# The recultivation of soils contaminated with oil using the vermiculture of earthworms and their probiotics

# The recultivation of soils contaminated with oil using the vermiculture of earthworms and their probiotics

**S B Chachina [1], N A Voronkova [1,2], M A Shadrin [1] and N S Evdokimov [1]**

[1] Omsk State Technical University, 11 Mira Pr., Omsk, 644050, Russian Federation
[2] Federal state budgetary scientific institution "The Omsk Agricultural research center", 26 Koroleva street, Omsk, Russia

E-mail: ksb3@yandex.ru

**Abstract.** The recultivation technology for soils contaminated with oil in the concentration of 50 g/kg in Western Siberia with the use of microbiological preparation received from coprolites of earthworms Eisenia fetida and vermiculture of earthworms E. fetida, E. andrei, D. veneta was developed. The coprolites microflora composition of the earthworms E. fetida was studied. The microbiological preparation from coprolites of the earthworms was received. Microorganisms – oil destructors were isolated, which were used as the basis of the microbiological preparation "Muck" containing Pseudomonas aeroginosa, Bacillus careus, Bacillus mucoides, Bacillus thuringiens, Pseudomonas putida, CFU – $3.3*10^8$. The following characteristics were determined: the remediation effectiveness for the soil contaminated with oil in the concentration of 50 g/kg when using the microbiological preparation "Muck" and earthworms E. fetida, E. Andrei and D. veneta.

## 1. Introduction

Currently, for the recultivation of oil-contaminated soils quite a lot of ways have been developed and are widely used to clean the soil from oil spills, all differing in efficiency and complexity. There is a well-known technique of technical recultivation including removal of the contaminated soil layer, transportation and storage of it in specially designated dumps, in other words, the replacement of the soil [1].

As recent research (Jacobo Rodriguez-Camposa, 2014) has shown, a higher efficiency of soil bioremediation can be achieved with the introduction of earthworms in the contaminated soil, i.e. when using the method of vermiremediation. Earthworms accelerate the removal of pollutants from the soil and improve the physical and chemical properties of the soil, mixing it with organic matter and improving soil aeration, making pollutants available to microorganisms [2].

Some authors noted the positive impact of earthworms on the removal of pollutants such as oil, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls [3-6]. Earthworms have been proven to dig holes in soil and mix it in their intestines. This process contributes to changes in the physical and chemical properties of the soil. It is known that earthworms contribute to an increase in contacts between pollutants and soil microorganisms and, accordingly, more intensive removal of pollutants [7]. Contreras-Ramos et al., 2008 registered the removal of polycyclic aromatic hydrocarbons in soil with earthworms: anthracene – by 91 %, phenanthrene – by 99% and Benz-(a)-pyrene – by 16% [8].

Microorganisms capable of decomposing polyaromatic hydrocarbons (PAHs) are of particular interest, since PAHs, due to their properties, are sufficiently resistant to various types of exposure. This resistance combined with possible carcinogenicity and toxicity makes PAHs particularly dangerous environmental pollutants.In nature, there are microorganisms that are adapted to use PAH as a single resource of carbon and energy. But many of them destroy only PAHs with a small molecular weight, and only a few microorganisms can dispose of PAHs, which are characterized by the presence of more than four rings. Naphthalene can be destroyed by the bacteria of the genus Pseudomonas sp. (Ps. putida, Ps. fluorescens), Rhodococcus sp., Moraxella sp., Streptomyces sp., Bacillus cereus) [9-11]; acetonaphthone by Beijerinckia sp., Pseudomonas putida, Pseudomonas cepacia [12-13]; anthracene by Beijerinckia sp., Mycobacterium sp., Pseudomonas paucimobilis, Pseudomonas cepacia, Flavobacterium sp) [14]; phenanthrene by Aeromonas sp., Alcaligenes faecalis, Alcaligenes denitrificans, Micrococcus sp.; and pyrene by Alcaligenes denitrificants, Rhodococcus sp., Mycobacterium sp. [15-16].

Bacteria are able to degrade PAHs. The broadest range of PAHs can be degraded by Sphingomonas paucimobilis (11 PAHs: naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthen, pyrene, benzopyrene, chrysene, benzopyrene, dibenzoanthracene). However, the absolute leader in the destructible substances range are bacteria of the genus Pseudomonas (13 substances naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthen, pyrene, benzopyrene, chrysene, benzopyrene, dibenzoanthracene, fluoren, acenaphthylen). The widest spectrum of PAHs is decomposed by Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas putida [17].

The aim of this work is to study the possibility of using muckworm (E. fetida), Californian worm (E. andrei) and Dendrobaena veneta (D. veneta) and the intestinal microflora for cleaning soil from oil and petroleum pollution in a concentration of 50 g/kg. Probiotic microorganisms – oil-destructors from the earthworm E. fetida coprolites were isolated, microbiological preparation "Muck" was developed on the basis of the named microorganisms and it was tested for the effectiveness of oil-contaminated soils remediation with the preparation and vermiculture of the earthworms Eisenia fetida, Dendrobaena veneta, Eisenia andrei.

## 2. Study subject

### *2.1. Earthworm Species*

Eisenia fetida. Worm mean mass was 0.41-0.92 g. Earthworm is cold-resistant, able to digest aggressive substrates, such as poultry manure, cattle dung, and substrate with high concentration of bark and sawdust. Eisenia andrei. Red Californian worms can digest any organic matter (cattle dung, kitchen waste, last-year foliage, paper, etc.), they have high production rate (100 times higher than other species) and live 4 times longer than other worms. Worms mean mass was 0.5-0.9 g. 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.

### *2.2. The Microorganisms Identification with MALDI-TOF Mass Spectrometry*

The composition of microorganisms in microbiological preparations and worm coprolites was determined by MALDI-TOF (matrix laser desorption mass spectrometry) using the system BIOMERIEUX (FRANCE). Determination of the coprolites' microflora composition was carried out at the research Institute of Epidemiology and Microbiology of Pasteur's. During the study, the microbial composition of the microbiological preparation "Muck": Pseudomonas aeroginosa, Bacillus careus, Bacillus mucoides, Bacillus thuringiens, Pseudomonas putida, Pseudomonas aeruginosa (microbial number UFC – $3.3*10^8$ per ml).

### *2.3. Soil substrate*

All the experiments were carried out with the earth substrate represented by a sterilized meadow soil with the brand name "Living Earth (Terra Vita) Universal Nutrient Ground" manufactured by MNPP

FART Ltd. (St. Petersburg, Russia). The soil had the following characteristics: humus content 46%, pH 5.9-6.0, and adsorptive capacity 28-40 mg-eq per 100 g soil. Chemical composition of the soil was 150 mg nitrogen ($NH_4$ + $NO_3$) kg-1, 270 mg phosphorus ($P_2O_5$) kg-1, 300 mg potassium ($K_2O$) kg-1, and 6.9 % (C) of organic matter (determined by Tyurin method). The organic matter was assessed by GOST 26213-91. Soil. Methods for determination of organic matter.

Before the experiment, the soil was prepared in compliance with ISO (ISO 11268-1, 1993; ISO 11268-2, 1998). The soil was dried to a constant weight, sieved using 5 mm mesh filter, and homogenized by hand; this was followed by the addition of calcium carbonate to bring pH to 6.0±0.5 and distilled water to obtain a soil moisture content of 60%. 1 kg of the prepared soil was placed in 2 L polypropylene containers. In the course of experiments, a water loss due to evaporation was monitored once a week, and the soil was humidified to bring the soil moisture content to 60%.

A series of oil contaminated soil samples was prepared. To obtain the samples, oil was added to the soil at concentration of 50 g/kg. The oil was obtained from the Samotlor (Russia) oil field, and had the following characteristics: relative density (ρ) 0.934, M=367, V20 = 63.13, pour point 25 °C, the temperature of processing 22 °C, and flash point in closed crucible 120 °C. The composition of crude oil was as follows: 2.3% of paraffins, 0.96% of sulfur, 0.12% of nitrogen, 14% of sulfurous resin, 10% of silica resin, 1.36% of asphaltenes, 1.99% coking ability, 0.01% of ash, 0.01% of naphthenic acids, and 0.006% of phenols. The elemental composition was as follows: C - 85.9%, H - 12.93%, O - 0.15%, S - 0.92%, and N - 0.1 %

*2.4. Laboratory earthworm toxicity test*

1 kg of sterile soil was put into the 2 L plastic containers. 10 adult earthworms were added to each container. Over the entire experiment, the soil was humidified once a week by introducing 100 mL of distilled water into each container. Containers with the soil were covered by plastic cover 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. Although E. fetida exhibit the maximum activity at a temperature of 20–25 °C, all the earthworms were incubated at 15±1 °C for 22 weeks because such temperatures are more typical for Siberia. The control soil samples and the experimental samples of contaminated soil with the introduced earthworms and microbiological preparation "Muck" were incubated for 22 weeks, from December 2016 to May 2017. For each sample were repeated three times.

## 3. Results

*3.1. Definition of the earthworms' microflora*

In the course of the study, pure cultures of microorganisms were isolated from earthworm coprolites, and the specific composition of microorganisms was defined by mass spectrometry. Eisenia fetida UFC $=3.3*10^8$.

Microorganisms – oil destructors of the earthworms (nutrient medium Raymond) are Psedomonas putida, Pseudomonas aeruginosa, Klibsiella pneumonia, Bacillus cereus, Bacillus mycoides, Bacillus thurinigiensis

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Microorganisms of earthworms, selected on Blood agar are Aeromonas hidrohila, Pseudomonas oleovorans, Leclercia adacarboxylata

Microorganisms of earthworms isolated on Endo medium are Aeromonas hidrohila, Pseudomonas oleovorans, Pseudomonas putida, Leclercia adacarboxylata.

Fungi of earthworms, isolated on Saburo medium are Candida guillirmondii, Aspergillus fumigatus, A. Niger, A.flavus, Fusarium, Mucor (M. hiemalis, M. rasemosus, Mucor sp.), Penicilliiim, Rhizopus oryzae, Trichoderma (T. harzianum, T. koningi, T. viride, Trichoderma sp.), Candida vartiovaare, Candida sp., Trichosporon.

Microorganisms of earthworms, isolated on Ashby medium are Raoultella ornithinolytica, Pseudomonas putida, Leclercia adacarboxylata

The microbiological preparation "Muck" includes Pseudomonas aeroginosa, Bacillus careus, Bacillus mucoides, Bacillus thuringiens, Pseudomonas putida, Pseudomonas aeruginosa (microbial number UFC – $3.3*10^8$ per ml). The preparation was obtained from the microflora of coprolites of earthworms and tested in soils contaminated with oil 50 g/kg.

*3.2. The survival of the earthworms E. fetida, E. andrei, D. veneta in the oil-contaminated substrate*

The total number of E. fetida in the control variant (clean soil), increased by 19 times and equaled 191 worms per container. When the microbiological preparation "Muck" was introduced, the total number of E. fetida increased by 23 times and equaled 230 worms per container. The survival of earthworms in the variant with oil concentration of 50 g/kg without the biopreparation was 20%, the total number of E. fetida amounted to 15 worms per container. In the variant with oil and the microbiological preparation, the survival of earthworms was 100%, and the total number increased by 5 times and equaled 52 worms per container (table 1).

The total number of E. andrei in the control variant (clean soil), increased by 10 times, and when the microbiological preparation "Muck" was introduced, the total number of E. andrei increased by 14 times and equaled 141 worms per container. The survival of the earthworms in the variant with oil concentration of 50 g/kg without the biopreparation was 10%, and the total number of E. andrei equaled 12 worms per container. In the variant with oil and the microbiological preparation, the survival of the earthworms was 90%, and the total number increased by 4 times and equaled 40 worms per container (table 1).

The total number of D. veneta in the control variant (clean soil) increased by 2 times and equaled 19 worms per container. When the microbiological preparation "Muck" was introduced, the total number of D. veneta increased by 4.5 times and equaled 46 worms per container. The survival of the earthworms in the variant with oil concentration of 50 g/kg without the biopreparation was 5%, the total number of D. veneta equaled 12 worms per container. In the variant with oil and the microbiological preparation, the survival of the earthworms increased up to 50%, and the total number increased by 2 times and equaled 21 worms per container (table 1).

*3.3. Recultivation of oil-contaminated soils using preparation "Muck" and vermiculture of earthworms*

The concentration of oil was significantly reduced in the soil samples containing the earthworms compared to the soil samples without the worms. The efficiency and the rate of destruction of oil hydrocarbons depend on the concentration in the soil and the presence of the biological preparation "Muck" in the soil. The significant decrease in the control variant, with the oil concentration in the soil of 50 g/kg, the hydrocarbon concentration in the soil was not observed. The oil content in the soil decreased to 46 g/kg (8% efficiency) within five months. With the introduction of the biological preparation "Muck", the oil concentration decreased to 34.7 g/kg (efficiency 26 %) (table 2). In the recultivation process of soil contaminated with oil (50 g/kg) and E. fetida, the oil concentration decreased to 21.7 g/kg (53% efficiency). Application of preparation "Muck" and E. fetida decreased oil concentration to 13 g/kg (efficiency 71 %). The process of remediation oil-contaminated soil with E. andrei decreased oil concentration from 50 to 18.3 g/kg (60% efficiency). In the variant with the biological preparation "Muck" and E. andrei oil concentrations decreased to 14.5 g/kg (efficiency of 68 %). In the variant with oil 50 g/kg and D. veneta oil concentration decreased to 21 g/kg (efficiency 54 %). Introduction of the biological preparation "Muck" to the variant with D. veneta oil concentration decreased to 12.3 g/kg (efficiency 74 %) (table 2).

**Table 1.** Survival, total number of earthworms at oil concentration in soil 50 g / kg.

| | Experiment 1. *E. fetida* | | | | Experiment 2. *E. andrei* | | | | Experiment 3. *D. veneta* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | variant | Total population ex./box | Total productivity cocoons/box | Individual productivity | variant | Total population ex./box | Total productivity cocoons/box | Individual productivity cocoons/individual | variant | Total population ex./box | Total productivity cocoons/box | Individual productivity cocoons/individual |
| 1 | control | 190±6 | 26.25 | 2.70 | control | 102.60±0.86 | 13 | 2.70 | control | 19±0.20 | 3.50 | 0.66 |
| 2 | preparation "Muck" | 230±4.10 | 22.16 | 2.47 | preparation "Muck" | 141.66±1.40 | 13.50 | 2.40 | preparation "Muck" | 46.66 | 17.41 | 2.14 |
| 3 | Oil 50g/kg | 15.30±0.20 | 2.53 | 0.34 | Oil 50 g/kg | 12.30±0.40 | 3.92 | 0.54 | Oil 50 g/kg | 8±0.40 | 2 | 0.22 |
| 4 | Oil 50 g/kg+ preparation "Muck" | 52.30±1 | 17.86 | 1.63 | Oil 50 g/kg+ preparation "Muck" | 40±0.50 | 9.05 | 1.01 | Oil 50 g/kg+ preparation "Muck" | 21.66±0.57 | 3.08 | 0.40 |

**Table 2**. Remediation of oil-contaminated soils.

| Variant | control | preparation "Muck" | *E. fetida* + water | *E. fetida* + preparation "Muck" | *E. andrei* + water | *E. andrei* + preparation "Muck" | *D. veneta* + water | *D. veneta* + preparation "Muck" |
|---|---|---|---|---|---|---|---|---|
| January | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| March | 49.10 | 46.70 | 34.80 | 35.64 | 36.50 | 29.77 | 41 | 47.90 |
| April | 48.20 | 40.10 | 25.60 | 24.92 | 24.30 | 18.58 | 25.70 | 17.58 |
| May | 46.26 | 34.70 | 21.70 | 12.91 | 18.33 | 14.57 | 21.03 | 12.28 |
| Efficiency,% | 8 | 26 | 52.80 | 71.70 | 60.20 | 68.40 | 54.34 | 74 |

## 4. Discussion of results

Earthworms have a great potential for removing hydrocarbons from the soil, even at high concentrations up to 50 g/kg. The introduction of the microbiological preparation increased the survival and reproductive potential of worms in oil-contaminated soils and contributed to a significant decrease in the concentration of hydrocarbons for 20 weeks in a laboratory experiment. These results suggest that earthworms may be recommended for bioremediation of even highly contaminated soils with oil concentrations up to 50 g/kg when combined with a microbiological preparation containing bacterial strains Pseudomonas aeroginosa, Bacillus careus, Bacillus mucoides, Bacillus thuringiens, Pseudomonas putida, (microbial number UFC - $3{,}3*10^8$ per ml). These strains of microorganisms – oil destructors are symbionts of earthworms.

In the process of recultivation for oil contaminated soil, the highest efficiency of the hydrocarbon decomposition is noted in the variant with E. fetida and microbial preparation "Muck" (71% efficiency) and D. veneta with microbial preparation "Muck" (74% efficiency). Also, the positive effect of using preparation "Muck" on the survival and growth in total productivity of all types of earthworms was noticed in the oil-contaminated substrate. The species-specific effect of the preparation on the growth of the earthworms' number was noted. The largest increase was observed in the total number of worms

E. fetida in the oil-contaminated substrate. Their total number has increased by 5 times. This is due to the fact that the preparation "Muck" contains probiotic cultures of microorganisms, symbionts of worm E. fetida.

Statistical analysis of the results was conducted as well. The normality verification for the distribution of quantitative traits was carried out using Kolmogorov-Smirnov, Lillifors and Shapiro-Wilk (W) criteria. The information was processed with the methods of descriptive statistics. To assess the significance of the differences, the Kruskal-Wallis (H) criterion was used for independent groups. The links were evaluated in the course of correlation analysis with calculation of Spearman's rank correlation coefficient (rs), modeling – by regression analysis with subsequent data visualization. The critical value of the statistical significance level when testing null hypotheses was taken to be 0.05.

When evaluating the results by the significance with Kruskal-Wallis criterion in several independent groups at soil contaminated by oil 50 g/kg with and without the biopreparation, no statistically significant differences were found. The differences were found between the following samples: the number of earthworms E. fetida in oil contaminated soil and samples of E. fetida with the biopreparation "Muck" (Kruskal-Wallis (H =to 11.56 p =0.02); Chi-square = 13.00 df = 4 p = 0.0013). Also, significant differences were found between the number of earthworms E. andrei in oil contamination soil and number of E. andrei with biopreparation "Muck" (Kruskal-Wallis (H =11.6 p =0.02); Chi-square = 13.00 df = 4 p = 0.0113.) The differences detected between the number of earthworms D. veneta in soil, polluted by oil 50 g/kg and samples of D. veneta with the biological product "Muck" (Kruskal-Wallis (H =10.01, p =0.018; Chi-square = of 6.66 df = 3 p = 0.008). The high degree of correlation was found between the number of earthworms E. fetida (R=0.97) and E. andrei in the variant with biopreparation "Muck", as well as between the number of earthworms D. veneta (r=0.93)

## 5. Conclusions

The recultivation process took 5 months. During recultivation in the control variant (without the preparation and worms) the oil concentration decreased by 8%, and in the variant with the microbial preparation, oil concentration decreased by 26%, while the combined use of the microbiological preparation and worms reduced the oil concentration by 70-74%.

The method of oil contaminated soil bioremediation with oil concentrations up to 50 g/kg in the presence of probiotic bacteria of E. fetida - Pseudomonas aeroginosa, Bacillus careus, Bacillus mucoides, Bacillus thuringiens, Pseudomonas putida, (UFC=$3.3*10^8$ per mL) and earthworms E. fetida, E. andrei, D. veneta is proposed. Decontamination and the ecological functions recovery of the oil- and petrochemicals-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 $m^2$ 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 80-85 %) in hydrocarbons content was registered in the soil with the earthworms and the biopreparation "Muck".

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