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The recultivation of the soils, contaminated with petrol and diesel fuel, with the help of earthworms *Dendrobena veneta* and the complex of microorganisms

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Abstract. The article investigates bioremediation efficiency of the soil contaminated with petroleum (20 to 60 g/kg) and diesel fuel (20 to 40 g/kg) with the help of earthworms *Dendrobena veneta* in the presence of bacteria *Paenibacillus pabuli*, *Azotobacter vinelandii*, *Lactobacillus casei*, *Clostridium limosum*, *Cronobacter sakazakii*, *Rhodotorulla mucilaginosa*, *Cryptococcus albidus*, eria, yeasts *Saccharomyces*, fungi *Aspergillus* and *Penicillium*, as well as Actinomycetales, all being components of biopreparation Baykal-EM. It was demonstrated that in oil-contaminated soil, the content of hydrocarbons decreased by 95 % after 22 weeks in the presence of worms and bacteria. The microbiological preparation introduction increased the earthworms' survivability in oil-contaminated substrate. Microbiological preparation introduction improved the earthworms' survival in contaminated substrate. The microbiological preparation introduction, therefore, allowed to use *Dendrobena veneta* for soil recultivation with higher oil concentrations that was impossible in the past. In petroleum-contaminated soil the content of hydrocarbons decreased by 97% after 5 months. The presence of the diesel fuel in the amount of 40 g per 1 kg soil had an acute toxic effect and caused the death of 90 % earthworm species in 14 days. Bacteria introduction enhanced the toxic effect of the diesel fuel and resulted in the death of 100 % earthworms after 7 days.

1. Introduction

Remediation of the soils contaminated with hydrocarbons is based on the chemical treatment or physical removal of the contaminants, but lately biostimulation, bioaugmentation and phytoremediation are used more and more often since they have less destructive effect on the environment [1-3]. The term 'vermiremediation' is applied when the earthworms are used in contaminants removal from the soil [4] and enhancement of PAHs compounds dissipation. Some authors reported positive effect of the earthworms on the removal of the contaminants such as oil, PAHs, PCBs, pesticides, and heavy metals [5-8].

Thus, the earthworms facilitate and enhance the contact between the contaminants and soil microorganisms [9]. *Lumbricus rubellus*, *Dendrobena octaedra* and *Aporrectodea caliginosa* [10] are the most common cultivated species in soils, contaminated with heavy metals and waste sites (ash, sludge).

Contamination of soil with oil is the most wide-spread problem in Russia and the whole world. The earthworms are proven to be able to lower the concentration of the oil in the soil [1]. M. Whitfield-



Aslund and his co-authors observed that oil-contaminated soils are not toxic for the earthworms, 90% survival was registered at low oil concentrations up to 25 g/kg, but the earthworm reproductive function disorder was also noted [11].

Dendrobena veneta has been used in agriculture for a long time. For the last 25 years the scientists of the Rothamsted Experimental Station investigated many aspects of the earthworms' application in soil recultivation and melioration. *Dendrobena veneta* is used in organic waste decomposition, and in recycling of animal and plant waste, sewage, and agricultural, household, urban and industrial sources [12]. There is no data on *Dendrobena veneta* usage in oil contaminated recultivation in scientific literature.

Erlacher et al. studied the influence of various oil concentrations on survivability of *Dendrobena hortensis*. At concentration 823 mg/kg the mortality of *Dendrobena hortensis* rose up to 100%. But after amendment with organic substrate, the mortality did not exceed 60% at higher oil concentrations from 1059 to 2241 mg/kg [13]. Hickman Z.A. and Reid noticed the decrease of 3-methylchloranthrene concentration in soil *D. veneta* cultivation [14].

Hickman Z.A. and Reid B.J. studied the effect of compost and earthworms *Dendrobena veneta* on the level of hydrocarbon catabolism in PAH-contaminated soil. The survival of the earthworm *D. veneta* was studied in the soil contaminated with PAHs 10 g/kg for 84 days. The variant with compost (1:0.5) but without the earthworm had PAHs residual concentration of 70%, and the variant without compost had 13%. The residual concentration of PAHs was 20% in the variant with the compost (1:2) and without the earthworms, and it was 8% in the variant with the earthworms. Compost and earthworms in PAHs-contaminated soil increases catabolic activity greatly [9, 15].

Therefore, the undertaken analysis of the publications shows that the information on *D. veneta* application in oil-contaminated soil recultivation is insufficient. The earthworms *D. veneta* were used in oil-contaminated soil bioremediation at hydrocarbons concentration of no more than 10 g per 1 kg of soil, and it was proved to be an efficient method. Taking into consideration the abovementioned, the present research is aimed to study oil-contaminated soil bioremediation with the help of *D. veneta* earthworms in the presence of bacteria such as *Pseudomonas*, nitrogen fixing bacteria *Azotobacter* and *Clostridium*, yeasts *Saccharomyces*, fungi *Aspergillus* and *Penicillium* as well as *Actinomycetales* and to evaluate the survivability of *D. veneta* earthworms in soil contaminated with various concentrations of oil (from 20 g/kg to 100 g/kg).

2. Material and methods

2.1. Earthworm species.

Earthworms *Dendrobena veneta*. They are usually found near human habitats, in gardens, vineyards, forests, and in high mountains. Worm mean mass was 0.9 - 1.42 g. Only the adult earthworms were used in the experiment. The adult earthworms were purchased from the Yermak farm (Saratov, Russia).

2.2. Microbiological preparation

Biopreparation Baykal- EM (NPO EM Center Ltd., Novosibirsk, Russia. License No. 226-19,156-1) served as a source of lactic acid and nitrogen fixing bacteria, and fungi. The microorganisms in biopreparation Baykal EM-1 were determined by MALDI-TOF (Matrix-assisted laser desorption ionization time-of-flight) mass spectrometry with the system BIOMERIEUX (FRANCE). Microorganisms composition of biopreparation Baykal EM-1 was as follows: bacteria *Paenibacillus pabuli*, *Azotobacter vinelandii*, *Lactobacillus casei*, *Clostridium limosum*, *Cronobacter sakazakii*, *Rhodotorulla mucilaginosa*, *Cryptococcus albidus*, yeast *Saccharomyces*, *Candida lipolitica*, *Candida norvegensis*, *Candida guilliermondii*, fungi *Aspergillus* and *Penicillium*, as well as *Actinomycetales* (KOE UFC=2*10¹¹ per mL).

2.3. Soil substrate

All the experiments were carried out with the soil substrate represented by a sterilized meadow soil with the brand name “Living Earth (Terra Vita) Universal Nutrient Ground” that was manufactured by MNPP FART Ltd. The soil had the following characteristics: organic substance content 46%, pH 5.9-6.0, base exchange capacity 28-40 mg-eq per 100 g soil. Chemical composition of the soil: nitrogen ($\text{NH}_4 + \text{NO}_3$) content 150 mg/l, phosphorus (P_2O_5) content 270 mg/l, and potassium (K_2O) content 300 mg/l. The composition of crude oil: paraffins and 2.3%, sulfur 0.96% for nitrogen and 0.12%, resin acid – 14%, resin silikagelya -10%, asphaltenes -1.36%, coking ability is 1.99%, ash content of -0.01%, naphthenic acids and 0.01%, phenols-0.006%. Elementary composition of oil: C-85.9%, H-12.93%, O-0.15%, S-0.92%, N-0.1%.

2.4. Chemical analyses (TPH concentration)

To analyze the petrochemicals and organic substance content in the samples, the collection of the soil samples was performed according to GOST of Russia 54039-2010 Soil quality. Quick method for the determination of oil products by NIR spectroscopy [15]. The content of crude or mineral oil in the soil was estimated by a technique developed at the Russian Institute of Experimental Metrology [MUK 4.1.1956-05]. The technique is based on measuring the amount of hydrocarbons extracted with carbon tetrachloride from oil-contaminated soil. The petroleum with the label AI-92 produced by company Lucoil was introduced in the amount of 20-60 g/kg into the soil samples of the 2nd series. The petroleum characteristics were in compliance with GOST of Russia 51105-97.

The diesel fuel with the label EURO GOST of Russia 52368-2005 (EH 590:2009) was introduced in the amount of 20-40 g/kg into the soil samples of the 3rd series.

3. Research results

3.1. Experiment 1. Laboratory earthworm petroleum toxicity test

Total population of *D. veneta*. Figure 1 showed an increase in the total population of the earthworms with 33 worms in the control variant and 95 worms in the variant *D. veneta* + microorganisms (biopreparation Baykal EM). The introduction of petroleum into the soil at concentrations of 20 (variant 3-6, Figure 1) resulted in 100% survival of *D. veneta* and a stable growth of the adult earthworms population both in the presence and in the absence of the microbiological preparation. As for variant 3 containing 20 g/kg of petroleum, the earthworms number increased to 112 worms, and as for the sample with the petroleum and the biopreparation it rose to 88 worms after a 22-week incubation. As the petroleum content in the soil was increased to 40 g/kg (variant 5), an increase in the number of earthworms was 126 after the incubation for 22 weeks. But introduction of the biopreparation Baykal EM resulted in the death of all worms in variant 6. An increase in the petroleum content in the soil to 60 g/kg (variant 7, Fig. 1) led to a decrease in the total number of *D. veneta*. The total population was 146 worms in variant 7 with the petroleum concentration of 60 g/kg, and with the microbiological preparation (variant 8) all earthworms died. Therefore, the presence of preparation Baykal EM enhances the petroleum adsorption in the earthworms digestive tract and causes an acute toxic effect.

Petroleum hydrocarbons decomposition. As figure 2 shows, in control variant contaminated with petroleum, the petrochemicals content was changing slightly within the entire incubation period. Light petroleum fractions evaporation was registered in the soil without the earthworms. As a result, petrochemicals concentration decreased by 70% after 22 weeks of the incubation and was equal to 6 g/kg in the control sample with petroleum concentration of 20 g/kg and with biopreparation -1.35 g/kg. Petrochemicals concentration decreased by 97% after 22 weeks of the incubation and was equal to 0.06 g/kg in the soil samples with petroleum concentration of 20 g/kg and the earthworms *D. veneta*, and in the presence of the microbiological preparation it was 0.02 g/kg. (figure 2a).

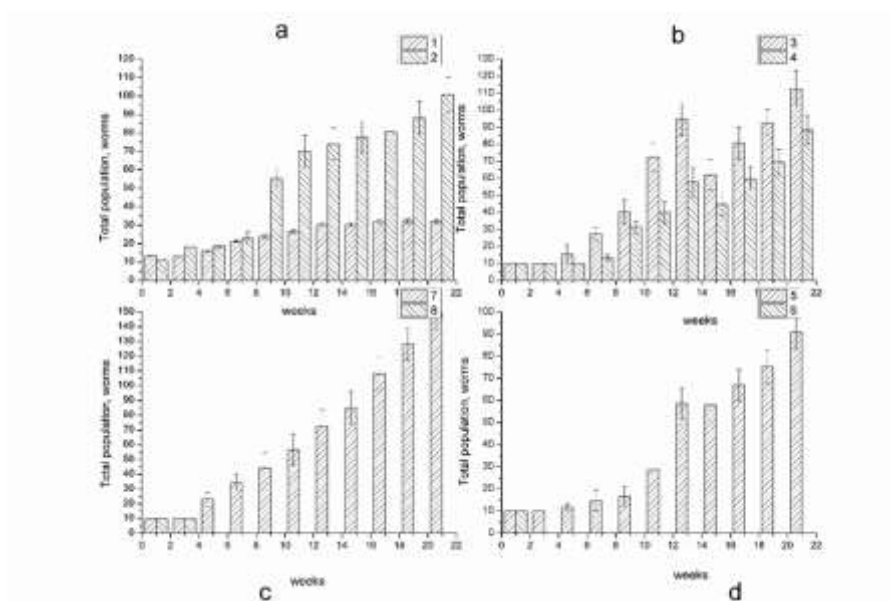


Figure 1. Total population of *D. veneta* with different petroleum concentrations ($p < 0.05$) including an absolute uncertainty value with 95% confidence. 1 – control variant *D. veneta* without petroleum, 2 – control variant *D. veneta* with Baykal EM-1, 3 – variant with 20 g petroleum kg⁻¹ and *D. veneta*, 4 – variant with Baykal EM-1 and 20 g petroleum kg⁻¹ and *D. veneta*, 5 – variant with 40 g petroleum kg⁻¹ and *E. andrei*, 6 – variant with Baykal EM-1 and 40 g petroleum kg⁻¹ and *D. veneta*, 7 – variant with 60 g petroleum kg⁻¹ and *D. veneta*, 8 – variant with Baykal EM-1 and 60 g petroleum kg⁻¹ and *D. veneta*.

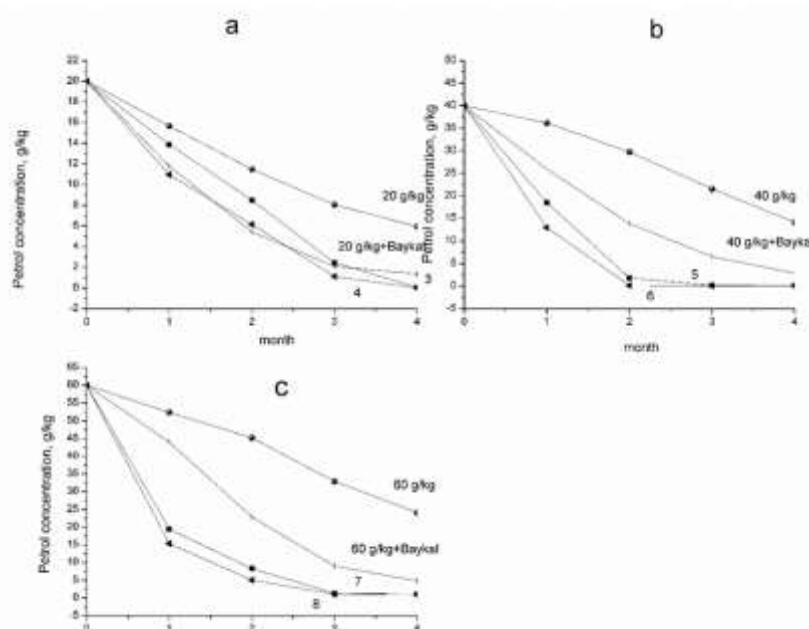


Figure 2. Changes in the concentration of petroleum hydrocarbons in the soil upon incubation of petroleum contaminated soil samples in the presence of earthworms *D. veneta* and biopreparation Baykal- EM in the laboratory experiment. 1 – control variant petroleum 20g/kg, 3 – variant with 20 g

petroleum kg-1 and *D. veneta*, 4 – variant with Baykal EM-1 and 20 g petroleum kg-1 and *D. veneta*, 5 – variant with 40 g petroleum kg-1 and *D. veneta*, 6 – variant with Baykal EM-1 and 40 g petroleum kg-1 and *D. veneta*, 8 – variant with Baykal EM-1 and 60 g petroleum kg-1 and *D. veneta*.

As for the control sample with 40 g/kg petroleum (Fig.2b), petroleum content decreased by 62% and was 15 g/kg after 22 weeks of the recultivation and with biopreparation -2.8 g/kg. The introduction of earthworm *D. veneta* resulted in the decrease of hydrocarbons concentration by 97 %, and it was equal to 0.16 g/kg and in the presence and in the absence of the microbiological preparation petroleum content decreased up to 0.09 g/kg. In the control sample, upon introduction of 60 g/kg petroleum, light fractions evaporation was 60%, resulting in the decrease of hydrocarbons concentration to 24 g/kg. Introduction of *D. veneta* earthworms led to the decrease of petroleum concentration to 1.7 g/kg (95% effectiveness), and after the microbiological preparation introduction it lowered to 0.9 g/kg (99% effectiveness).

3.2. Experiment 2. Laboratory earthworm diesel fuel toxicity test.

Total population of *D. veneta*. Figure 3 shows the changes in earthworm *D. veneta* amount in the diesel-contaminated soil samples with the concentration of 20-40 g/kg, after the soil samples having been incubated for 22 weeks at 15-17°C. As seen in Figure 3, the introduction of diesel fuel into the soil at concentration of 20 g/kg (variant 3,4 Fig. 3) resulted in 50% survival of *D. veneta* and stable growth of the earthworms' population. The earthworms' population after 22 weeks of incubation increased 3.5 fold and was equal to 36 worms for variant 3, and it was equal to 18 worms for the sample with diesel fuel (20 g/kg) and biopreparation Baykal EM. An increase in the diesel fuel content in the soil to 40 g/kg (variant 5) led to the death of all specimen of *D. veneta*.

Diesel fuel hydrocarbons decomposition. Figure 4 shows, that hydrocarbons content changed significantly in control variant contaminated with diesel fuel during the entire incubation time. Light petroleum fractions evaporation was registered in the soil without the earthworms and with diesel fuel concentration of 20 g/kg. As a result, petrochemicals concentration decreased by 77% after 22 weeks of incubation and was equal to 4.5 g/kg. Petrochemicals concentration decreased by 97% after 22 weeks of incubation and was equal to 0.7 g/kg in the variant with earthworms *D. veneta* and diesel fuel concentration of 20 g/kg. The introduction of biopreparation Baykal EM had no significant effect on soil bioremediation process. Petrochemicals content lowered to 1.01 g/kg and recultivation efficiency was 95%. As for the control variant with 40 g/kg diesel fuel, the hydrocarbons content decreased by 82% and was 7 g/kg. Soil bioremediation proceeds more intensely in the samples with the earthworms and diesel fuel concentration of 40 g/kg. With earthworms *D. veneta* introduction diesel fuel concentration lowered to 2.1 g/kg (95% effectiveness), and with the microbiological preparation the concentration decreased to 3.24 g/kg (92% effectiveness).

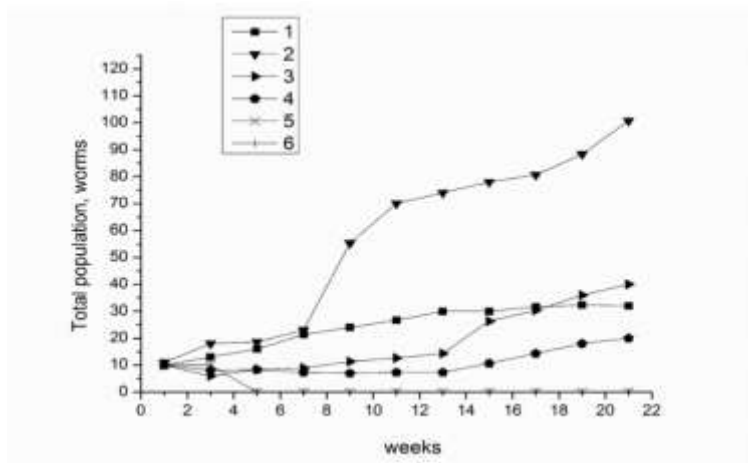


Figure 3. Total population of *D. veneta* with different diesel fuel concentrations ($p < 0.05$) including an absolute uncertainty value with 95% confidence. 1 – control variant *D. veneta* without diesel fuel, 2 – control variant *D. veneta* with Baykal EM-1, 3 – variant with 20 g diesel fuel kg-1 and *D. veneta*, 4 – variant with Baykal EM-1 and 20 g diesel fuel kg-1 and *D. veneta*, 5 – variant with 40 g diesel fuel kg-1 and *D. veneta*, 6 – variant with Baykal EM-1 and 40 g diesel fuel kg-1 and *D. veneta*.

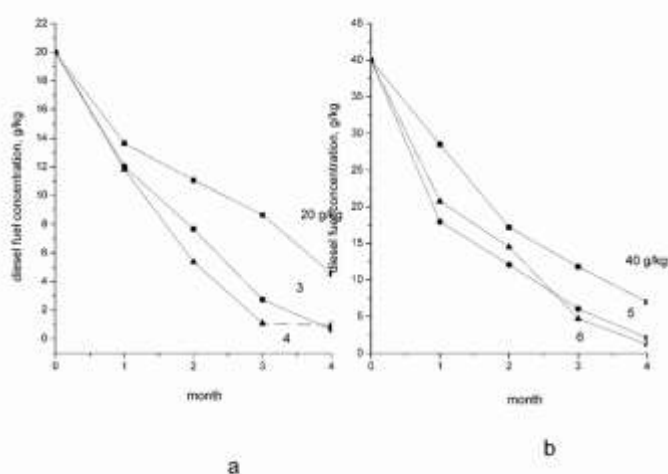


Figure 4. Changes in the concentration of diesel fuel hydrocarbons in the soil upon incubation of diesel fuel contaminated soil samples in the presence of earthworms *D. veneta* and biopreparation Baykal- EM in the laboratory experiment. 1 – control variant diesel fuel 20g/kg, 3 – variant with 20 g diesel fuel kg-1 and *D. veneta*, 4 – variant with Baykal EM-1 and 20 g diesel fuel kg-1 and *D. veneta*, 5 –variant with 40 g kg-1 diesel fuel and *D. veneta*, 6 – variant with Baykal EM-1 and 40 g diesel fuelkg-1 and *D. veneta*, 8 – variant with Baykal EM-1 and 60 g diesel fuelkg-1 and *D. veneta*.

4. Discussion of results

Petroleum hydrocarbons concentration was considerably lowered in the soil with the earthworms in contrast to the soils without the earthworms. The efficiency and the rate of the oil degradation depend on the petrol concentration. At introduction of low oil concentrations (20-40 g/kg), soil recultivation took 4 months, during which petrol concentration decreased by 97-99%. The addition of the microbiological preparation had no significant effect on the oil degradation process. At introduction of high petrol concentrations (60 g/kg), the oil degradation process took 5-6 months and called for compulsory addition of microbiological preparation. Light petroleum fractions evaporation was registered in the soil without the earthworms. As a result, petrochemicals concentration decreased by 60-70% in 5 months. In all versions recultivation process took 3 months, and petrol concentration decreased by 98-99%. Therefore, one may conclude that oil (at low concentrations) is not a toxic substance for the earthworms and it simulates the population growth of *D. veneta*.

Diesel fuel has more acute toxic effect on *D. veneta*. The introduction of diesel fuel at concentration of 20 g/kg led to the death of 50% earthworms after 14 days of the experiment. The addition of microbiological preparation intensified the toxic effect of the diesel fuel. Diesel fuel concentration of 40 g/kg had an acute toxic effect and caused the death of 100 % species after 14 days. Microbiological preparation increases the toxic effect of diesel fuel and resulted in 100% death after 14 days. Isabella Gandolfi and Matteo Sicolo noticed vast toxic effect of diesel light hydrocarbons on the earthworms, but the compost presence enhanced HC degradation. Compost introduction to diesel-contaminated soil resulted in diesel fuel remediation increase by four rings of PAHs and soil toxicity decrease, its effect on soil genotoxicity being relatively low [17].

We carried out statistical analysis of the obtained results. Verification of normality of distribution of quantitative traits was performed using the Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilk (W). We are using normality tests (Lilliefors and Shapiro-Wilk tests) the resulting value was less ($p < 0.05$), therefore, an alternative hypothesis was formulated, i.e. the characteristic distribution differs from the normal one. When evaluating the results by the significance with Kruskal-Wallis criterion in several independent groups at soil contamination of 20 g/kg and 40 g/kg with and without the biopreparation, no statistically significant differences were found. In evaluating the results of significance of differences criterion Kruskal -Wallis several independent groups of soil contamination with petrol 20-60 g/kg in introducing microbial drug, and without him was not statistically significant differences (Kruskal-Wallis (N) (7, N= 80) =11.446 $p=0.1203$) (median test = 62.00; Chi-square = 6.400 df = 7, $p=0.4939$).

5. Conclusions

The method of oil contaminated soil bioremediation with petrol concentrations up to 60g/kg and diesel fuel concentrations up to 40g/kg, in the presence of bacteria *Paenibacillus pabuli*, *Azotobacter vinelandii*, *Lactobacillus casei*, *Clostridium limosum*, *Cronobacter sakazakii*, *Rhodotorulla mucilaginosa*, *Cryptococcus albidus*, yeast *Saccharomyces*, *Candida lipolitica*, *Candida norvegensis*, *Candida guilliermondii*, fungi *Aspergillus* and *Penicillium* as well as Actinomycetales (KOE UFC)= 2×10^{11} per mL) and *D. veneta* is proposed. Decontamination and the ecological functions recovery of the oil-contaminated substrates is carried out due to the following method: the substrate is treated with biopreparation, ploughed and put under the steam for 1 month for recultivation; afterwards, the earthworms are added in the amount of 1000 per 1 m² of the soil; and then, cow dung is added as a nutritional medium in the amount of 1 t per 1 ha. During the experiment lasting for 5 months, a significant lowering (by 95-97%) in hydrocarbons content was registered in the soil with the earthworms and the biopreparation.

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