, 36.583056, using the envelope method, followed by mixing and cleaning of remnants of plant rhizomes, fallen branches, and leaves. Isolation of microorganisms from the soil sample was carried out by the method of serial dilutions on 3% peptone agar [19]. Pure cultures were obtained using the depletion streak method; after 3 days, individual colonies were passaged onto plates with 3% peptone agar using a microbiological streak.

The selection of isolates for further research was carried out according to visual qualitative characteristics—pigmentation and active growth.

The ability to biodegrade petroleum products was studied by determining the ability to use diesel fuel as a carbon source in a liquid mineral nutrient medium (g/L): KNO_3—4;

KH_2PO_4—0.6; Na_2HPO_4—1.4; $MgSO_4$—0.8; carbon source—10 [19]. Isolates were passaged on this liquid nutrient medium and cultivated for 4–5 days. The ability to biodegrade diesel fuel was determined visually by the turbidity of the culture liquid at the end of cultivation.

The assessment of the introduction of microorganisms capable of destroying toxic compounds on seed germination was carried out as follows. The previously collected soil sample was divided into 5 parts weighing 800 g each. Two parts were sterilized with steam under pressure to obtain more reliable data on the effect of the contaminant. Samples were placed in identical plastic trays with 1 L volume. Then diesel fuel in an amount of 5% (*w*/*w*) was added to 2 sterile and 2 non-sterile trays with soil and mixed until homogeneous. Also, 13 mL of a mixture of suspensions of 13 bacterial cultures was added to 1 sterile and 1 non-sterile tray with soil, obtained by mixing suspensions of each bacterial culture grown to 1.5 OU (600 nm), 1 mL each. Trays were covered with cling film to prevent drying and placed at room temperature for 1 month, moistening the soil every day with settled water at room temperature.

Seed germination and soil toxicity in each tray were determined at time zero, after 1, after 3, and after 4 months according to the recommendations of the "Methods for determining the toxicity of water, water extracts from soils, sewage sludge and waste by changes in the level of chlorophyll fluorescence and the number of algae cells"; FR 1.39.2007.03223 [20].

The soils selected for toxicological analysis were first loosened manually with a metal spatula and freed from material known to be foreign (random) mechanical inclusions (possible industrial, construction, household waste, etc.), as well as pebbles, stone fragments, rhizomes, and branches. Before biotesting, the samples were brought to an air-dry state and sifted through a sieve with a mesh size of 1 mm.

For this, the sample was dried in a fume hood or in a well-ventilated room, placing it (depending on its weight and natural humidity) on glass. The soil samples placed in this way were kept open for at least 2 h at room temperature and air humidity.

A 20 g sample of soil was placed in a 250 mL flask, and 4 times the amount of distilled water was added. The resulting mixture was then shaken for 2 h on a shaker and then left to settle for 30 min. The supernatant was transferred to 15 mL Falcon conical tubes and centrifuged at 5000 g for 5 min, and then the natant, which was a soil extract, was collected.

To assess the overall contamination of the soil by determining the seed germination rates, wheat seeds were used as a test object. The method is based on determining germination—the number of germinated seeds in the studied soil extract sample compared to seed germination in distilled water.

Filter paper was placed in Petri dishes. In total, 15 mL of distilled water was poured into the control Petri dishes, and the same volume of the analyzed soil extract was poured into the experimental Petri dishes. A total of 50 wheat seeds were placed in each Petri dish. The dishes were covered with lids and incubated in a thermostat at a temperature of 27 °C. After 24 h, the number of germinated seeds was determined: the seeds were considered germinated if the rootlet broke through the seed coat. After calculating the seed germination, the average percentage of seed germination in the experiment was determined compared to the control samples.

The toxicity index was determined by the following formula:

$$J = (B_c - B_{ex})/B_c \quad (1)$$

where J is the toxicity index, B_c is the seed germination in the control, and B_{ex} is the seed germination in the experimental variant.

Based on the results of seed germination and the toxicity index, the degree of contamination of the samples was determined [20] according to Table 1.

Table 1. Values of the degree of soil contamination.

Indicators	Degree of Pollution			
	No Pollution	**Light Pollution**	**Average Pollution**	**Heavy Pollution**
Germination rate, %	90–100	65–90	30–65	<30
Toxicity index	<0.1	0.1–0.35	0.36–0.7	>0.71

3. Results and Discussion

The soil sample was selected at the coordinates 50.534106 and 36.583056 and was mixed and cleaned. After the initial isolation and purification of the isolates, the presence of 45 microorganisms in the sample was discovered, of which 13 cultures showed the ability to pigment and actively grow. Further, only these 13 cultures are shown in the study. These same crops showed the ability to use diesel fuel as a carbon source, which suggests their ability to biodegrade petroleum products.

In a month, wheat was sown in these trays with soil samples in the amount of 50 seeds in each tray to determine the percentage of seed germination, followed by daily spraying with water for a month. In this case, the germination of seeds in the test samples was 32% in tray no. 2, 86% in tray no. 3, 34% in tray no. 4, and 88% in tray no. 5.

As part of the experiments to assess the introduction of microorganisms capable of destroying toxic compounds on seed germination, the following indicators of seed germination (Table 2), toxicity indices (Table 3), and the degree of contamination of soil samples subjected to various types of treatment were determined.

Table 2. Seed germination.

	Zero Moment	**In 1 Month**	**In 3 Months**	**In 4 Months**
Sample 1. Non-sterile soil (control)	90%	94%	92%	92%
Sample 2. Sterile soil + diesel fuel	88%	90%	82%	91.8%
Sample 3. Sterile soil + diesel fuel + bacterial suspension	96%	90%	96%	95.9%
Sample 4. Non-sterile soil + diesel fuel	88%	84%	90%	89.8%
Sample 5. Non-sterile soil + diesel fuel + bacterial suspension	94%	92%	84%	91.8%

Table 3. Toxicity index.

	Zero Moment	**In 1 Month**	**In 3 Months**	**In 4 Months**
Sample 1. Non-sterile soil (control)	0.1	0.04	0.08	0.08
Sample 2. Sterile soil + diesel fuel	0.12	0.1	0.18	0.08
Sample 3. Sterile soil + diesel fuel + bacterial suspension	0.04	0.1	0.04	0.04
Sample 4. Non-sterile soil + diesel fuel	0.12	0.16	0.1	0.102
Sample 5. Non-sterile soil + diesel fuel + bacterial suspension	0.06	0.08	0.16	0.08

According to the results obtained and the table values for determining the degree of contamination of the samples, we have the following results: at the zero moment, samples No. 2 and No. 4 showed weak contamination; after a month, samples no. 2, no. 3, and no. 4 showed weak contamination; after 3 months, weak contamination of the samples was observed for samples no. 2, no. 4, and no. 5. After 4 months, only sample no. 4 had slight contamination; the rest had no contamination.

Thus, the introduction of 5% of diesel fuel by weight of the soil in the tray showed slight contamination throughout the experiment. The growth rates of wheat on soil treated with diesel were 32% and 34% with sterile and non-sterile soil, respectively, while the growth rates on soil treated with diesel and bacterial suspension were 86% and 88%, respectively.

Thus, it can be said that the bacterial suspension has a positive effect on the condition of the soil throughout the experiment while reducing toxicity. This can be seen in the results of the seed germination and toxicity index. It can also be judged that after 4 months there was slight contamination in non-sterile soil with diesel fuel, while in sterile soil with diesel fuel and a bacterial suspension there was no contamination, and the toxicity index was lower than that of the control. From this, we can judge either the positive effect of the bacterial suspension on soil restoration and the stimulation of seed growth.

4. Conclusions

Thus, several key conclusions can be drawn about the effect of the bacterial suspension on the condition of the soil and its toxicity in the context of the experiment:

1. Reduced soil toxicity: The introduction of a bacterial suspension into the soil with diesel fuel led to a noticeable decrease in toxicity. This was manifested in a higher percentage of seed germination in the variants with a bacterial suspension compared to the control and soil contaminated only with diesel fuel.
2. Efficiency of bacterial remediation: In sterile soil treated with diesel fuel and bacterial suspension, contamination was significantly lower, and toxicity was below control levels. This indicates the effective role of microorganisms in decomposing pollutants and reducing their harmful effects on the soil.
3. Seed resistance to environmental conditions: Higher seed germination in the presence of a bacterial suspension may indicate that microorganisms create favorable conditions for their germination, possibly by improving soil structure and reducing the concentration of toxic compounds.
4. Difference between sterile and non-sterile soil: Observation of mild contamination in non-sterile diesel soil after 4 months indicates that natural microflora also plays a role in the degradation of contaminants. However, in sterile soil supplemented with a bacterial suspension, there was no contamination, highlighting the importance of specially introduced microorganisms for effective remediation.
5. Factors influencing results: Possible fluctuations in germination rates may be due to uneven seed germination or varying resistance to toxic conditions. This means that improved germination is not always directly related to reduced toxicity and also depends on the characteristics of the seeds.

Thus, the introduction of a bacterial suspension demonstrates a promising approach for the bioremediation of diesel-contaminated soils, increasing their recovery and reducing toxicity. This highlights the importance of using targeted microbial cultures for soil cleanup and improvement.

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