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Bioremediation Possibilities of Oil-Contaminated Soil by Biosurfactant Based on *Bacillus* Strain

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ABSTRACT
This paper represents the results of the investigation of soil phytotoxicity with a high level of oil pollution. The artificially contaminated soils were exposed to an oil-destroying biosurfactant based on the strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis.* The main objective of the work was to evaluate the effectiveness of a consortium of microorganisms in reducing the phytotoxicity of soils with high levels of oil contamination. The determination was performed on the reactions of test organisms to the pollution of the soil environment. The test system in the experiment comprised *Sorghum bicolor subsp. Drummondii*, *Phleum pretense*, *Galéga officinális*, *Trifolium pretense*, *Medicágo sativa,* and *Sinapis arvensis*. The variety of the applied indicator plants has provided a comprehensive analysis of the results of oil destruction and allowed an assessment of the sensitivity of the seeds of each species to toxic substances.

Keywords: bioassay, biodegradation, bioremediation, biosurfactant, petroleum, phytotoxicity, soil.

INTRODUCTION

Soil is one of the main ecological objects being the central link between the biotic and abiotic components of the biosphere. To assess the quality of soils and soil cover, the understanding and application of a range of analytical and theoretical methods available in the inventory of soil science are required (Lim et al. 2016).

Soils are considered contaminated when the concentration of petroleum products in these soils reaches a value at which environmental negative ecological changes begin. The ecological balance in the soil is being disturbed, soil biota is dying, productivity is falling, or plants are withering, the morphology, water, and physical properties of soils are changing, their fertility decreases, and there is a risk of groundwater and surface water pollution. A dangerous level of soil contamination is the one that exceeds the limit of self-cleaning potential (Nizamzade 2014; Romaniuk et al. 2016).

A large number of components of pollution in each individual case and their variability in contrast to most other anthropogenic pollutants are common features of oil pollution (Dubrova et al. 2013).

Development of new technologies and improvement of existing ones relating to the restoration of oil-contaminated lands, neutralization, and utilization of oil-sludge waste are important measures to solve the environmental pollution problems. Currently, the methods that lead to the complete decomposition of organic pollutants and allow soil cleaning without significant production costs are considered the most environmentally friendly and promising (Robichaud et al. 2019; Khalid et al. 2021). Bioremediation technology is quite common among such methods. Increasing microbial density through the introduction of organic matter helps accelerate the decomposition of pollutants (Sui et al. 2021).

Bioremediation can be carried out by using biostimulation of microflora or by introducing specialized microbial preparations designed to decontaminate the ecosystems (Bakhonina et al. 2013). Restrictions and complications associated with certain petroleum products that are part of the soil suite composition may occur during the implementation of bioremediation with the application of biological products. These may be hydrocarbons that are poorly oxidized or toxic to the microflora, sulfur-containing compounds represented by mercaptans, hydrogen sulfide, sulfides, and thiophene derivatives. To date, there has been no unified algorithm of actions for a set of measures aimed at the recultivation of oil-contaminated soils. Balancing environmental safety and the economic efficiency of applied technologies also remains unresolved (Kirieieva 2010; Borodin 2016).

The development of the environmental standards for soils lags far behind creating the standards for other media (atmosphere, water systems). This is due to the complexity and heterogeneity of the object of investigation – the soil consists of four phases: solid, liquid, gaseous, and biotic ones (Gorova 2001). This soil property, which is distinct from other systems, greatly complicates the regulation of the content of pollutants in soil and the adequate environmental assessment (Stankevych 2012).

A review of published data shows that the microorganisms of the genus *Bacillus* have high resistance to changes in pH, temperature, salinity, and effective emulsifying properties when interacting with petroleum hydrocarbons (Alekseev 2012; Nayak et al. 2020). High adhesion of microorganisms, reduction of surface tension, emulsifying activity, production of bioactive substances even under extreme environmental conditions, decreased viscosity and high rate of degradation indicate the potential of these microorganisms for biodegradation of oil pollution (Zhang et al. 2020; Mambwe et al. 2021).

Previous investigations confirm that the microorganisms of the genus *Bacillus* can coexist with the microbial ecosystem of petroleum hydrocarbons of the same oil field from which they were isolated. At the same time, they have an increased ability to biodegrade light fractions of petroleum products (Wang et al.2012). Microorganisms can degrade alkanes, naphthenes, and aromatic components, while lighter fractions of these compounds can be completely cleaved. Cyclic hydrocarbons produce heavier fractions resistant to such bacterial effects (Xiaoli et al. 2021).

On the basis of the results of experiments, it was found that to increase the biodegradation efficiency, it is advisable to use mixed cultures consisting of two or more microorganisms. Clear criteria for the formation of artificial associations of oil destructors have not yet been produced, and the composition of biosurfactant includes the strains selected on the principle of their compatibility and high oil decomposition activity (Kumar et al. 2015; Bhaskar et al. 2020).

MATERIALS AND METHODS

There are many modern methods for assessing soil toxicity, but their application is usually limited due to their high labor intensity and cost. At the same time, an effective and relatively inexpensive approach to assessing the toxicity of contaminated soils is based on applying biotesting methods using test systems. Bioassay is aimed at assessing the overall toxicity of the whole complex of pollutants by using the investigated objects (Cherniak et al. 2021). The main advantages of this method are demonstrativeness, convenience, and simplicity of experiments, repeatability and reliability of results, cost-effectiveness, as well as objectivity (Bakina et al. 2004). To identify the toxicity of soil and aquatic environment, phytotests are used. In these tests plants are able to respond to contamination, which is established by seed sprouting parameters, root and shoot growth rate, i.e. indicators of soil toxicity (Hubachov 2010; Han et al. 2016).

Determination of the effectiveness of the biosurfactant has been carried out using the standard method of the "Growth Test" (Hrytsak 2017). Several different vegetational test systems were used in the experiment, namely Sudan Grass (*Sorghum bicolor subsp. Drummondii*), Meadow Grass (*Phleum pretense)*, Goat's-Rue (*Galéga officinális)*, Meadow Clover (*Trifolium pretense)*, Brasilian Clover (*Medicágo sativa*)*,* Field Mustard (*Sinapis arvensis*). The variety of the applied indicator plants has provided a comprehensive analysis of the results of oil destruction, as well as allowed an assessment of the sensitivity of the seeds of each species to toxic substances.

The investigations have been conducted on the basis of the Environmental Laboratory of the Admiral Makarov National University of Shipbuilding. The tested biosurfactant was based on microbial strains of *Bacillus amyloliquefaciens subsp. plantarum* NSh-2, *Bacillus amyloliquefaciens* NSh-3 and *Bacillus subtilis* NSh-4. These microorganisms were isolated from the drilling oil sludge of Semirenkivskyi gas condensate field of the Myrhorod district in the Poltava region. Physiological, metabolic, and destructive properties of the strains, resulting in combining an association capable of degradation of oil and petroleum products in a wide range of temperatures and pH, have been taken into account. The samples of artificially contaminated soil mixture have been prepared in the ratio of the mass of oil sludge to the uncontaminated part in the proportions of 1:1, 2:1, and 4:1, respectively.

To ensure stable oil destruction processes, the biosurfactant was applied in an amount calculated on the basis of the sorption capacity of the soil samples. The total weight of each individual sample of soil mixture was ~ 70 grams (2.47 ounces). Given the environmental conditions, primarily temperature (20-24°C) and light intensity (6000–9000 Lux), sprinkling with biological preparation took place twice a week at a concentration of 4%. Between sprinklings with the biological preparation, as the soil dried, it was moistened with plain water.

The concentration of the biosurfactant was selected on the basis of preliminary testing of a similar consortium of microorganisms. On the basis of the results of authors' previous investigations, the selected concentration provided the soil with the best growth-initiating properties, despite oil pollution. In this amount, the *Bacillus* microorganisms have no adverse effect on plant growth and the resulting mass of organic substances (Nedoroda et al. 2021).

The measurement results are processed using mathematical statistics methods as the mean ± standard deviation. In order to identify priority factors and assess the consistent patterns of the values observed, the analysis was performed by identifying the main components using a correlation matrix.

RESULTS AND DISCUSSION

The experiment was performed in identical plastic beakers with a capacity of 180 ml. The samples of oil-contaminated soil mixture (the appropriate amount of sludge was added to the soil up to the desired concentration and mixed), were placed in beakers in equal amounts. The surface was leveled and moistened with the same (5 ml) volume of water. Vegetation test systems sprouted in clean soil (Figure 1), contaminated soil (with various oil concentrations) without biosurfactant, and the soil samples exposed to biosurfactant effect.

The following test parameters were investigated in the ecotoxicological analysis: seed germination, shoot height, root length and mass of an organic substance. The results of phytotesting of contaminated soils under the influence of *Bacillus* strain were compared with the control results of identical plants in the absence of contamination.

Germination of the first sprouts was recorded in the control groups on both 3–4 and 9–10 days of the experiment. Different plants yield different results. In the beakers with a high concentration of oil, the germination occurred later, on 6–7 and 14–15 days, respectively. It should be noted that the intensity of germination changed significantly on the days of sprinkling with the biosurfactant. Such results confirm its growth-initiating properties.

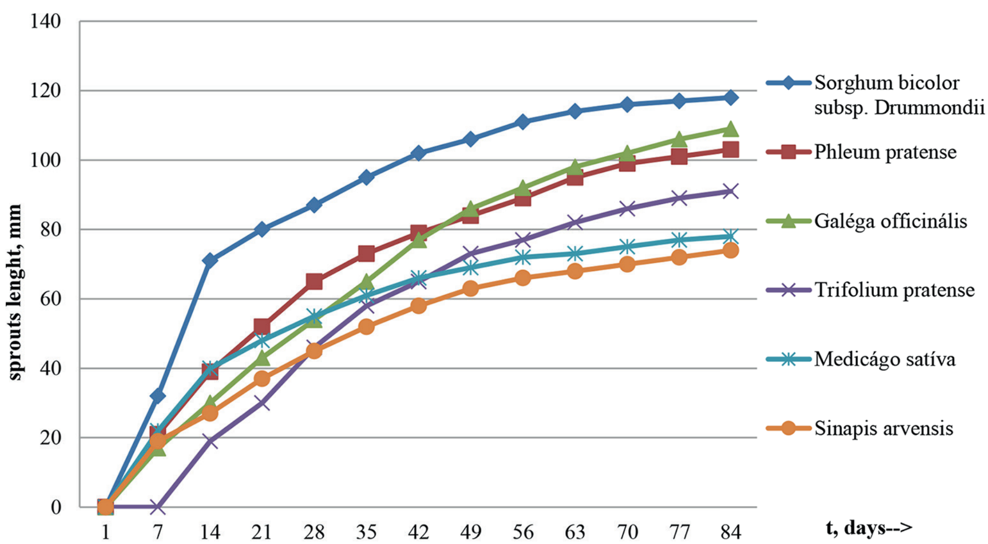

Figure 1. Dynamics of growth of bioindicator plants in uncontaminated soil (control), mm

Reactions of test systems were detected at different stages of the experiment, depending on the nature and intensity of contamination. Figure 2 and Figure 3 show that in the plants grown in the soils contaminated with oil, some changes were revealed in the linear dimensions of the roots, and the aboveground part. For example, in the control, the length of the aboveground part of *Sorghum bicolor subsp. Drummondii* at the age of 84 days was 120 mm, whereas in the version with 50% oil sludge contamination, the length of the plant was only 62 mm.

According to the results of biotesting of oil-contaminated soil samples, presented in Figure 2, it was found that the doses of applying oil in a ratio of 1: 1 cause acute high toxic effects only within the first 14 days of the experiment. The obtained results can be explained by the fact that in this period, the application of the biosurfactant has not yet had a sufficient effect on the contaminated samples (growth of the mass of microorganisms). Therefore, the first shoots are exposed to a significant phytotoxic impact, resulting in wilting. However, starting from the third week of research (after 4–5 sprinklings), soil samples had a reduced toxic effect on the subsequent vegetative period.

Control was performed on the samples not exposed to the impact of oil destructor for each test system at each level of oil sludge concentration. Without the application of microorganisms, in most cases, the seeds do not germinate.

In some samples, namely *Sorghum bicolor subsp. Drummondii* and *Sinapis arvensis*, the seeds take roots. There are stable wilting processes in such cases, and plants do not germinate above 4–5 mm. In all the controls without the use of oil-destroying microorganisms, the plants completely wilted before the end of the experiment.

In other samples, during the whole period of the investigation – in the cases with *Bacillus* microorganisms – there was a tendency to increase the biometric parameters of test culture sprouts. On the basis of the obtained samples, it is possible to conclude the significant initiating effect of the biosurfactant on the biological indicators of vegetation test systems, even with high (up to 70%) oil sludge contamination.

Each bioindicator plant showed different resistance to oil sludge contamination and the impact of the oil destructors. The analysis of indices of seed germination, number of sprouts, the linear size of stems and roots, weight of organic substance obtained, i.e. total productivity of plant growth in the laboratory environment, allowed establishing the relationship between different levels of oil pollution and the biosurfactant impact. Thus, when soil contamination exceeds 80% (4:1), no seed germination occurs in the laboratory environment. At lower levels of pollution, the most stable germination processes take place using *Sorghum bicolor subsp. Drummondii* and *Sinapis arvensis* as tests. However, given that these are the only tested cultures that germinated even in the absence of a biosurfactant, it can be concluded that these plants are less sensitive to oil pollution. The general results of germination of test cultures are summarized in Table 1.

The mass wilting of young plants a time after their germination in oil-contaminated soil, noted

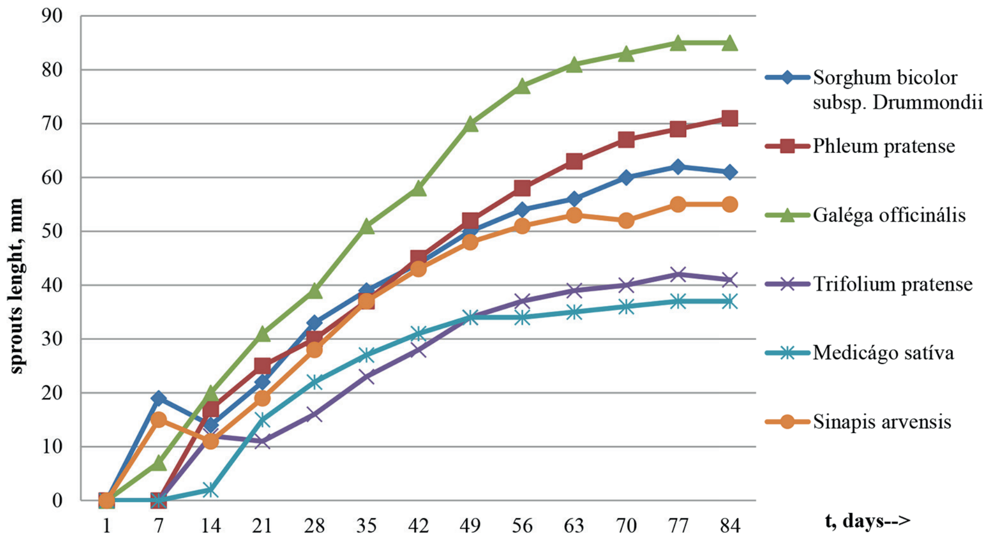

Figure 2. Dynamics of growth of bioindicator plants in soil contaminated with oil sludge in the ratio 1:1 (50%), mm

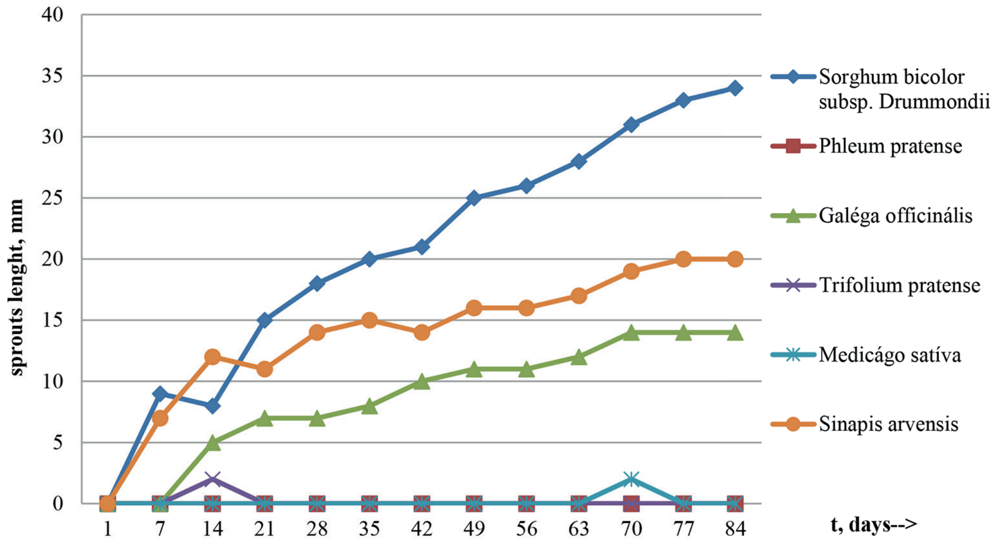

Figure 3. Dynamics of growth of bioindicator plants in soil contaminated with oil sludge in the ratio 2:1 (70%), mm

in the conducted experiment, was also observed by many authors. This effect is not associated with the deterioration of the physical properties of oil-contaminated soil, as it is observed when applying to the soil with oil composite the mixtures that restore the leaching and air regime of the soil. Another explanation is likely the slow penetration of oil hydrocarbons into plants which eventually causes their suppression. The period of primary toxic action in oil pollution is not so long, because it is due to light fractions of hydrocarbons which are relatively quickly destroyed by the applied oil destructor

Table 1. Average results of seed germination at the end of the experiment, mm

Bioassay test-system	The mass ratio of oil sludge and uncontaminated soil					
	Control		1:1		2:1	
	Shoot height	Root length	Shoot height	Root length	Shoot height	Root length
Sorghum bicolor subsp. Drummondii	120.16 ± 19.07	95.79 ± 10.68	62.54 ± 8.40	71.27 ± 5.35	34.96 ± 5.70	42.59 ± 7.37
Phleum pratense	104.64 ± 13.75	81.87 ± 6.29	72.76 ± 9.62	60.18 ± 7.34	-	-
Galéga officinális	112.35 ± 20.92	94.16 ± 8.13	85.09 ± 15.72	70.97 ± 11.26	14.71 ± 3.19	31.79 ± 5.42
Trifolium pratense	92.08 ± 23.52	85.97 ± 18.60	43.82 ± 10.07	45.95 ± 9.52	-	-
Medicágo satíva	79.61 ± 12.24	98.49 ± 14.87	37.71 ± 7.96	59.67 ± 12.75	-	-
Sinapis arvensis	74.36 ± 10.77	68.59 ± 7.04	56.31 ± 8.78	49.32 ± 6.69	20.93 ± 3.37	35.42 ± 6.48

Table 2. Phytotoxic effect of contaminated soil when applying oil destructors, %

Bioassay test-system	The mass ratio of oil sludge and uncontaminated soil		
	1:1	2:1	4:1
Sorghum bicolor subsp. Drummondii	26.45	71.91	100
Phleum pratense	14.92	100	100
Galéga officinális	19.86	81.05	100
Trifolium pratense	46.73	100	100
Medicágo satíva	48.16	100	100
Sinapis arvensis	24.32	70.27	100

microorganisms (Bryanskaya et al. 2014). The toxicity of the investigated soils was evaluated based on the difference between the control and affected samples. The results are shown in Table 2. The soil samples in the ratio of the mass of oil sludge to the uncontaminated part 4:1 were the most toxic ones. The same is true for all controls containing sludge without using *Bacillus* microorganisms.

It should be noted that *Phleum pretense* is the most resistant to phytotoxic effects at 50% of oil sludge in the investigated samples. However, with further increases in pollution, the growth processes of this bioindicator plant do not occur at all. This may indicate the high sensitivity of *Phleum pretense* when approaching maximum concentrations of oil pollution. On the one hand, such sensitivity can effectively control phytostimulation processes, i.e. the development of symbiotic organisms that take part in the processes of soil purification. However, on the other hand, the lack of growth processes reduces the effect of rhizodegradation (microbiological destruction in the root zone of the plants).

CONCLUSIONS

It has been established that the application of biosurfactant based on a consortium of strains of oil-destroying microorganisms *Bacillus amyloliquefaciens subsp. plantarum* NSh-2, *Bacillus amyloliquefaciens* NSh-3, and *Bacillus subtilis* NSh-4 are rational approaches in bioremediation methods. According to the obtained samples, an initiating effect on the biological parameters of seedlings of vegetation test systems has been revealed. The test results showed that introducing a consortium of microorganisms of the *Bacillus* strain significantly increases the efficiency of the biodegradation process of petroleum products.

Applying this biosurfactant on the investigated samples of oil-contaminated soils has provided a reduction of phytotoxic effect at the level of 73.55–85.48% at low and 18.95–29.73% at significant (up to 70%) levels of oil sludge contamination.

The oil-contaminated soil without applying a *Bacillus*-based solution had a more significant negative impact on the growth and development of indicator plants than the contaminated soil with *Bacillus* solution within the same oil doses. Such data indicate a positive effect of *Bacillus* microorganisms on oil degradation processes.

During the experiment, within the first 10-14 days, there was a significant percentage of wilting of young plants due to the phytotoxic impact of contaminated soil. At the same time – after several weeks of regular sprinkling with a solution of oil destructors – the wilting of test cultures in the samples stopped. This result indicates the need for further research with the preliminary introduction of the biosurfactant into the soil to reduce the primary toxicity.

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