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Oil-Contaminated Soil Bioremediation with *Eisenia fetida* in the Presence of Lactic Acid and Nitrogen-fixing Bacteria in Laboratory Experiments

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In this study, the survival, reproductive potential of earthworm species *Eisenia fetida* in oil-contaminated soil and the effect of earthworms in combination with a biopreparation Baykal EM-1 (Reg. N 226-19-156-1) on the process of total petroleum hydrocarbons degradation have been investigated. In particular, the earthworms were incubated in crude oil-polluted soil containing 20,000 to 100,000 mg/kg of total petroleum hydrocarbons or a reference soil for 22 weeks. The laboratory studies demonstrated high level of the *E. fetida* survival in the oil-contaminated samples to which the Baykal EM-1 biopreparation was added. The oil content decreased more intensely (by 95–97%) in the soil samples containing earthworms as compared to the samples without them. The decomposition efficiency and the rate of oil hydrocarbons removal depended on the oil content and the presence or absence of the Baykal EM-1 biopreparation. The highest value (97–99%) was registered in the sample with the oil concentration of 40,000 mg/kg. The reproductive characteristics of the earthworms were shown to improve in the presence of Baykal EM-1. The obtained results are statistically important and demonstrate that microorganisms occurring in the Baykal EM-1 biopreparation have a positive effect on the earthworms' survival, their reproductive potential, and capacity of hydrocarbon degradation.

*Key words***:** bioremediation, earthworms, oil, biopreparation.

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Oil spills often accompany oil recovery and transportation. Every year, 20,000–23,000 of oil spills with petrochemicals concentrations of up to 50,000–100,000 mg/kg are registered in Russia (https://www.rbc.ru/economics/10/04/2012/5703f5c09a7947ac81a66c05). They suppress the vital activity of the soil biota and cause irreversible changes in the soil microbiological properties affecting air and water conditions in it. Therefore, the bioremediation of oil-contaminated soil is becoming increasingly important. By now, various methods for soil bioremediation have been developed and widely used; these methods differ in their efficiency and labor intensity. For example, technical bioremediation involves the removal of a contaminated soil layer, its transportation and storage at special dump sites [1]. Certainly, it is only advisable for small oil spills in limited areas with the depth of oil penetration not greater than 10 cm.

Hydrocarbons combustion is another widely spread method for the elimination of oil pollution [1]. This method, however, cannot ensure a complete oil removal; in addition, it exerts pronounced

Abbreviations: H, Kruskal–Wallis test; GC/FID, Gas Chromatography – Flame Ionization Detector analysis; ISO, International Standardization Organization; K-St, Kolmogorov–Smirnov test; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; TPH, total petroleum hydrocarbons; U, Mann–Whitney test; W , Shapiro–Wilk test.

detrimental effects on the environment: it destroys soil ecosystems, kills plants and leads to the accumulation of toxic and carcinogenic substances.

Peat, sapropel, sand, and polymeric materials are some of natural or synthetic sorbents that can be used to control oil spreading and remove oil spills [2]. Washing, leaching, extraction, and other physicochemical methods of soil treatment and contaminants removal are known [3]. Oil contaminants are commonly removed with water, acetone, ethyl acetate, liquid hexane, and CO_2. The removal rate can be increased by the ultrasonic or microwave treatment. However, all the listed methods are not able to restore biological balance in the treated soil.

The use of organisms for which the hydrocarbon utilization is a part of their vital activity is a more promising method for the oil-contaminated soil bioremediation [4]. The process permits to convert crude or mineral oil carbon into carbon dioxide that further proceeds into cell biomass, transforms into humus and gets fixed in soil. This method is efficient at the mineral oil concentration below 20 mg/kg soil [5, 6]. At higher hydrocarbon content, the number of bacteria decreases and soil bioremediation fails to occur.

Recent studies have shown that so-called *vermiremediation* results in a higher efficiency of soil biorestoring [7]. This method implies for the introduction of earthworms into contaminated soil. The Organization for Economic Cooperation and Development (OECD) has proposed *Eisenia fetida* as the reference earthworm species to be used in toxicity tests because they are easily cultivated under laboratory conditions, become mature in 8 weeks and have a high reproduction rate. Laboratory studies have demonstrated that *E. fetida* facilitate the removal of such contaminants as pesticides, polychlorinated biphenyls, PAHs and crude and mineral oil hydrocarbons from soil [8–10].

The effect of three earthworm species (*Lumbricus terrestris, Allolobophora chlorotica* and *E. fetida*) on the microbial community of the oil-contaminated soil (10,000 mg/kg) during 28 days was investigated [11]. The microbial biomass concentration increased during the experiment, and taxonomic groups of the microorganisms changed. The hydrocarbons concentration decreased by 30–42% in the samples with *L. terrestris*, by 31–37% with *E. fetida*, and by 17–18% with *A. chlorotica* (*vs*. 9–17% in the control samples) [11].

As the crude oil content increased up to 15,000 mg/kg, the survival of *E. fetida* was reduced by 40% after 15 days, whereas the population of *L. terrestris* remained unchanged. Other authors observed a high mortality of *E. fetida*, *A. chlorotica* and *L. terrestris* cultivated for 28 days in the soil without bovine manure at 9,500 mg crude oil per kg [12].

It was also reported [13] that *E. fetida* are resistant to the oil contamination of not higher than 50,000–80,000 mg/kg. Therewith, it was demonstrated that light are more toxic to earthworms than medium and heavy oil fractions [13].

Some authors also noted the positive effect of earthworms on the removal of such contaminants as oil, PAHs and PCBs [10, 12, 14–16]. During the burrowing process, earthworms mix soil in their intestines, thus changing its physical and chemical properties. [17]. The earthworms increase the number of contacts between contaminants and soil microorganisms and facilitate the contaminants removal [18, 19].

If introduced in soil with earthworms, some microorganisms can decompose PAHs [20–22]. And the complete removal of the latter can be achieved by the introduction of *E. fetida, L. rubellus,* and the above microorganisms into the contaminated soil [23, 24]. PAHs decomposition rate, however, depends on both structure of polycyclic aromatic hydrocarbons and type of microbial soil communities.

It was reported that the survival of *E. fetida* cultivated for 56 days in the soil contaminated with a mixture of PAHs (2 to 6 rings) at a concentration of 773 mg/kg reached 97%; however, no cocoons were found [25]. The experiments carried out at high oil concentrations (45,000–80,000 mg/kg) showed that the toxicity of the oil-contaminated soil decreased during 10 months. The fall already started after 90 days; and after 240 days there was no acute toxicity in the soil primarily contaminated with medium and heavy hydrocarbons. Light fractions of mineral oil manifested much higher toxicity than medium and heavy oils [13].

The rate of total petroleum hydrocarbons degradation was enhanced by the occurrence of earthworms and wastewater sludge [26].

Thus, the analysis of the reported data shows that the use of *E. fetida* for the oil-contaminated soil bioremediation is an efficient method for the decontamination at the hydrocarbon concentration inferior to 4000 mg/kg. It provides a high level of earthworms' survival in oil-contaminated soil; therewith, the hydrocarbons concentration decreases to an acceptable level in the soil owing to the *E. fetida* activity. Another factor that enhances the bioremediation efficiency of oil-contaminated soil is the presence of various microorganisms that also improve the earthworms' survival.

The present investigation was aimed at the selection of a microbial consortium improving *E. fetida* survival in a substrate with oil concentrations up

to 100,000 mg/kg and supporting the oil-contaminated soil bioremediation. To this end, the survival and efficiency of *E. fetida* 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)* and the representatives of the family of *Actinomycetales* (total preparation CFU = $2 \cdot 10^{11}$ per mL) was investigated. Another aim was to develop a method for the oil-contaminated soil bioremediation by microorganisms and *E. fetida* in a climate of Western Siberia at the temperature of 15 °C.

EXPERIMENTAL

Soil substrate

All the experiments were performed with a sterilized meadow soil (brand name of Living Earth (Terra Vita), Universal Nutrient Ground) manufactured by MNPP FART Ltd. (St. Petersburg, Russia) as a substrate. The soil had the following characteristics: humus content 46%, pH 5.9–6.0, and adsorptive capacity 28–40 mg-equ/100 g soil. Chemical composition of the soil was as follows, mg/kg: nitrogen (NH_4 + NO_3), 150; phosphorus (P_2O_5), 270; potassium (K_2O), 300; and 6.9% (by carbon) of organic matter (determined by the Tyurin method). The latter was assessed according to GOST 26213-91 («Soil. Methods for determination of organic matter»).

The substrate was prepared in advance in compliance with ISO (ISO 11268-1, 1993; ISO 11268-2, 1998). The soil was dried up to a constant weight, sieved using 5 mm mesh filter, and homogenized manually; this was followed by the addition of calcium carbonate (2.5 g/kg; Ecros, Russia) to bring pH to 6.0±0.5 and distilled water to obtain a soil moisture content of 60%. The prepared soil (1 kg) was placed in 2-L polypropylene containers (disposable tableware). In the course of the experiments, water losses due to evaporation were monitored once a week, and the soil was humidified to adjust its moisture content to 60%.

A series of oil-contaminated soil samples was prepared in the following way: oil was added to the soil up to the concentration of 20,000–100,000 mg/kg. The oil was obtained from the Samotlor (Russia) oil field, and had the following characteristics: relative density (ρ), 0.934; M = 367; V_{20} = 63.13; pour point, 25 °C; temperature of processing, 22 °C; and flash point in closed crucible, 120 °C. The composition of the crude oil was as follows, %: paraffins, 2.3; sulfur, 0.96; nitrogen, 0.12; sulfurous resin, 14; silica resin, 10; asphaltenes, 1.36; coking ability, 1.99; ash, 0.01; naphthenic acids, 0.01; and phenols, 0.006. The elemental composition was as follows, %: C, 85.9; H, 12.93; O, 0.15; S, 0.92; and N, 0.1.

Earthworms

Oil contaminated soil vermiremediation was carried out with *Eisenia fetida* earthworms. As was said, a compost worm *Eisenia fetida* (Savigny, 1826) is a standard organism for the earthworm toxicity test (ISO guideline 11268). *E. fetida* are epigeic species found mainly in compost heaps and litter [27]. Their maximum activity is observed at 20–25 °C which is manifested in the increasing number of offsprings [28].

Only adult earthworms were used in the experiment; they were received from the farm Yermak (Saratov, Russia) and had the average weight 0.41–0.92 g.

Identification of Baykal EM-1 preparation composition. The biopreparation of Baykal EM-1 (EM Center Ltd., Novosibirsk, Russia. License No. 226-19, 156-1) served as a source of lactic acid, nitrogen-fixing bacteria, yeast and fungi. A biopreparation in the amount of 1.5–2 mL was taken from the vessel and mixed at 15,000 rpm for 20 min. After that, the matter was suspended in 1 mL of distilled water and the suspension was mixed at 5000 rpm for 10 min and sedimented. The supernatant was removed, and the residual matter was resuspended and centrifuged once more. Next, the chalky residue was collected, dissolved in 1 mL of distilled water and centrifuged at the maximum speed (24088 g) for 10 min. The pellet was resuspended in 1 mL of 80% ethanol and centrifuged at 24088 g for 10 min. The newly formed residue was dissolved in a mixture of 15 μL of deionized water and 35 μL of formic acid. After the addition of 50 μL of acetonitrile, the sample was centrifuged at the maximum speed for 2 min. The resulting supernatant was applied to a chip and coated with 1 μL of a matrix consisting of alpha-cyano-4-hydroxycinnamic acid in the form of saturated solutions in the mixture of 50% acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonics, Germany). The mass-spectrometric identification of the microorganisms was performed on a mass spectrometer MicroFlex (VITEK MS, Biomerieux, France). Each sample was tested 4 times. The spectra were registered automatically; the detection mode was standard MBT_FC. The spectra range started from 2–20 kDa. 240 spectra were obtained from each sample. The identification was performed using a database of Biotyper 3 (Bruker, Germany). The identification precision was almost 100% [29].

Using MALDI-TOF mass spectrometry (VITEK MS), the microorganisms in biopreparation Baykal EM-1 were identified as: bacteria *Paenibacillus pabuli, Azotobacter vinelandii*, *Lactobacillus casei, Clostridium limosum, Cronobacter sakazakii, Rhodotorulla mucilaginosa, Cryptococcus albidus;* yeast *Saccharomyces*, *Candida lipolitica, Candida norvegensis, Candida guilliermondii;* and fungi *Aspergillus* and *Penicillium,* as well as *Actinomycetales* (total CFU = $2 \cdot 10^{11}$per mL).

Laboratory earthworm toxicity test was conducted according to the ISO 1998 guideline. Sterile soil (1 kg) was placed into the 2-L plastic containers. 10 adult earthworms were added to each container. During the entire experiment, the soil was humidified once a week by introducing 100 mL of distilled water into each container. The containers with the soil were closed by plastic covers with small-size air holes and then sealed to prevent excessive moisture loss and earthworm escape. The earthworms were fed with 5 g of fresh grated potato once a week each. Regardless that *E. fetida* exhibit the maximum activity at 20–25 °C, the earthworms were incubated at 15±1 °C because this temperatures are more typical for Siberia. The control soil samples and the experimental samples of contaminated soil with the introduced earthworms and biopreparation Baykal EM-1 were incubated for 22 weeks, from September 2013 to February 2014.

Model test on vermiremediation with *E. fetida* species earthworms. Adult earthworms (with visible clitellum) ranging from 400 to 900 mg were used in all the experiments. To measure the body weight, earthworms were sorted from the test soil by hand, washed with tap water, dried on absorbent paper, and then weighed in groups of ten on an electronic balance. The numbers of cocoons, adult and juvenile representatives were measured once 2 weeks [30].

A series of oil-contaminated soil samples was prepared. To this purpose, the oil from the Samotlor (Russia) oil field was added to each experimental soil sample at a concentration of 20,000–100,000 mg/kg. Ten adult earthworms were added into each container with contaminated soil, and some containers were supplemented with biopreparation Baykal EM-1 in the amount of 1 mg/kg (1 mL). Along with the contaminated soil samples, control samples were prepared in each series of the experiments; they were free of the oil contaminant but contained the earthworms and biopreparation Baykal EM-1 (total CFU = $2 \cdot 10^{11}$ per mL). The compositions of all the samples used in the study are listed in Table 1. Three experiments were performed simultaneously with each sample described in Table 1.

Table 1

Composition of control and oil-contaminated soil samples to which *E. fetida* earthworms were added (10 species per container)

Sample No.	Oil content, g/kg	Presence of biopreparation Baykal EM-1 (1 mL)
1	–	–
2	–	+
3	20	–
4	20	+
5	40	–
6	40	+
7	60	–
8	60	+
9	80	–
10	80	+
11	100	–
12	100	+

To estimate the concentration of oil and organic substances in the soil, the latter was sampled according to GOSTs 28168, 17.4.3.01, and 17.4.4.02. The soil was ground in a mortar, a 3–5-g sample was additionally milled to obtain the particle size inferior to 0.3 mm and then sieved using 0.25 mm screen. After that, the TPH content was assessed.

Analyses of total petroleum hydrocarbons concentration

IR spectroscopy of TPH. 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) that is based on measuring the amount of hydrocarbons extracted with carbon tetrachloride from the oil-contaminated soil.

The analyses were performed at room temperature. In order to wet Al_2O_3, 3 mL of carbon tetrachloride was poured into a glass column (1 cm in diameter) filled with alumina (a 5-cm layer). As soon as CCl_4 was absorbed, a soil sample was added to the column. The soil was covered with 3–5 mm thick cotton wool, and carbon tetrachloride was poured in. The eluate of oil-contaminated soil dripped at the rate of 0.1–0.2 mL/min into a graduated cylinder mounted under the column. The first 3 mL of the eluate were discarded, and CCl_4 was poured into the column to obtain 10 mL of the eluate. The resulting eluate was placed into the cell of an IKH-025 IR spectrophotometer (IKN-025) to determine the amount of mineral oil in the eluate at the wavelength of 3.42 μ.

The mineral oil concentration in a sample (X, g/kg) was calculated by the formula:

$$X = CV\eta/m \cdot 1000,$$

where C is concentration of mineral oil in a sample according to the instrument readings, mg/L; V is eluate volume, mL; m is soil sample mass, g; and η is dilution ratio ($\eta = V_2/V_1$, where V_1 is the eluate volume, mL, and V_2 is the volume of CCl_4 taken for the dilution, mL).

Gas chromatography and mass spectrometry of TPH. The total hydrocarbon concentration was determined by GC/FID analysis. Ten ml of a hexane–acetone mixture (1:1, v/v) was added to 5 g of the test substrate. This mixture was shaken overnight at room temperature and then filtered (<0.45 mm) through a Teflon membrane (Agilent Technologies, USA) [12]. The clear filtrate (1 mL) was used to determine the TPH concentration by GC using mass-spectrometer 6890/5973N (Agilent Technologies) (column diameter of 0.25 mm, length of 30 m, thickness of stationary phase layer of 0.25 μ) equipped with a non-polar column (30 m × 0.25 mm (internal diameter) × 0.25 mm HP5) and FID. Helium (He) was a carrier gas. The initial column temperature was maintained at 70 °C for 2 min, and then raised in portions of 8 °C up to the final temperature of 230 °C. The total run time was 40 min. The analyses were calibrated with an alkane standard (C_{14}–C_{24}, C_{30}, C_{32} and C_{36}) with a medium fraction of $C_{18–20}$. The total area of the alkane peak was collected and used for the TPH concentration quantification. The range of the analyzed compounds included normal alkanes from C_{10} to C_{36}. To interpret the results obtained by the GC/MS, we used a standard library of mass spectra NIST MS Search 05 (>750,000 mass spectra of individual compounds) with structural formulas.

Statistical analysis. All quantitative estimations of toxicity parameters were based on three experimental repetitions, and the values were given as arithmetic average ± standard error. The software package of STATISTICA 10 was used for the analysis of the material. Normality testing for quantitative characteristics distribution was carried out with the help of the K-S, Lilliefors and W tests. The information was additionally processed by the methods of descriptive statistics. To evaluate the variance significance, the H and U tests for independent groups were used. The relations were evaluated by the correlation analysis with the calculation of the Spearman's rank correlation coefficient (r_s). The data on the earthworms' number in the contaminated soil with various oil concentrations were subjected to the H test, median one-way analysis, Van der Waerden, Siegel-Tukey, Ansari-Bradley one-way analyses, and Spearman correlation analysis.

RESULTS AND DISCUSSION

Laboratory earthworm toxicity test

The changes in earthworms number after soil contamination with oil

Total population of *E. fetida*. Fig. 1 illustrates the changes in the number of *E. fetida* in the oil samples contaminated with crude oil at the concentration of 20,000–100,000 mg/kg upon the incubation for 22 weeks at 15–17 °C.

As seen from Fig. 1, after the incubation for 22 weeks, control samples *1* and *2* showed an increase in the total number of earthworms: the number of adults reached 60 for sample *1* and 200 for sample *2*. It should be noted that in the first 10–12 weeks, an increase in the earthworms' number in sample *1* was similar with that in sample *2* and equal to 25–30. It can be seen from Fig. 1 that the addition of biopreparation Baykal EM-1 to the soil (sample *2*) accelerated the growth of the earthworms' number in comparison with the sample that was free of the microorganisms constituting the biopreparation.

The introduction of crude oil into the soil at the concentration of 20,000–40,000 mg/kg (samples *3–6*, Fig. 1) resulted in 100% survival of *E. fetida* and a stable growth in the adult earthworms' population either in the presence (samples *4* and *6*) or absence (samples *3* and *5*) of the microbial preparation. However, it should be noted that for samples *1* and *3* (without Baykal EM-1 biopreparation), an increase in the earthworms' amount was less pronounced than for samples containing this biopreparation (*2* and *4*) after the 22-week incubation: 275 for sample *3* and 310 for sample *4*. The difference was 25–30, which is higher than between the control samples, *1* and *2*.

As the oil content increased up to 40,000 mg/kg (samples *5* and *6*), the gain in the number of adults decreased in comparison with samples *3* and *4* making up 220–230 after the 22-week incubation.

An increase in the oil content in the samples without the biopreparation (samples *7*, *9* and *11*) led to the 100% death of earthworms in 3 days, which may be caused by the chemical burns of the earthworms residing on the surface of the contaminated substrate. Therefore, the corresponding data are not represented in figures.

However, when biopreparation Baykal EM-1 was added in the amount of 1 mL to the soil containing oil in the concentration of 60,000–100,000 mg/kg,

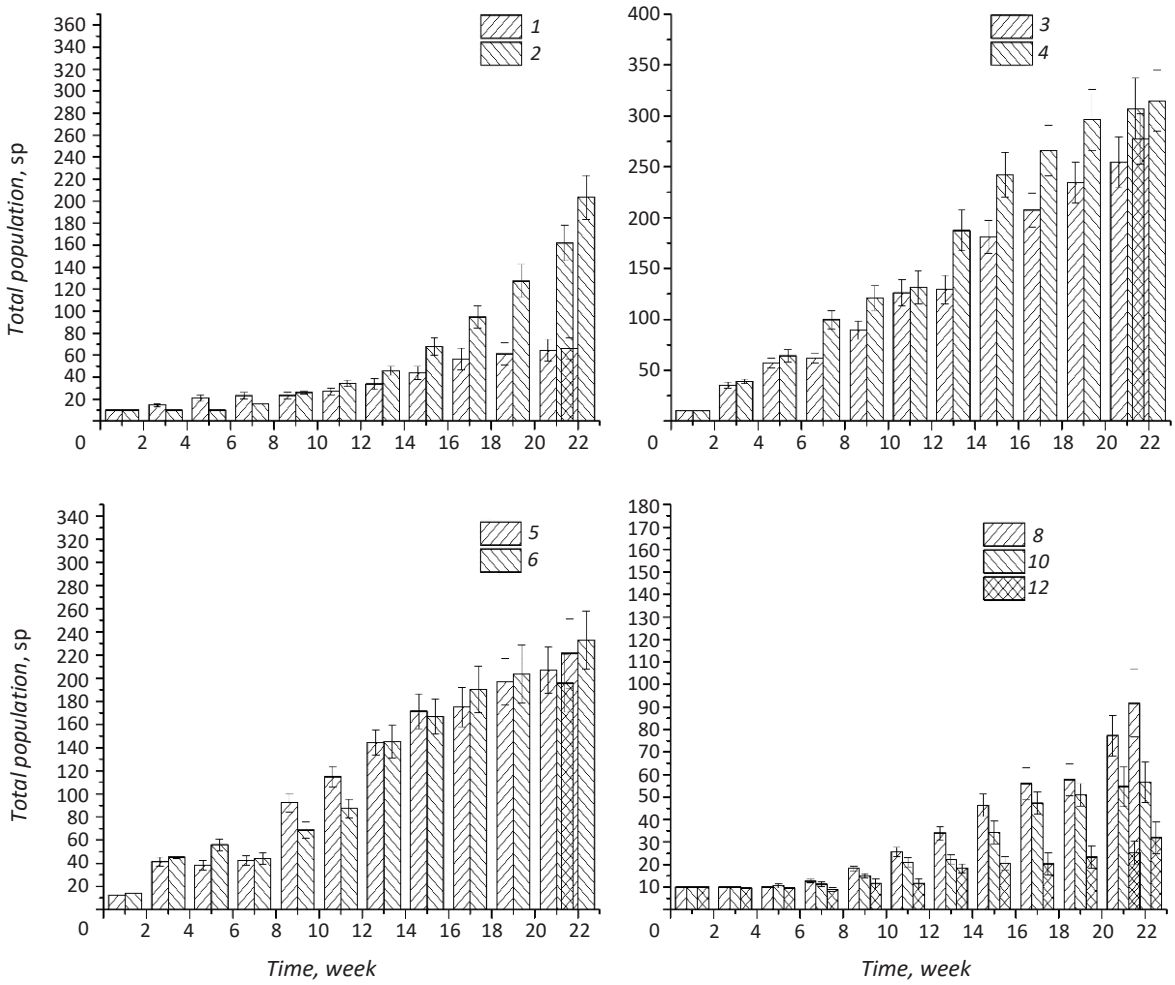

Fig. 1 Changes in the total number of *E. fetida* during incubation of soil samples. (*1*)–(*12*) are in consent with soil sample numbers in Table 1

the earthworms' survival increased (Fig. 1, samples *8*, *10* and *12*). During 4 weeks, the number of *E. fetida* adults remained unchanged. When the incubation period extended from 7 to 22 weeks, the earthworms' number grew in the soil samples. According to Fig. 1, the increase in the oil content reduced the earthworms' activity, and after the 22-week incubation their number was 91, 56 and 32 earthworms in soil samples *8*, *10* and *12*, respectively.

Whitfield [31] revealed the reproductive function disorders in *E. fetida* at the oil content of 0.5 to 25.0 g/kg soil, although their mortality did not exceed 10% [31]. In [16, 32], the *E. fetida* survival at the oil amount of 2.6–2.8 g/kg soil was studied: it was equal to 18% after 28 days and 8% after 56 days in the oil-contaminated soil. Rodriguez-Campos et al. [7] reported a high mortality of *E. fetida* after 28 days of incubation in soil containing 9.5 g crude oil per kg. Moreover, manure stimulated the soil microorganisms' activity, accelerated the oil contaminants removal and increased the earthworms' survival [7]. Our findings support the data reported in [7] and demonstrate that the addition of a microbial matter enhances the *E. fetida* survival in the oil-contaminated substrate [7]. Owing to the consortium of microorganisms, *E. fetida* survival was 70% at the oil content of 80–100 g/kg after 22 weeks (Table 2).This differs significantly from the previous research results.

Total cocoons. Fig. 2 shows changes in the total number of cocoons in the oil-contaminated soil samples at the concentration of 20,000–100,000 mg/kg over the entire time of incubation, i.e. 22 weeks. The highest total number of cocoons (35 on average) was observed for sample *4* containing oil in an amount of 20,000 mg/kg, earthworms and no additional preparations. For sample *3*, the total number of cocoons was lower, about 25. At the increased oil content of 40,000 mg/kg soil (sample *5*), the total number of cocoons decreased up to 15 per sample, while the introduction of biopreparation Baykal EM-1 (sample *6*) enhanced the total number of cocoons up to 22. The further increase in the crude oil

Table 2

***E. fetida* earthworm populations in control and oil-contaminated soil samples after 22-week incubation**

Sample No. (identical with Table 1)	Total number of earthworms per container*	Survival of earthworms, %	Total number of cocoons per container** (mean value)	Individual productivity*** (mean value)
1	66	100	4	5
2	200	100	7	7
3	275	100	25	2
4	310	100	35	3
5	220	100	15	1
6	230	100	22	2
7	0	0	0	0
8	91	90	17	2
9	0	0	0	0
10	56	80	13	1
11	0	0	0	0
12	32	70	5	1

*Manually counted; includes the number of cocoons, young and mature earthworms.
**Manually counted.
***Amount of cocoons per 1 mature earthworm.

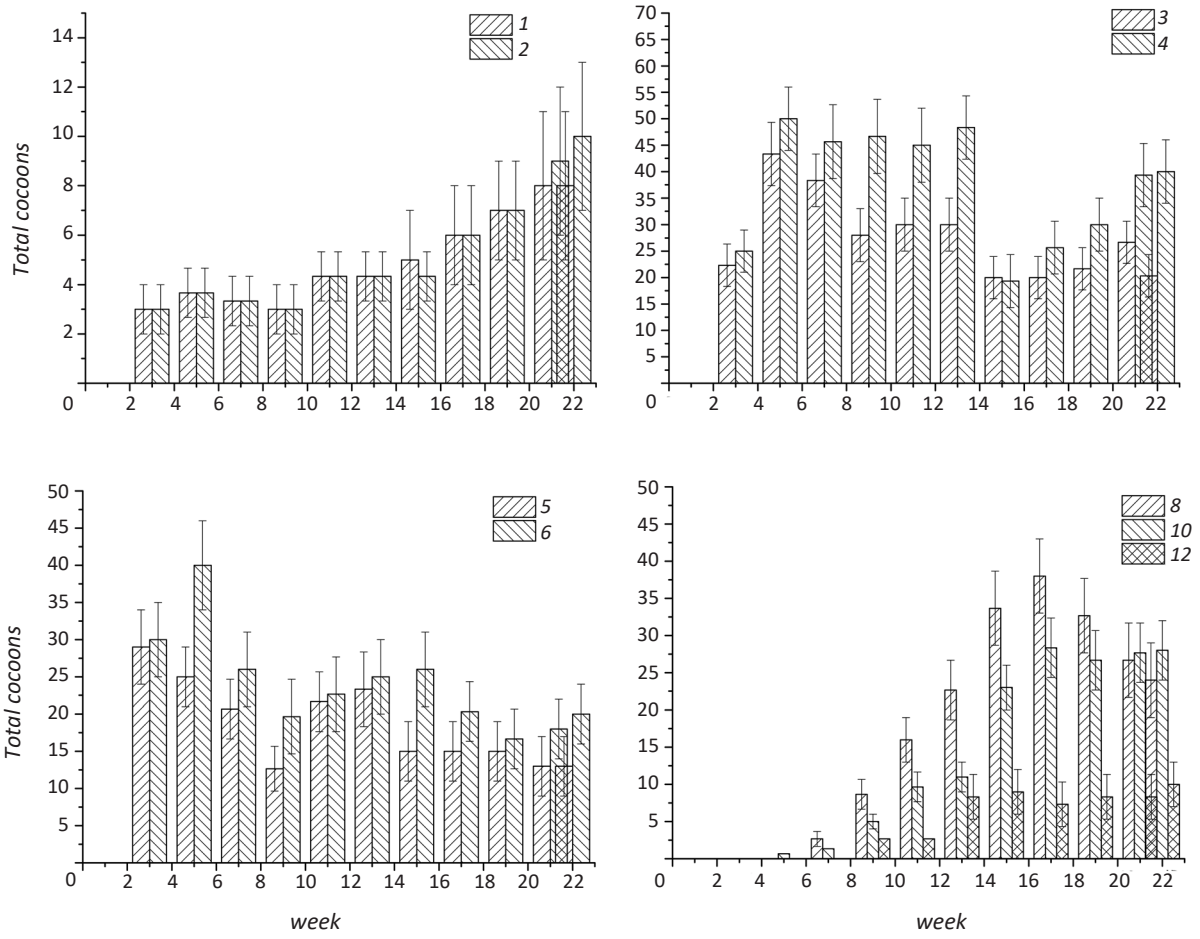

Fig. 2. Changes in total cocoon number of *E. fetida* during their incubation in oil-contaminated soil samples: (*1*)–(*12*) are in consent with soil sample numbers in Table 1

content up to 60,000, 80,000 and 100,000 mg/kg (samples *7*, *9*, and *11*, not represented in figures) caused the death of all the earthworms and the laying of cocoons was not observed. After adding crude oil in amounts of 60,000, 80,000 and 100,000 mg/kg with biopreparation Baykal EM-1(samples *8*, *10*, and *12*), the total number of cocoons amounted to 13, 17, and 5, respectively. The data on the total and individual productivity of earthworms during the 22-week incubation are listed in Table 2.

The individual productivity of earthworms (the number of cocoons per earthworm) was up to 8 for sample *1* and up to 10 for sample *2* after the 22-week incubation. The addition of oil in amounts 20,000–40,000 mg/kg soil led to a sharp growth in the individual productivity: the number of cocoons per earthworm in samples *3* and *4* was 2 and 3, respectively. Similar to the control samples, the introduction of Baykal EM-1 increased the total productivity to 3 cocoons in the soil samples with 20,000 and 40,000 mg crude oil per kg (samples *3, 4, 5* and *6*). A further increase in the oil content up to 60,000–100,000 mg/kg reduced the total productivity of earthworms up to 1 cocoon per earthworm (samples *8*, *10* and *12*) (Table 2).

Thus, the obtained results testify that concentrations of 20,000 and 40,000 mg oil per kg of soil exert a stimulating effect on the earthworms' productivity. Similar results were reported in [33], where it was demonstrated that low concentrations of toxic substances in soil increase the productivity of earthworms. This may relate to the self-preservative instinct of this biological subject under unfavorable conditions, implying an enhancement of the reproductive function to create resources by the time of death. An increase in the toxic substance concentration reduced the productivity and total population.

Earthworms can survive at high concentrations of contaminants; for instance, *E. fetida* survived in soil contaminated with 3,500 mg TPH per kg [34]. The introduction of the microbial preparation of Baykal EM-1 increased the cocoon laying of *E. fetida* per container at 60,000–100,000 mg oil/kg, and this evidence differs significantly from the previously published results.

Weight of *E. fetida*. The earthworm mass increased (K-St, $p<0.05$) after the introduction of biopreparation Baykal EM-1 (**Supplementary Information, Fig. S1**). The obtained results support the data of [23].

TPH degradation. The dynamics of the oil content in the contaminated soil samples containing earthworms *E. fetida* and biopreparation Baykal EM-1 at 15–17 °C was investigated. The maximum incubation time for all the samples was 22 weeks. The hydrocarbons content was found by IR spectroscopy on a monthly basis. The accelerated degradation of great oil amounts (up to 100,000 mg/kg) was observed in the occurrence of earthworms. The presence of the Baykal preparation permitted to intensify the loss of TPH, whereas the combined addition of Baykal and earthworms made this degradation even more pronounced (**Supplementary Information, Fig. 2S**).

Fig. 3 demonstrates the results of GC/MS for the soil contaminated by oil, untreated or subjected to the TPH degradation by the Baykal preparation or combination of Baykal and eartworms. It is seen that the addition of the biopreparation led to the decrease in the oil concentration by 60% after 22-week incubation, whereas in the soil with the biopreparation and *E. fetida*, the TPH concentration lowered by 98% after 22 weeks of incubation. The obtained results are in agreement with those in [12].

The laboratory study showed that the TPH concentration decreased more intensely in the soil samples containing the earthworms as compared to the samples without them. The decomposition efficiency and the TPH concentration removal rate depended on the oil content and the presence of biopreparation Baykal EM-1 in the soil. As a result of the addition of oil in the amount of 20,000–400,000 mg/kg, the bioremediation took 22 weeks and the hydrocarbons concentration decreased by 97%. In this case, the addition of biopreparation Baykal EM-1 had no significant effect on the oil hydrocarbons degradation (the effectiveness was deduced on the basis of the Fig. S2 data).

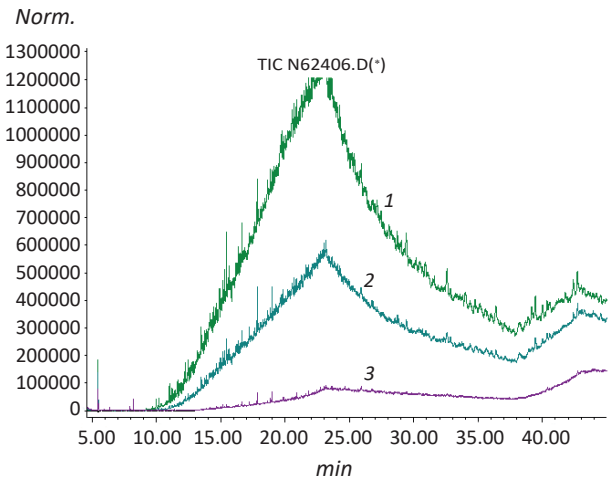

Fig. 3. GC/MS profiles of oil-contaminated soil (100,000 mg/kg) after various treatments: without treatment (curve *1*); treated with biopreparation Baykal EM-1 (curve *2*), and treated with biopreparation Baykal EM-1 + earthworms of *E. fetida* (curve *3*)

With 60,000–100,000 mg oil per kg, the TPH decomposition and the soil bioremediation took 22 weeks and occurred only in the presence of Baykal EM-1. Therewith, the TPH concentration decreased by 94% after the 22-week incubation (the effectiveness was deduced on the basis of the **Fig. S2** data).

The data obtained on the survival of *E. fetida* in the oil-contaminated soil disagree with the data reported in [7, 11, 12, 35]. This may be a result of the introduction of biopreparation Baykal EM-1, which decreased the oil content by 30% after one month, and of the soil substrate composition (it was rich in nitrogen, phosphorus and potassium compounds). Therefore, the laboratory study showed that the oil-contaminated soil bioremediation can be successfully performed when the oil concentration does not exceed 20,000–100,000 mg/kg and earthworms *Eisenia fetida* are used in the ocurrence of lactic acid, nitrogen-fixing and photosynthetic bacteria, the components of biopreparation Baykal EM-1.

The results of the normality tests (Lilliefors and Shapiro-Wilk tests) with $p < 0.05$ permitted to put forward an alternative hypothesis that the characteristic distribution differs from the normal one. At the initial stage of the investigation, the relations between soil contamination and the number of *E. fetida* were evaluated using the Spearman's correlation analysis. As a result of the investigation, strong inverse correlations were determined between the contamination level and the earthworms' number.

When evaluating the dependence of the earthworms' numbers in the samples without the biopreparation incubated for 22 weeks, the dependency of the earthworm numbers on the oil concentrations was observed, namely, the enhancement in the oil concentration resulted in the reducing earthworms' number. The earthworms died in the absence of the biopreparation when the oil concentrations reached 60,000–100,000 mg/kg. The effect of the soil contamination provided the worms survival of 70% (Table 2). The obtained model is highly significant, $R = -0.84$ (Fig. 4).

The addition of biopreparation Baykal EM-1 to the soil contaminated with oil at a content of 60,000–100,000 mg/kg enhanced the earthworms' survival by 70% (Table 2). The contribution of soil contamination with oil was more than 78%. The obtained model is highly significant, $R = -0.84$ (Fig. 4).

The intensity of the correlation relationships was evaluated by the Spearman's rank correlation coefficient between the characteristics "Total number of earthworms" and "Oil concentration, g/kg" and is represented in Supplementary Information (**Table S1**), along with other statistical assessments.

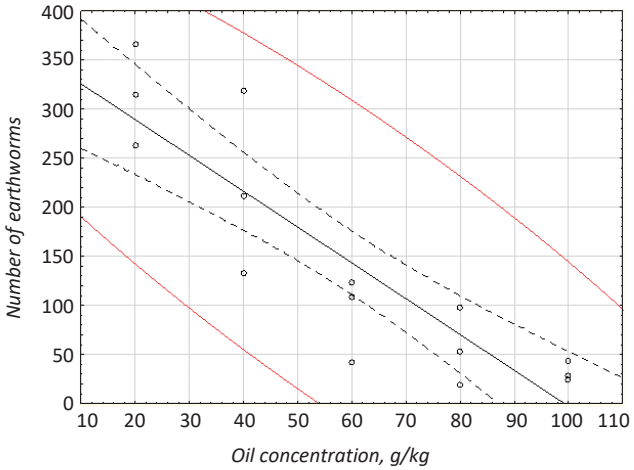

Fig. 4. Regression analysis of the dependency *Oil contamination–Number of earthworms* in samples without biopreparation in the 22nd week of incubation; $y = 332.6 - 3.8667*x$; where x is soil contamination level; force (R) = −0.84; contribution (adjusted R2) = 71%; significance, $p = 0.00008$

Thus, a method for the bioremediation of soil containing oil in an amount of up to 100,000 mg/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* and *Actinomycetales* (CFU=$2 \cdot 10^{11}$ per mL), and in the occurrence of earthworms of *E. fetida* has been suggested. During the experiment lasting for 5 months, a significant lowering (by 95–97%) in hydrocarbons content was registered in the soil to which earthworms and the biopreparation were added.

The online version of this paper contains Supplementary Material available free of charge at www.biotechnology-journal.ru

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