Effect of benzo(a)pyrene on the number of soil microorganisms of the genus *Pseudomonas* and *Rhodococcus*

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Abstract. The paper presents the results of a study of the effect of benzo(a)pyrene on the activity of hydrocarbon-oxidizing soil bacteria of the genus *Pseudomonas* and *Rhodococcus*. The objects of research are samples of agrosod-calcareous soil, exposed to oil pollution, located near an oil refinery with an increased concentration of benzo(a)pyrene. Studies carried out over three years from 2017 to 2019 showed that in all soil samples that are exposed to oil pollution, the concentration of benzo(a)pyrene exceeds the MPC. It was found that an increase in the concentration of benzo(a)pyrene in the soil is accompanied by the suppression of the activity of hydrocarbon-oxidizing bacteria of the genus *Rhodococcus* and *Pseudomonas*. The presence of an inverse relationship between the content of benzo(a)pyrene and the number of microorganisms was confirmed by correlation analysis carried out using Spearman's rank correlation coefficients (ρs) and τb-Kendall. The data presented in this work can be used in organizing bioremediation of oil-contaminated agrosod-calcareous soils. In the case of a high level of soil pollution with oil products, for its effective restoration, additional support for the activity of hydrocarbon-oxidizing microorganisms is needed, including by loosening.

1. Introduction

The ecological problem of anthropogenic pollution of soil with oil products does not lose its relevance now. The consequence of this pollution is the violation of the stability of ecosystems and the biosphere in general, and, first, soil degradation. Oil products disrupt the natural ratio of the number of soil microorganisms, reduce the soil's ability to self-purify and contribute to the accumulation of toxic and highly stable compounds in it. We have previously shown that soil pollution with oil products leads to an increase in the concentration of benzo(a)pyrene in it, and is also accompanied by the accumulation of pathogenic micromycetes of the genus *Fusarium, Rhizoctonia, Phytophthora* [1].

One way to solve this problem is the reclamation of oil-contaminated lands. At the same time, a variety of methods are used, such as mechanical cleaning, application of adsorption materials of organic and inorganic nature to soils, the introduction of chemicals and biological preparations, as well as organic components of waste from the logging industry. Bioremediation is an economically profitable, highly specific and environmentally friendly treatment; it has huge potential and competitive advantages, primarily due to environmental safety and low cost. One of the main methods
of bioremediation in situ (at the place of contamination) is the introduction of microorganisms into the place of contamination [2].

In work [3] it is shown that four groups of bacteria are most often found among oil-oxidizing microorganisms: gram-positive coryne-like bacteria (Rhodococcus, Bravobacterium, Arthrobacter, Micrococcus), gram-positive spore-forming bacteria (Bacillium), gram-negative oxidase-positive bacteria (Flavromobacterium, Chromobacterium) and sticks (Acinetobacter).

Currently, preparations containing strains of hydrocarbon-oxidizing bacteria are increasingly used to restore soil. In works [4-14] it is shown that actinobacteria of the genus Rhodococcus promote oxidative biotransformation of petroleum hydrocarbons and xenobiotics. Bacteria of the genus Pseudomonas have a similar activity [9-10].

It is known that the agronomically useful microflora plays a significant role in the processes of cleansing soils from oil; therefore, it is important to determine which components of oil products have a depressing effect on it.

The aim of this work is to study the effect of benzo(a)pyrene on the number of hydrocarbon-oxidizing bacteria Pseudomonas and Rhodococcus.

The danger of accumulation of high concentrations of benzo(a)pyrene in agricultural soils is due to the high stability of this xenobiotic, the possibility of migration into the feedstock for food production, high toxicity, and the ability to have a carcinogenic effect on the human body [8]. Solving the problem of anthropogenic pollution of the environment with benzo(a)pyrene is one of the priority environmental problems.

The objects of research are samples of agrosod-calcareous soil, exposed to oil pollution, located near an oil refinery with an increased concentration of benzo(a)pyrene.

2. Materials and methods
The work was carried out by order of an agricultural enterprise located in the Perm region of the Perm region. The objects of study are seven soil samples, which were taken in the territory adjacent to the oil refinery LLC LUKOIL-Permnefteorgsintez.

Point samples were taken from one or more layers on the trial plot using the envelope method so that each sample was a part of the soil typical of genetic horizons or layers of a given soil type. Spot samples were taken from the holes with a spatula. A pooled sample was obtained by mixing at least 5 incremental samples that were taken from one sample site. Soil samples were packed in polyethylene bags with labeling (accompanying coupons). A pooled soil sample was prepared from spot samples by mixing them. The combined sample mass was at least 1 kg [4-5].

The benz(a)pyrene concentration was determined by high performance liquid chromatography using an Agilent 1100 instrument with a fluorometric detector in accordance with the FR.1.31.2005.01725 method [6]. According to the Russian standard GN 2.1.7.2041-06, the MPC of benzo(a)pyrene in the soil is not more than 0.02 mg / kg in total with the background level [7].

The quantitative content and taxonomic composition of the complex of hydrocarbon-oxidizing microorganisms was determined by a standard method using the method of inoculation from a thousandth dilution in 5 replicates on agarized glucose-peptone-yeast medium and Czapek's medium with the addition of hexadecane. The samples were preliminarily subjected to homogenization using an UZDN-1 ultrasonic disperser (22 kHz; 0.44 A; 2 min). To suppress the development of fungi, nystatin was added to the nutrient medium in an amount of 50 mg per 0.5 L of the medium. The inoculations were incubated at room temperature for 3-7 days and then the growing colonies were counted and microscopic. The total number of microorganisms was determined by a direct microscopic method in a Goryaev chamber using a dye acridine orange [13]. Bacteria were identified based on the study of cultural and micro morphological characteristics [16].

3. Results and discussion
The results of determining the concentration of benzo(a)pyrene and the number of bacteria of the genera Pseudomonas and Rhodococcus in the samples of oil-contaminated soil are shown in table 1.
Table 1. The number of microorganisms (CFU / g) in the 0-20 cm soil layer contaminated with benzo(a)pyrene, 2017 – 2019.

| Sample No. | Concentration of benz(a)pyrene, mg/kg *** | Bacteria content, CFU * / g soil |
|------------|--------------------------------------------|--------------------------------|
|            |                                            | Pseudomonas | Rhodococcus | Pseudomonas | Rhodococcus | Pseudomonas | Rhodococcus |
| 1          | 0.0006                                    | 10^6        | 10^6        | 10^6        | 10^6        | 10^6        | 10^6        |
| 2          | 0.004                                     | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        | 10^4        |
| 3          | 0.002                                     | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        | 10^4        |
| 4          | 0.005                                     | 0           | 0           | 0           | 0           | 10^0        | 10^2        |
| 5          | 0.003                                     | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        |
| 6          | 0.004                                     | 0           | 10^2        | 10^2        | 10^2        | 0           | 0           |
| 7          | 0.001                                     | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        | 10^2        |

*colony-forming unit; **not detected; ***MPC of benzo(a)pyrene in soil no more than 0.02 mg/kg [9]

Studies conducted over three years from 2017 to 2019 showed that all soil samples exposed to oil contamination contain benzo(a)pyrene. It is known that this compound belongs to the class of polycyclic aromatic hydrocarbons, is a product of incomplete combustion of organic compounds and is present in the products of coal and oil processing [15].

The lowest concentration of benzo(a)pyrene is found in soil sample no. 1, and has a tendency to decrease over three years. At the same time, the total number of bacteria *Rhodococcus* and *Pseudomonas* is increasing, which indicates the active course of the soil recovery process. A similar pattern is observed in samples no. 2, no. 3 and no. 4.

Thus, we have established that an increase in the concentration of benzo(a)pyrene in the soil leads to a decrease in the number of microorganisms of the genus *Rhodococcus* and *Pseudomonas*.

To confirm the correlation of the declared indicators: the concentration of benzo(a)pyrene and the number of microorganisms, we carried out a correlation analysis of the empirical data presented in table 1. Let $X_1$ be the content of benzo(a)pyrene, measured in mg/kg of soil; $X_2$ - the number of microorganisms (CFU/g) in the soil layer 0-20 cm contaminated with benzo(a)pyrene (*Pseudomonas*); $X_3$ is the number of microorganisms (CFU/g) in the 0-20 cm soil layer contaminated with benzo(a)pyrene (*Rhodococcus*). Since the experimental data contain only seven observations, we have small samples for each variable. In addition, the samples for variables $X_2$ and $X_3$ have outliers and related ranks, the data are heterogeneous, and therefore, the use of the Pearson correlation coefficient is inappropriate.

We carried out a correlation analysis using Spearman's rank correlation coefficients ($\rho_s$) and $\tau_b$-Kendall according to the algorithm described in [11].

1. The correlation coefficients were found separately for years between the content of benzo(a)pyrene ($X_1$), measured in mg/kg of soil, and the number of microorganisms (CFU/g) in the 0-20 cm soil layer contaminated with benzo(a)pyrene 2017-2019 (respectively $X_2$, $X_3$) and checked their significance. Next, the results were interpreted.

2. Found descriptive statistics, such as arithmetic mean and median mean, for each of the 7 passive experiments for 2017-2019 for the variables $X_1$, $X_2$, $X_3$, which are denoted, respectively $\bar{X}_1, \bar{X}_2, \bar{X}_3(i)$ where $i = 1,2,3$.

3. Found the general coefficients of correlation, by summarizing the data for years, using the arithmetic mean and median mean between $X_1$, $X_2$, $X_3$, checked the statistical reliability of the results and given their interpretation. The first and third steps of the algorithm are related to testing the null
hypothesis that the general correlation coefficient of Spearman and Kendall is equal to zero at a significance level of 0.05.

Because of performing 1 step of the algorithm, the following results were obtained (table 2).

| Correlation coefficients between X1 and X2 | Correlation coefficients between X1 and X3 | Correlation coefficients between X2 and X3 |
|------------------------------------------|------------------------------------------|------------------------------------------|
| ρₘ = -0.88                              | ρₘ = -0.72                              | ρₘ = -0.72                              |
| τₘ = -0.90                              | τₘ = -0.81                              | τₘ = -0.81                              |
| Tₚ = 0.63                               | Tₚ = 0.63                               | Tₚ = 0.63                               |
| Tᵥ = -0.90                              | Tᵥ = -0.91                              | Tᵥ = -0.91                              |

According to the results of step 1, it can be seen that all the found Kendall correlation coefficients determine a close inverse relationship between the content of benzo(a)pyrene (X₁), measured in mg/kg of soil, and the number of microorganisms (CFU/g) in the soil layer 0-20 cm, contaminated with benzo(a)pyrene 2017 - 2019 (respectively X₂, X₃), and all correlation coefficients are significant. Thus, the conclusions of the qualitative analysis made by the authors have been fully confirmed.

Another approach to determining the relationship between the features using the Spearman's rank correlation coefficient showed almost the same result, since all the rank correlation coefficients are greater than 0.7, which indicates a noticeable correlation of features. Signs for all coefficients are negative, which once again confirms the inversely proportional relationship between the signs.

To perform step 3 of the algorithm, average values were found that characterize the averaged values for each of the seven experiments over 3 years. For quantitative analysis, two statistics were selected: the arithmetic mean, as well as the median, which in this case is more consistent with the typical mean due to the heterogeneity of the samples (table 3).

| Benzopirene arithmetic mean over 3 years | Benzopirene 3-year median | Pseudomonas arithmetic mean over 3 years | Pseudomonas - median 3-year mean | Rhodococcus arithmetic mean over 3 years | Rhodococcus median 3-year mean |
|------------------------------------------|--------------------------|------------------------------------------|---------------------------------|------------------------------------------|-------------------------------|
| 0.0003666667                            | 0.0003                   | 340000000                               | 1000000                        | 340000000                               | 1000000                       |
| 0.0027                                  | 0.00276                  | 67                                       | 100                            | 3400                                     | 100                           |
| 0.0014566667                            | 0.00122                  | 3400                                     | 100                            | 3400                                     | 100                           |
| 0.00361                                 | 0.00379                  | 33.66666667                              | 1                              | 33.333333333                            | 0                             |
| 0.0035                                 | 0.00325                  | 66.66666667                              | 100                            | 66.66666667                              | 100                           |
| 0.0038833333                            | 0.00396                  | 33.33333333                             | 0                              | 33.33333333                            | 0                             |
| 0.00152                                 | 0.00122                  | 100                                      | 100                            | 100                                      | 100                           |

Further, the rank correlation coefficients were found for the averaged values (table 4).

The rank correlation coefficients found from the averaged values over three years also indicate a close relationship between the studied characters and confirm the conclusions about the inverse relationship between the content of benzo(a)pyrene and the number of bacteria of the genus Rhodococcus and Pseudomonas in the 0-20 cm soil layer contaminated with benzo(a)pyrene in 2017 - 2019.
Table 4. Rank correlation coefficients for median arithmetic mean values.

| Correlation coefficients between $X_1$ and $X_2$ based on the median values of observations over 3 years | Correlation coefficients between $X_1$ and $X_3$ based on the median values of observations over 3 years | Correlation coefficients between $X_1$ and $X_2$ based on the arithmetic mean of observations over 3 years | Correlation coefficients between $X_1$ and $X_3$ based on the arithmetic mean of observations over 3 years |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| $\rho_S = -0.74$ | $\rho_S = -0.71$ | $\rho_S = -0.91$ | $\rho_S = -0.77$ |
| $\tau_b = -0.81$ | $\tau_b = -0.82$ | $\tau_b = -0.9$ | $\tau_b = -0.67$ |

4. Conclusion

Benz(a)pyrene, as one of the representatives of polycyclic aromatic hydrocarbons, is one of the dangerous pollutants due to its toxic, carcinogenic and mutagenic properties, as well as due to their slow decomposition in ecosystems.

Based on the studies carried out, it was found that an increase in the concentration of benz(a)pyrene in the soil leads to a decrease in the number of hydrocarbon-oxidizing bacteria of the genus *Rhodococcus* and *Pseudomonas*.

Most of the areas affected by oil pollution require reclamation work. These works must be scientifically justified, which ensures the quality and cost of their implementation.

The results of our studies can be used in organizing bioremediation of oil-contaminated agrosoddy-carbonate soils [2]. In the case when there is a high level of soil pollution with oil products, in order to increase the efficiency of its restoration, it is necessary to organize additional support for the activity of hydrocarbon-oxidizing microorganisms, including by loosening.

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