

COMPARATIVE EVALUATION OF THE EFFICACY OF *Pseudomonas putida* IN THE BIOREMEDIATION OF DIESEL FUEL CONTAMINATED DERNO-PODZOLUIVISOLIC SOIL OF DIFFERENT HORIZONS

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ABSTRACT

This study was carried out to compare the efficacy of *Pseudomonas putida*, contained in the biopreparation «Pseudomin» in the bioremediation of diesel fuel contaminated derno-podzoluivisolic soil of two different horizons. By analyzing the Total Petroleum Hydrocarbons (TPH) content using IR-spectrometry method under laboratory conditions, results of the research showed that the inoculation of *Pseudomonas putida* in to the diesel fuel contaminated derno-podzoluivisolic soil samples in only 12 weeks was able to reduce the diesel fuel content up to 99.2% in the humus-accumulative horizon; and up to 92.8% in the eluvial horizon. Comparative evaluation of the contaminated variants inoculated with *Pseudomonas* of both horizons revealed a significantly higher rate of diesel fuel degradation in the humus accumulative horizon of up to 8.7 times, compared to the eluvial horizon, which shows a slightly slower rate of degradation.

KEYWORDS: Bioremediation, petroleum hydrocarbon, humus-accumulative horizon, eluvial horizon, derno-podzoluivisolic soil, *Pseudomonas putida*, biological preparation «Pseudomin»,

INTRODUCTION

The problem of oil spill is one of the major challenges of modern ecology [Atlas, 1988; Bola et al, 2006; Halimov et al, 1996; Stankevich, 2002]. In recent years, due to the increase in the production of oil, construction of new oil pipelines and refineries, there has been a sharp increase in the occurrence and scale of crude oil contamination, leading to a shift in the ecological balance, and the deterioration of the environment [Atlas, 1988; Nwachukwu, 2001; Stankevich, 2002; Vasenev et al, 2010].

With an annual volume of world crude oil production of more than 2.5 billion tons, about 50 million tons of crude oil per year is lost to the environment as a result of accidents, leaks, bunkering, etc. Creating serious environmental challenges in many countries including Nigeria and Russia [Atlas, Bartha, 1992; Bola et al, 2006; International Petroleum Monthly, 2011].

The bioremediation of petroleum hydrocarbon contaminated soils using petroleum hydrocarbon-oxidizing microorganisms is presently accepted worldwide as the most cost effective and environmental friendly means of cleaning up oil contaminated soil and water [Kireeva, 1996; Stankevich, 2002; Tyagi, 2010; Vidali, 2001].

Bio-augmentation, which is one of the techniques of bioremediation, involves the introduction / inoculation into the contaminated medium of a single / consortium of highly specialized petroleum hydrocarbon-oxidizing microorganisms in the form of biological preparations, one of which is Pseudomin [Nwachukwu, 2001; Stankevich, 2002].

Pseudomin was developed at the Department of

Microbiology of the Russian State Agrarian University – MTAA named after K.A. Timiryazev, using strains of petroleum hydrocarbon-oxidizing microorganisms of the genus of *Pseudomonas putida*, isolated from derno-podzoluivisolic soils long exposed to contamination near a filling station. Pseudomin possesses a prolonged degrading effect on petroleum products remaining in the soil after mechanical and (or) physical-chemical treatment. It has been successfully tested under conditions of the southern taiga zone of the European part of Russia and in a number of oil fields in Siberia [Stankevich, 2002].

However, little research has been carried out on its efficacy in the bioremediation of petroleum hydrocarbon contaminated soils of different horizons, hence the **aim** of the research – to make comparative assessments on the efficacy of *Pseudomin* in the bioremediation of diesel contaminated humic-accumulative ($A_1+A_1A_2$) and eluvial (A_2) horizons of derno-podzoluivisolic soils of the central region of Russia.

MATERIALS AND METHODS OF RESEARCH

Materials for this research included:

1. **Soil sample:** mixed samples of the humic-accumulative and transition horizons ($A_1+A_1A_2$); and samples from the eluvial (A_2) horizons of a loamy, completely podzolized derno-podzoluivisols, were collected for the research. The samples were collected from Area 3, of the ecological monitoring site, located in the experimental forest reserve of the Russian State Agrarian University – MTAA named after K.A. Timiryazev (tab. 1).

Table 1: Summary of the properties of the understudy derno-podzoluvisolic soil of different horizons [Burinova, 2011]

Topography and location (coordinates) of the area of sample collection	Horizon	Depth	C _{org} , %	pH _{H₂O}	pH _{KCL}	H, mg-eqv/100g soil
Watershed of the moraine hills (N55° 49' 11.8" E037° 33' 08.6")	A ₁ + A ₁ A ₂	0-30	1,91	4,21	3,81	12,80
	A ₂	30-44	0,31	4,32	4,93	8,50

- Pollutant:** Diesel fuel was used as a contaminant in the model laboratory experiments, which had a density of about 840 kg/m³. With boiling point of 40° C; and freezing point -35 °C.
- Microorganism:** For this study pure culture strains of petroleum hydrocarbon oxidizing microorganisms were used, which belong to the genus *Pseudomonas putida*, strain 91-96 (first isolated from oil contaminated derno-podzoluvisolic soils by the Department of Microbiology and Immunology of the Russian State Agrarian University – MTAA named after K.A. Timiryazev).

Methods of research:

To conduct this study, analysis of the Total Petroleum Hydrocarbons (TPH) content using IR-spectrometry method on a liquid photometric analyzer «Model №: *Fluorat-02*» instrument, was carried out at regular intervals [PND F 16.1; 2.2.22-98, 2001].

The research was carried out in the laboratory of the agro-ecological monitoring, modeling and ecosystems forecasting; and in the microbiology laboratory of the Russian State Agrarian University-

MTAA named after K.A. Timiryazev. Statistical analysis of the research results were performed by «Statistic 8.0».

Experimental Design:

The two horizons (humus-accumulative and eluvial) of the understudy derno-podzoluvisolic soil having been dried and sieved (through hand held sieves with diameter of 1 mm) were placed into 2 different containers for mixing. 2.7 kg of soil was placed in each bucket.

According to the experimental scheme, the variants to be contaminated were polluted by the addition of diesel fuel at a concentration of 18.2 mg/g soil, which corresponds to 5% by weight of air-dry soil. In the variants to be inoculated with hydrocarbon oxidizing microorganism, a 10 ml suspension of *Pseudomonas putida* was added, with titer value of 1.6×10^7 CFU/ml.

Finally the soil was mixed and placed in plastic containers of 270 g each, the Total number of experimental variants – 8, replications – 3 (tab. 2). The soil moisture content was fixed at 60%, and maintained daily by gravimetric methods.

Table 2: Experimental Scheme

№ of Variant	Experimental Series (derno-podzoluvisolic soil)	
	Horizon A ₁ + A ₁ A ₂	Horizon A ₂
1	Uncontaminated soil	Uncontaminated soil
2	Contaminated Soil	Contaminated Soil
3	Uncontaminated soil + <i>Pseudomonas</i>	Uncontaminated soil + <i>Pseudomonas</i>
4	Contaminated Soil + <i>Pseudomonas</i>	Contaminated Soil + <i>Pseudomonas</i>

RESULTS

Analysis of the total petroleum content in the two horizons of the understudy derno-podzoluvisolic soil is displayed in the table below:

Table 3: The rate of diesel fuel degradation in the understudy derno-podzoluivisolic soil of different horizons

Experimental Variants			Initial Petroleum Hydrocarbon Content, mg/g	Amount of Petroleum Hydrocarbon Added, mg/g	Total Petroleum Hydrocarbon, mg/g (Weeks)					
					1	2	3	4	9	12
Horizon A ₁ +A ₁ A ₂	1	Contaminated Soil	0.01	18.2	8.54	7.02	5.01	3.16	0.76	0.34
	2	Contaminated Soil + <i>Pseudomonas</i>			7.25	6.15	4.75	2.58	0.57	0.15
Horizon A ₂	3	Contaminated Soil	0.01	18.2	11.39	9.49	7.90	6.32	3.02	1.87
	4	Contaminated Soil + <i>Pseudomonas</i>			10.75	9.14	7.55	5.72	2.59	1.31
Approximate Safe Exposure Level			4mg/g							

P₀₅ – 0.16

DISCUSSIONS

The scale of soil pollution by crude oil and its derivatives sometimes reaches several lower layers of the soil profile, bringing about the destruction of the soil's properties and ecological balance, therefore, it is necessary to study and evaluate the effectiveness of the biological preparation *Pseudomin* in the clean-up of oil contaminated soils of different horizons. To carry out this experiment, the humus-accumulative (A₁+A₁A₂), and eluvial (A₂) horizons of derno-podzoluivisolic soil were used.

After only 12 weeks of experiment, results of the research on the effectiveness of the biological preparation *Pseudomin* in the clean-up of oil contaminated humus-accumulative (A₁+A₁A₂), and eluvial (A₂) horizons of derno-podzoluivisolic soil, revealed a sharp decline in the total petroleum hydrocarbon content in the soils contaminated by diesel fuel of up to 25-48 times the initial amount after the 1st week of experiment in the mixed sample of horizons A₁+A₁A₂; and up to 6-8 times the initial amount after the 1st week of experiment in the soil sample from horizon A₂ (tab. 3).

As reported by many researchers in their studies [Halimov et al, 1996; Kireeva, 1996; Stankevich, 2002], the initial stage of the bioremediation process of diesel fuel contaminated soils, is characterized by the evaporation of the volatile fractions (light fraction of petroleum hydrocarbons) as a result of physical and chemical weathering and evaporation, i.e. about 50% of diesel fuel decomposes without the action of hydrocarbon oxidizing microorganisms. This trend was also observed in all the variants of the experiment, i.e., by comparing the residual petroleum hydrocarbon content at the 1st week of the experiment to the total amount of diesel fuel added, about half the content had already evaporated.

Comparative evaluation of the contaminated variants inoculated with *Pseudomonas* of both horizons (variants 2 & 4) reveals a significantly higher rate of diesel fuel degradation in the humus accumulative horizon of up to 8.7 times, compared to the eluvial horizon, which shows a slightly slower rate of degradation. This phenomenon can be explained by the presence of humus, which provides an additional source of energy (co-substrate), for the soil microbial communities, thus increasing their population and microbial activity (Figure 1).

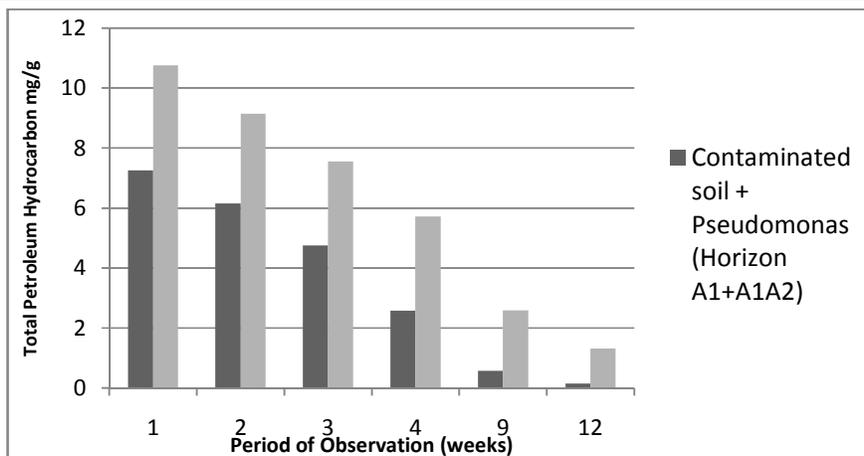


Fig. 1: Graphical representation of the efficacy of Pseudomin in the bioremediation of the diesel contaminated derno-podzoluivisols of two different horizons

It should also be noted that results of the experiment showed a significant difference in the rate of decomposition of diesel fuel between the variants with the inoculation of *Pseudomonas putida* (variants 2 & 4) and without inoculation (variants 1 & 3). The inoculated variants showed the most impressive result in the reduction of the amount of diesel fuel, as the residual content of petroleum hydrocarbon at the 12th week of experiment was 2.3 times lower in the case of the humus accumulative horizon; and 1.4 times lower in the eluvial horizon, when compared with the variants without inoculation. This provides evidence of the potency of the biological product (Pseudomin) in accelerating the decomposition of petroleum products in the soil, at an average of 1.9 times

The substantial decrease in the total petroleum hydrocarbon content observed in the variants without the inoculation of *Pseudomonas putida* (variants 1 & 3), was as a result of the conducive environment created for the indigenous microbial community (biostimulation), by the constant action of stirring (ploughing) of soil samples in the containers, which improved the aeration and percolation of the soil; as well the constant monitoring of the soil moisture content.

In general, results of the experiment have shown a very high efficacy and potency of the *Pseudomonas putida* contained in the biological product Pseudomin in the biodegradation of petroleum hydrocarbons, as evident in the over-all scale of total hydrocarbon reduction in the experimented soils, where in only 12 weeks the inoculation of Pseudomin into the derno-podzoluivisolic soil samples, was able to reduce the diesel fuel content in the: humus-accumulative horizons up to 99.2%, showing a 1.1% increase over the non-inoculated variant, which had an efficiency rate of 98.1%. While the efficiency rate of the non-inoculated variant was quite significant, as a result of biostimulation, the addition of Pseudomin had a more impressive impact on the rate of degradation, which was 2.3 times faster as evidenced by the residual petroleum hydrocarbon content at the 12th week of experiment. The residual amounts of petroleum hydrocarbon in the soil after the experiment were significantly lower than the approximate safe exposure levels for soils of the mid

and southern taiga of Russia [Russian State Epidemiological Sanitary Standard .2.1.7. Soil, 2010].

In the eluvial horizon, the rate of efficiency for the inoculated variant was 92.8%, showing a 3.1% increase over the non-inoculated variant, which had an efficiency rate of 89.7%.

Cost-wise the inoculation of *Pseudomonas putida* contained in the biological product Pseudomin into the soil is minimal, as pure culture strains of *Pseudomonas putida* can be directly introduced into the soil, at no significant cost.

In conclusion the application of bioaugmentation as a technique of bioremediation using pure culture strains of *Pseudomonas putida* has proven to be very effective and efficient for the clean-up of petroleum hydrocarbon contaminated soils of various horizons of the soil profile and can be used for the bioremediation of petroleum hydrocarbon contaminated soils no matter the depth and scale of pollution provided all other factors are optimal.

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