

Microbiological Landscape of Oil-Contaminated Soil and its Bioremediation by Microorganisms

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ABSTRACT

The composition of microbial contaminants of soil samples polluted with oil and oil products from oil depots of ports in southern Ukraine was investigated, and the possibility of their bioremediation by microorganisms present in the soil was determined. The microbiological landscape of the soil contaminated with oil and oil products was established, the quantitative and qualitative characteristics, group and dendrological composition of microorganisms as well as their potential ability to biodegrade petroleum hydrocarbons were determined. The degree of sanitary and ecological contamination of the samples was characterized by the number of the main groups of microorganisms – mesophilic aerobic and facultative anaerobic microorganisms (MAFAnM), molds, yeasts, as well as the dominance of MAFAnM by 3-5 orders among the studied groups of microorganisms. According to MAFAnM, the number of thermophilic bacteria, titers of nitrifying bacteria, *E. coli*, *Clostridium perfringens*, bacteria of the genus *Proteus*, and the degree of oil contamination, the soil samples studied are characterized as contaminated and heavily contaminated. According to the study of morphological, tintorial, cultural, biochemical properties, 130 species were identified and 9 morphogroups of bacteria in oil-contaminated soil samples were determined. A dendrogram was constructed based on the set of studied properties of the isolated microorganisms. According to the results of the screening, the microorganisms isolated from the contaminated soil samples are capable of biodegradation of long-chain alkanes of petroleum hydrocarbons. The identified groups of microorganisms can be arranged in the following order of increasing indicator: *Bacillus subtilis* and *Paenibacillus macerans* < *Paenibacillus polymyxa* < *Bacillus licheniformis* < *Bacillus thuringiensis* < *Bacillus megaterium* < *Bacillus pumilis* < *Bacillus cereus* < *Paenibacillus circulans*. *Paenibacillus circulans* and *Bacillus cereus* were identified as the most promising strains, biotransforming up to 48 percent of the total amount of hydrocarbons.

Keywords: oil-contaminated soils, ecological biotechnology, biodegradation, microorganisms-biodegraders of petroleum hydrocarbons.

INTRODUCTION

In line with the principles of the circular economy, environmental technologies are progressively evolving towards the use of more natural, environmentally friendly and sustainable bioremediation methods (Ehiosun et al., 2022). The biodegradation of hydrocarbons is heavily dependent on microbial metabolism, which generates carbon

dioxide and water as end products. Therefore, the metabolic potential of microorganisms and the release of hydrocarbon-producing microorganisms (Jang et al., 2009, Chaillan et al., 2004, Verma et al., 2006) are important for hydrocarbon biodegradation. This has led to a constant search for microorganisms with appropriate metabolic capabilities for hydrocarbon degradation (Das et al., 2014, Ron and Rosenberg, 2014, Mishra et al., 2001).

Microorganisms are considered to be the best utilizers in nature due to their ability to extract carbon and energy from numerous compounds, and the oil-contaminated environment is an ideal place for the isolation of bacteria capable of hydrocarbon degradation (Abatenh et al., 2017, Abdulsalam et al., 2011, Abousnina et al., 2016). This is because the presence of hydrocarbons in the environment selectively promotes the growth of bacteria that are resistant to hydrocarbon toxicity and/or capable of degrading them due to the lack of other carbon and energy sources (Ehiosun et al., 2022), thereby affecting the diversity of the bacterial community (Yadav et al., 2017, Adams et al., 2015).

It has been reported that the bacterial species belonging to the classes *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, etc. degrade petroleum hydrocarbons using different metabolic strategies (Varjani et al., 2017). Some bacteria can efficiently degrade certain oil fractions and hydrocarbon classes. For example, *Nocardia-soli* Y48 and *Rhodococcus erythropolis* YF28-1 were reported to grow on the n-alkane fraction (C8-C36) of crude oil (Margesin et al., 2003), while *Bacillus cereus* uses hydrocarbons of different structures as a carbon source (Varjani et al., 2015). This may be due to the fact that the bacterial strains have different catabolic enzymes, bioavailability, and bioaccessibility to hydrocarbons.

Alkanes are a major component of oil, while their water solubility and biodegradability decrease with increasing molecular weight. During aerobic decomposition, alkanes are typically activated by the enzymatic addition of molecular oxygen or, in rare cases, peroxides (Varjani, S., Upasani, V., 2017, Yang et al., 2017) by terminal or subterminal oxidation to form the corresponding primary or secondary alcohols. Primary alcohols are oxidized to aldehydes, which are further converted to fatty acids or ω -hydroxy fatty acids and dicarboxylic acids by ω -hydroxylation (Yang et al., 2015, Yeung, 2010).

Ketones are formed by the oxidation of secondary alcohols and are further converted to esters as well as hydrolyzed into fatty acids. Fatty acids are converted to acetyl-CoA (acetyl coenzyme A) by β -oxidation and further metabolized in the tricarboxylic acid cycle to produce CO_2 as well as reducing equivalents, which are sent to the respiratory chain for complete oxidation to produce energy (Varjani et al., 2015).

The low solubility of hydrocarbons in water limits their availability and use by bacteria as substrates (Head et al., 2003). Hence, it requires specific assimilation mechanisms. Bacteria produce surface-active compounds known as biosurfactants to increase the solubility and availability of hydrocarbons through emulsification, thereby facilitating hydrocarbon uptake and degradation (Al-Zubaidi and Al-Tamimi, 2018; Agarry and Latinwo, 2015).

Recent studies have shown how biosurfactants contribute to the emulsification and solubility of hydrocarbons during biodegradation (Head et al., 2003; Agarry, Latinwo, 2015). Another important mechanism that bacteria use to overcome poor hydrocarbon solubility is biofilm formation. A biofilm is defined as microbial aggregates that are often embedded in a self-produced matrix of extracellular polymeric substances (EPS) as well as adhere to each other and/or to a surface (Sharma, 2012). EPS serve as a building block for the three-dimensional architecture of the biofilm and as an anchor material for attachment to the surface.

Thus, the literature review shows that many species of bacteria belonging to different classes are capable of degrading petroleum hydrocarbons. At the same time, the composition of microbial contaminants of the most common types of soil, the impact of hydrocarbon chemical pollutants on the composition of microorganisms present in it, and the possibility of remediation processes by microorganisms present in such soil remain unclear.

The purpose of the study was to establish the microbiological landscape of the soil contaminated with oil and oil products, to provide quantitative and qualitative characteristics, group and dendrological composition of microorganisms, as well as to determine their potential ability to biodegrade petroleum hydrocarbons. To achieve this goal, it is necessary to solve the following tasks:

- study the composition of microbial contaminants of individual soil samples contaminated with oil and oil products;
- assess the sanitary and ecological condition of the soil by some bacterial indicators and the degree of contamination;
- detect and identify potential species of microorganisms that biodegrade petroleum hydrocarbons.

METHODS

Soil sampling

Soil samples contaminated with crude oil were collected from two oil depots in Odesa Oblast: the Odesa and Chornomorsk ports. The soil samples were collected using the selective method, the “envelope method” with the selection of 50 point samples in the field, each sample was taken in five replicates to obtain two combined samples (samples), using a soil auger at a depth of 0–10 cm in the area contaminated with crude oil, soils – typical for the identified regions of low humus. The soil samples were stored in a clean glass container, soil pH was measured by suspending the soil in water (1:5 w/w), the suspension was stirred for 1 min and allowed to settle, pH was measured using a pH meter (Hanna Instruments HI 5221, Germany) at 25 °C.

Determining the content of oil products in the soil

Two soil samples were taken from the soil sample. The weight of the sample was 30–100 g, depending on the estimated oil content. The soil sample was placed in a 150 ml flask and moistened with chloroform until it was wet. Then, the extraction was carried out several times by adding 10–15 ml of chloroform until the last portion was colorless. The time of each extraction was 5–10 minutes. The extracts were filtered into a conical flask through a red ribbon filter. The remaining soil in the extraction flask was washed with 5 ml of chloroform. The combined chloroform extract was evaporated in a water bath fume hood or chloroform was removed by distillation. For this purpose, the extract was placed in a 250-ml flask connected to a refrigerator and placed in a water bath for evaporation. When 10–15 ml of liquid remained in the flask, the distillation was stopped. The contents of the flask were poured into a 50 ml beaker, and the flask was rinsed twice with 10 ml of chloroform. These two portions of chloroform were poured into a beaker, which was placed in a fume hood for evaporation. The precipitate remaining in the beaker after evaporation of the chloroform was dissolved in 5–10 ml of hexane. The resulting solution was passed through an aluminum oxide column to remove polar compounds. After a 1–2 cm layer of solution

remained above the aluminum oxide, the column was washed with 2–3 portions of hexane (2–3 ml each), after rinsing the beaker with it. The hexane was evaporated in a stream of air at room temperature. After the hexane was completely removed, the beaker was weighed on an analytical balance, kept for half an hour in the laboratory, and re-weighed. The weighing was repeated until a constant weight was reached.

When isolating organic compounds of anthropogenic origin (oil and oil products) by chloroform extraction, the content of organic compounds, which was also determined by using the gravimetric method of chloroform extraction, in conditionally uncontaminated (background) soil was taken into account by subtracting the corresponding indicators.

Preparation of dilutions of the soil samples for determining the microbiological landscape

The soil samples were freed from large inclusions of various nature and sieved through a sterile sieve. All dilutions were prepared in sterile tap water. For the first dilution (1:10), 10 g of soil was added to the water and after mixing, subsequent tenfold dilutions were prepared from the resulting soil suspension without settling. Several tenfold dilutions were prepared from the initially prepared suspension (1:10): up to 4–6 for the analysis of contaminated soils (1:10 000; 1:1 000 000).

Determination of the microbiota composition of samples

The composition of the microbiota of the samples was studied by standardized methods: mesophilic aerobic and facultative anaerobic microorganisms (MAFanM) and fungi were counted by sowing on meat-peptone agar (MPA) and agarized wort (AW), respectively (Persianova, 2010). Using the deep seeding method, 1 ml of the suspension was taken from each dilution and incubated for 24 to 48 hours at 28–30 °C. The number of colonies was counted per 1 g of soil.

For a complete sanitary and bacteriological analysis of the soil, the presence of anaerobes (in particular, *Clostridium difficile*), *Escherichia coli* (*E. coli*), *Proteus vulgaris*, nitrifying bacteria, and pathogenic microorganisms (*Salmonella*, *Shigella*) was also determined (Harley and Prescott, 2002).

The titer of *E. coli* to assess the degree of fecal contamination was determined by inoculating 1 ml of different dilutions of soil from 1:1 000 to 1:1 000 000 into modified Kessler medium. Crops were thermostated at 43 °C for 24 hours. In the presence of gas formation and turbidity or turbidity only, the cultures were streaked onto Endo medium and cultured at 37 °C for 24 hours. The morphological and tintorial signs of microorganisms from colonies characteristic of *E. coli* (dark red with a metallic sheen and colorless colonies on Endo medium) were determined. The detection of gram-negative short non-spore bacilli and characteristic colonies in the smears when they were inoculated on glucose-peptone medium indicated the presence of *Escherichia coli*.

The titer of nitrifying bacteria was determined by inoculating dilutions of soil suspension from 1:100 to 1:10 000 into Vinogradsky's liquid mineral medium, thermostating at 28 °C for 14–15 days. The formation of nitrite or nitrate acids was checked by a qualitative test with diphenylamine. A blue color indicates the presence of nitrates.

The titer of *Clostridium perfringens* was determined by inoculating 1 ml of different dilutions of soil (from 1:1 000 to 1:1 000 000) into iron sulfite agar (Wilson-Blair medium) after preheating to 80 °C for 15 min to free it from non-spore-bearing microbiota. First, 1 ml of the appropriate dilution was poured into a test tube with molten and cooled to 45 °C Wilson-Blair medium; then the material was distributed evenly. Afterwards, it was thermostated at 37 °C and during the first 18 hours, the appearance of black colonies was observed in the depths of the agar, which often broke the medium due to gas formation, indicating the reduction of sodium sulfite to sodium sulfate (Na_2S) by bacteria, which, when interacting with chlorine iron, forms black ferrous sulfate (FeS), i.e., the presence of *C. perfringens* was noted.

The degree of fecal contamination of the soil was determined by the number of thermophilic bacteria, the temperature optimum of which is 58–60 °C, by sowing dilutions of the soil suspension (from 1:10 to 1:1 000 000) on MPA. Crops were incubated at 60 °C for 24 hours and the number of colonies of thermophilic bacteria was counted.

Bacteria of the genus *Proteus* were detected by using the Shukevich method by sowing 0.1 ml of dilutions of soil suspension up to and including 1:10 000 into the condensate of mowed MPA. The tubes were incubated at 30 °C for 24–48 hours. Bacteria of the genus *Proteus* grow in the form of a thin veil-like film on the surface of agar.

Media enrichment and detection of salmonella and shigella

The enrichment for the accumulation of microorganisms was carried out on a special RCM medium at pH 8.0, since the soil sample was characterized by an alkaline pH of the medium. The special medium (RCM) has the following composition (g/l): casein (2.0), glycerol (2.5), asparagine (0.10), sodium propionate (4.0), K_2HPO_4 (0.50), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.10), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.001) and was sterilized by autoclaving at 121 °C and 1 bar for 20 minutes. For the test for the detection of Salmonella and Shigella, 0.2 ml of the medium after accumulation was used, which was inoculated with a loop or Drigalski spatula onto solid selective media (Ploskirev, Wilson-Blair). Crops were grown in a thermostat at 37 °C.

Further study of the morphological, tintorial, cultural and enzymatic properties of the isolated cultures was carried out according to the generally accepted methodology based on the growth pattern on solid and liquid nutrient media such as meat-peptone broth (MPB), MPA, enriched with starch, nitrates, etc. Sucrolytic properties were determined by inoculation into semi-liquid Hiss media. Proteolytic properties were determined by inoculation into milk and meat-peptone gelatin (MPG). Indole was determined using a paper indicator impregnated with oxalic acid solution, catalase – by reaction with hydrogen peroxide; acetone formation – by reaction with egg yolk; hemolytic activity – by the ability of microorganisms to break down hemoglobin by direct inoculation of the culture on blood agar (Persianova, 2010, Harley and Prescott, 2002, Connor et al., 2010). The quantitative characterization was established as the proportion (%) of rod-shaped microorganisms in the total number of detected microorganisms (Connor et al. 2010, Public Health England, 2015).

Screening of isolates for hydrocarbon biodegradation

To identify the isolates capable of utilizing hydrocarbons, 1 ml of cell suspension of each isolate was added to 5 ml of IMM (incomplete mineral medium), on n-hexadecane and 50/50 n-decane and n-hexadecane models, and incubated at 30 °C and 75 rpm. Isolates were also incubated in 5 ml of IMM supplemented with propionate (4 g/L) and asparagine (0.1 g/L) as a positive control. Growth was monitored by the residual amount of hydrocarbon additives after 10 days.

Biodegradation of n-hexadecane

For the biodegradation study, five milliliters of IMM nutrient medium were used, inoculated with 1 ml of a suspension of growing cells of the selected bacteria and supplemented with n-hexadecane (0.1% v/v) as a carbon source. In parallel, 0.4% (w/v) propionate as the sole carbon source was used as a positive control, while the medium supplemented with n-hexadecane without inoculation served as a negative control. Three replicates were incubated at 30 °C and 50 r/m. Experimental replicates of the test samples and control were used on day 10 to monitor residual n-hexadecane.

Five milliliters of n-hexane containing 0.1% n-dodecane as an internal standard was added to the test samples (5 ml) to extract residual n-hexadecane. The mixture was stirred at 50 m⁻¹ for 20 min. The organic phase was separated from the aqueous phase by freezing at -20 °C and analyzed by GC-FID. In the non-biodegradation mode, 1 ml of the sample was injected into a GC-FID (6850 Network GC System, Agilent Technologies, Santa Clara, CA) equipped with an HP-1 column (30 m × 0.320 mm × 0.25 µm) and nitrogen as a carrier gas. The temperature program was as follows: initial temperature: 50 °C, gradient at 20 °C/min to 130 °C, 1.5 °C/min to 150 °C, 4 °C/min to 172 °C with a hold for 5 min. The response coefficient was calculated as the ratio of the peak area of n-hexadecane to the peak area of 0.1% n-dodecane (internal standard) of each sample. Subsequently, the residual hexadecane was expressed as a percentage as the ratio of the response factor of the test samples to the response factor of the abiotic control at each sampling time to account for potential abiotic losses of n-hexadecane during incubation.

The research results were processed using the methods of mathematical statistics.

RESULTS AND DISCUSSION

The soil samples used for strain isolation contained a high diversity of bacteria and were characterized by varying degrees of oil contamination. The microbiological landscape, the presence of certain morphological groups of microbial contaminants, and the degree of sanitary and environmental contamination of the samples are shown in Table 1.

As it can be seen from the results presented in Table 1, the studied samples differ significantly in the number of different morphological groups of microorganisms and contamination. The dominant group is bacteria, including a significant number of thermophilic microorganisms and clostridia. It should be noted that the soil containing a lot of *Escherichia coli* and a small number of thermophiles is considered to be contaminated with feces, as the human and animal intestinal biota is very poor in thermophiles. Conversely, the soils that contain large numbers of *Escherichia coli* and thermophiles have been fertilized with manure, compost, or other organic contaminants. No microorganisms characterizing sanitary and epidemiological hazards were found in the soil samples.

The pH of the soil, which is characterized by lower rates of oil pollution, is shifted to a more alkaline side, which can be explained by more intense oxidation processes that occur in more contaminated soil with the production of acidic compounds.

For agricultural land (soils) (Prykhodko, 2015), the first level of pollution (slightly contaminated) is characterized by a soil pollutant content of 300–1000 mg/kg; the second level (moderately contaminated) – 1000–5000 mg/kg, the third level (highly contaminated) – more than 5000 mg/kg. For non-agricultural land, these limits are as follows: Level 1 – 1000–5000 mg/

Table 1. Sanitary and ecological condition of the soil by main indicators

Indicators	Sample 1	Sample 2
MAFANM, [CFU/g]	7.6·10 ⁸	4.1·10 ¹⁰
Mold fungi and yeasts, [CFU/g]	9.9·10 ⁴	8.1·10 ⁵
Number of thermophiles, [CFU/g]	950 000	3.4·10 ⁶
Titre of nitrifying bacteria, [g]	0.001	0.0001
Titre <i>Clostridium perfringens</i> , [g]	0.0001	0.00001
Titre <i>E. coli</i> , [g]	0.01	0.001
Bacterial titer of the genus <i>Proteus</i> , [g]	0.001	0.001
Mass fraction of oil products, [mg/kg]	2085	38375
The degree of contamination	Contaminated	Heavily contaminated

Table 2. Characterization of acid-forming soil bacteria and their potential for hydrocarbon biodegradation

Indicators	Sample 1	Sample 2
MAFANM, CFU/g	$7.6 \cdot 10^8$	$4.1 \cdot 10^{10}$
Mold fungi and yeasts, CFU/g	$9.9 \cdot 10^4$	$8.1 \cdot 10^5$
Number of thermophiles, CFU/g	950 000	$3.4 \cdot 10^6$
Titre of nitrifying bacteria, g	0.001	0.0001
Titre <i>Clostridium perfringens</i> , g	0.0001	0.00001
Titre <i>E. coli</i> , g	0.01	0.001
Bacterial titer of the genus <i>Proteus</i> , g	0.001	0.001
Mass fraction of oil products, mg/kg	2085	38375
The degree of contamination	Contaminated	Heavily contaminated

kg, Level 2 – 5000–10000 mg/kg, Level 3 – over 10000 mg/kg. In accordance with this classification, the samples under study are classified as slightly contaminated and highly contaminated.

After isolation of pure bacterial cultures from oil-contaminated soil, their properties, in particular the ability to biodegrade hydrocarbons, were studied. To facilitate the description of microorganisms, they were divided into morphotypes. Table 2 shows a description of the morphophysiological and biochemical properties of 76 isolated bacillus strains the spores of which do not modify the vegetative cell and which utilize arabinose, mannitol, and xylose to form acid without gas (6 morphotypes). These bacterial cultures had the following general properties: medium-sized rods $(0.6\text{--}0.8) \times (1.5\text{--}3.0) - (1.0\text{--}1.2) \times (3.5\text{--}5.0)$ mkl with elliptical spores located in the center and not exceeding the size of the cell. They are also gram-positive; 27 isolates had marked motility in overnight culture.

All 76 isolate species were tested for growth with 0.2% (w/v) crude oil and 0.2% (w/v) 50/50 mixture of n-decane and n-hexadecane (Table 2).

On the basis of the results above, only the representatives of morphotype I were able to grow inactively on crude oil or a mixture of n-alkanes as the only source of carbon and energy. Bacteria of morphotypes III–VI grew quite actively on aliphatic carbohydrates of different structure or a mixture of n-alkanes as the only source of carbon and energy.

According to the set of morphological and cultural features of the isolated cultures of the first and second morphotypes studied, it can be concluded that, despite the general coincidence of most individual indicators, they differ from each other in a few indicators (colony relief – representatives of morphotype I on MPA formed small grayish shiny colonies, and representatives of morphotype II – blurred; representatives of the

I morphotype produced crater-like gelatin liquefaction when inoculated, representatives of the II morphotype produced bag-like liquefaction, and representatives of the I and II morphotypes on MPB formed opacity and a thin film, but in the second case the broth was clarified; representatives of the I morphotype alkalized milk during peptonization) and can be grouped into the subtilis-licheniformis group. These cultures accounted for the largest proportion of the detected bacilli.

From the characteristics of the bacilli of the *Subtilis licheniformis* group presented in Table 2 shows that eight cultures from the third morphological group were characterized by smoother, whitish-shiny colonies on MPA that grew into the substrate, the formation of a thin film on MPA and opacity; peptonization of milk without coagulation; crater-like liquefaction of gelatin during inoculation into test tubes; lack of amylase and tyrosinase activity and the ability to reduce nitrates. Presumably, they were attributed to the species *Bacillus pumilis*, the number of which was insignificant and amounted to no more than 10% of the total number of bacilli contaminating the samples. In the fourth group of cultures, the microorganisms formed smooth grayish-white colonies on the MPA, caused turbidity of the MPA and the formation of a precipitate, did not change the appearance of milk, did not liquefy gelatin when sown in tubes, forming a shiny coating on the surface; they broke down maltose, did not break down mannitol. In the early stages of growth on glucose agar, the cells contained fatty balls. Spores were formed rapidly. All cultures exhibited lecithinase activity on yolk agar, formed acetoin and characteristic ruby colonies on salt agar with 2,3,5-triphenyltetrazolium chloride and indole, which confirmed their difference from *B. pumilis* and microorganisms of the

Subtilis licheniformis group. This allowed their identification as *B. cereus*.

Colonies of group V rods are rounded, grayish-white, pasty, with a matte surface, as in *B. cereus*, with a slightly wavy edge. Presumably, this group is formed by *B. thuringiensis* strains. *B. thuringiensis* species were not numerous in the soil samples studied.

The size, cellular structure, and folded macrorelief of the colony were important guidelines for identifying group VI bacilli, which distinguish them from the species described above.

Colonies on MPA are round, thick, convex, whole, shiny, and mucous. With the age of the culture, the substrate turns brown. On MPA, growth is scanty in the form of a haze, which creates a grayish surface coating on the gelatin medium, when sown in a column – a liquefaction in the form of a crater. Milk does not curdle, it is peptidized. In old cultures, when grown in MPA, fat was found. Representatives of the sixth group were identified as *B. megaterium*. The reactions of tyrosine cleavage and nitrate reduction varied depending on the age of the culture. The number of heat-resistant strains of these bacilli in soil samples does not exceed 9–14%.

Table 3 shows the characterization of the properties of 54 species of isolates of acid- and gas-producing bacteria from soil samples.

From the data presented in Tables 2 and 3, it can be concluded that the studied microorganisms exhibit significant activity in the biodegradation of the hydrocarbon complex of oil and oil products, which is expressed as a percentage of preservation of the initial hydrocarbon concentration on the 10th day of incubation of microorganisms in the environment of petroleum hydrocarbons. Thus, it can be noted that *Bacillus subtilis* and *Paenibacillus macerans* break down only 10% of petroleum hydrocarbons, *Bacillus licheniformis*

– 17%, *Bacillus pumilis* – 33%, *Bacillus cereus* – 47%, *Bacillus thuringiensis* – 28%, *Bacillus megaterium* – 31%, *Paenibacillus polymyxa* – 11%, *Paenibacillus circulans* – 48% of hydrocarbons. The strains of the studied microorganisms can be ranked in the following order of increasing biodegradability *Bacillus subtilis* and *Paenibacillus macerans* < *Paenibacillus polymyxa* < *Bacillus licheniformis* < *Bacillus thuringiensis* < *Bacillus megaterium* < *Bacillus pumilis* < *Bacillus cereus* < *Paenibacillus circulans*.

Thus, according to the ability to grow on model samples of n-alkanes, i.e., to use the biodegradation of petroleum hydrocarbons, out of 130 studied microorganism isolates, it is possible to identify the microorganisms of the IX morphogroup, which are identified as strains of *P. circulans*, and strains of the IV morphogroup, namely *Bacillus cereus*, which split 48% and 47% of hydrocarbons, respectively, were also characterized by similar activity.

The bacteria described in Table 3 are Gram-positive motile rods the spores of which are larger in diameter than cell thickness and are subterminal or terminal. They produce catalase, but are able to grow on IPA under anaerobic conditions, as well as hydrolyze starch, casein, reduce nitrates to nitrites, and do not produce indole, lecithinase, or tyrosinase. In contrast to the bacilli described in Table 2, when cultivated on media with arabinose, xylose, and mannitol, they form gas together with acid.

Group VII of bacilli consists of the microorganisms that grow poorly on IPA in the form of thin round beige colonies. They cause turbidity of the MPA and form a mucous precipitate. Gram staining of cells when cultivated in different media showed variability. These bacilli do not decarboxylate tyrosine and do not form acetylmethylcarbinol, and they do not liquefy gelatin. They

Table 3. Characteristics of acid- and gas-producing soil bacteria and their potential for hydrocarbon biodegradation

Indicators	Sample 1	Sample 2
MAFANM, CFU/g	7,6·10 ⁸	4,1·10 ¹⁰
Mold fungi and yeasts, CFU/g	9,9·10 ⁴	8,1·10 ⁵
Number of thermophiles, CFU/g	950 000	3,4·10 ⁶
Titre of nitrifying bacteria, g	0,001	0,0001
Titre <i>Clostridium perfringens</i> , g	0,0001	0,00001
Titre <i>E. coli</i> , g	0,01	0,001
Bacterial titer of the genus <i>Proteus</i> , g	0,001	0,001
Mass fraction of oil products, mg/kg	2085	38375
The degree of contamination	Contaminated	Heavily contaminated

form a weak surface coating on gelatin medium and do not cause liquefaction during sowing. Starch is completely hydrolyzed to mono- and disaccharides. Milk coagulates with the formation of gas, glucose, lactose, maltose are utilized with the formation of acid. The set of identified properties of this group basically coincides with the description of bacilli of the species *B. macerans* (formerly *B. aerosporus*), which are currently classified as *Paenibacillus* (Dzhej Dzh. M. et al., 2005, Bergey's Manual, 2005).

A distinctive feature of group VIII bacilli is the formation of mucus on dense and liquid substrates and slow liquefaction of gelatin. On MPA they form grayish shiny large colonies, on MPB – turbidity, sediment, grayish surface film. Milk does not coagulate and does not form a precipitate. Starch is hydrolyzed, gelatin is slightly liquefied (baggy liquefaction). These rods can probably be attributed to *P. polymyxa* species. Group IX includes the bacilli that form thin, spreading colonies on the surface of MPA. They cause weak acid formation in milk (slow coagulation). On gelatin media, they grow as a slight surface coating; when inoculated with a prick, growth is absent. Glucose, lactose, sucrose are converted to acid. Three isolates of this group after cultivation on different substrates were variably colored by Gram stain, the rest were positive. According to the type of respiration, they are classified as

facultative anaerobic microorganisms. They do not form acetoin, slowly liquefy gelatin, and hydrolyze casein. In most respects, the description corresponds to *B. circulans*, a number of strains belonging to the genus *Paenibacillus* (Bergey's Manual, 2005). This species is considered to be mesophilic, but thermophilic variants are reported in the literature (Doyle, 2006).

In the detection of bacilli of VII-IX morphotypes, it should be noted that the morphophysiological, cultural and biochemical properties of the studied cultures were not always convincing. On different media, some R-form colonies transformed into S-form colonies, which made it difficult to identify them by cultural and tinctorial characteristics. Difficulties in identification did not allow including the characteristics of some cultures in the tables and clearly determining the proportion of the studied isolates in the total number of bacilli detected on the raw materials under study. The study showed that accurate identification of rod-shaped microorganisms by classical methods is not only time-consuming and laborious, but also often difficult to identify accurately.

On the basis of the results of the description of the microorganisms found in the soil samples, a dendrogram (Fig. 1) was constructed using the hierarchical divisional cluster analysis method.

The dendrogram of microorganisms of soil samples shown in Figure 1, the dendrogram of

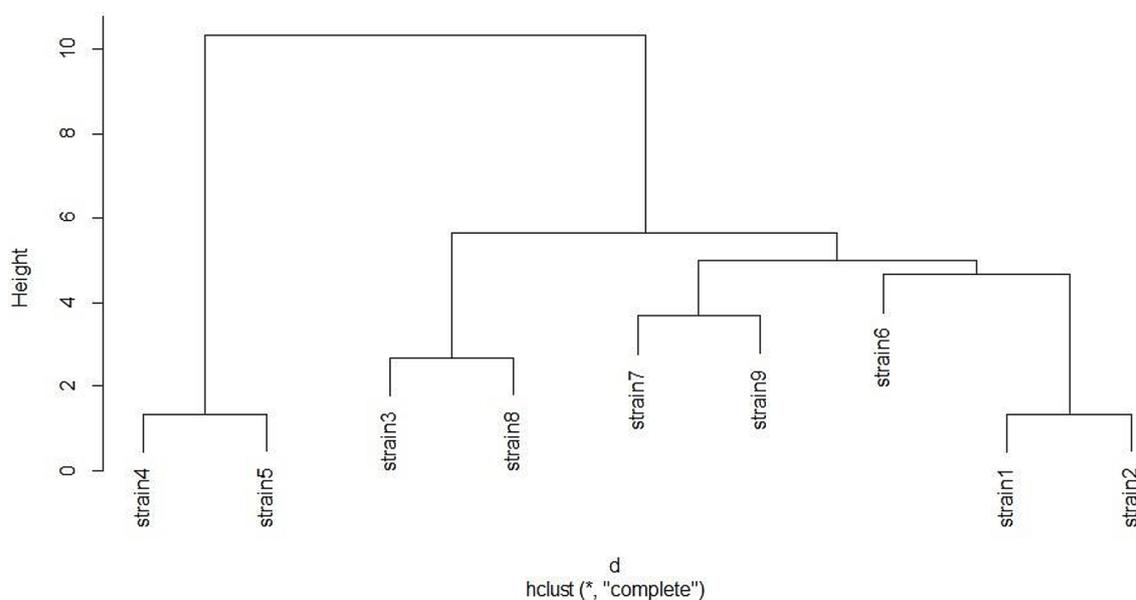


Figure 1. Dendrogram of the microorganisms of soil fractions contaminated with naphtha products (strain1 – *Bacillus subtilis*, strain2 – *Bacillus licheniformis*, strain3 – *Bacillus pumilis*, strain4 – *Bacillus cereus*, strain5 – *Bacillus thuringiensis*, strain6 – *Bacillus megaterium*, strain7 – *Paenibacillus macerans*, strain8 – *Paenibacillus polymyx*, strain9 – *Paenibacillus circulans*)

microorganisms of soil samples, which is based on their morphotintorial and biochemical properties, shows the presence of two clusters. The first cluster includes microorganisms belonging to the *Bacillus cereus* (strain 4) and *Bacillus thuringiensis* (strain 5) groups. The second cluster has branches and includes other defined groups of microorganisms. According to the screening of isolates for the ability to biodegrade petroleum hydrocarbons on day 10, the isolated strains can be arranged in the following sequence in descending order of this indicator: *Paenibacillus circulans* > *Bacillus cereus* > *Bacillus megaterium*.

CONCLUSIONS

The microbial contamination of oil-contaminated soil samples from oil depots at ports in southern Ukraine was characterized. It was found that bacteria dominate among the identified groups of microorganisms, the number of which is 3–5 orders of magnitude higher than the number of molds and yeasts. According to quantitative sanitary and hygienic, ecological indicators as well as titers of *E. coli*, *Clostridium perfringens*, nitrifying bacteria, and microorganisms of the genus *Proteus*, the soil samples can be classified as contaminated and highly contaminated.

On the basis of the study of morphological, tintorial, cultural, biochemical properties, 130 species were isolated and 9 morphogroups of bacteria were identified in the oil-contaminated soil samples. A dendrogram was constructed based on the set of studied properties of the isolated microorganisms. The screening of the isolated microorganisms for the ability to biotransform petroleum alkanes on the models of n-hexadecane and a mixture of 50/50 n-decane and n-hexadecane showed the ability to assimilate up to 48% of hydrocarbons by *Paenibacillus circulans* and up to 47% by *Bacillus cereus* strains.

Thus, the studied microorganisms of oil-contaminated soils are effective biodestructors of oil and oil products that can be used to eliminate oil spills and clean up environmental components from petroleum hydrocarbons.

REFERENCES

1. Abatenh, E., Gizaw, B., Tsegaye, Z., Wassie, M. 2017. Application of microorganisms in bioremediation

– review. J. Environ. Microbiol., 1(1), 2–9. Application of microorganisms in bioremediation-review (pulsus.com)

2. Abdulsalam, S., Bugaje, I., Adefila, S., Ibrahim, S. 2011. Comparison of biostimulation and bioaugmentation for remediation of soil contaminated with spent motor oil. Int. J. Environ. Sci. Technol. 8, 187–194.
3. Abousnina, R.M., Manalo, A., Shiau, J., Lokuge, W. 2016. An overview of oil contaminated sand and its engineering applications. Int. J. GEOMATE 10 (1), 1615–1622.
4. Adams, G.O., Fufeyin, P.T., Okoro, S.E., Ehinomen, I. 2015. Bioremediation, biostimulation and bioaugmentation: A review. Int. J. Environ. Bioremediat. Biodegrad. 3(1), 28–39. DOI: 10.12691/ijebb-3-1-5
5. Agarry, S., Latinwo, G.K. 2015. Biodegradation of diesel oil in soil and its enhancement by application of bioventing and amendment with brewery waste effluents as biostimulating-bioaugmentation agents. J. Ecol. Eng., 16(2), 82–91. doi: 10.12911/22998993/1861
6. Al-Zubaidi, I., Al-Tamimi, A. 2018. Soil remediation from lubricating oil. Environ. Technol. Innov. 9, 151–159. doi: 10.1016/j.eti.2017.11.004
7. Bergey's Manual of systematic bacteriology. 2005. The proteobacteric. Part A, Bergey's Manual Trust Department of Microbiology and Molecular Genetics Michigan State University, 2. 1 (springer.com)
8. Chaillan, F., Flèchi, A.L., Bury, E., Phantavong, Y., Grimont, P., Saliot, A., Oudot, J. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Res. Microbiol., 155, 587–595. doi: 10.1016/j.resmic.2004.04.006
9. Connor N., Sikorski J., Rooney P. et al. 2010. Ecology of speciation of the genus *Bacillus*, Appl. Environ. Microbiol., 76, 1349–1358. doi: 10.1128/AEM.01988-09
10. Das, N., Chandran, P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: An overview. Sage-Hindawi Biotechnol. Res. Int., 13, 941810. <https://doi.org/10.4061/2011/941810>
11. Doyle, M.E. 2006. Food spoilage microorganisms – losses and control strategies. Food Research Institute, University of Wisconsin–Madison Madison, WI 53706. Microsoft Word - Brief_Spoilage_7_07.doc (wisc.edu)
12. Dzhej, Dzh. M., Lessner, M. Dzh., Gol'den, D.A. 2005. Modern food microbiology, 7 edition, Springer, 886. <https://doi.org/10.1007/BF03174975>
13. Ehiosun, K., Godin, S., Urios, L., Lobinski, R., Grimaud R. 2022. Degradation of longchain alkanes through biofilm formation by bacteria isolated from oil-polluted soil. International Biodeterioration

- & Biodegradation, 175, 105508. <https://doi.org/10.1016/j.ibiod.2022.105508>
14. Harley, J.P., Prescott, L.M. 2002. Laboratory Exercises in Microbiology, The McGraw Hill Companies, N.Y., p. 466. laboratory-exercises-in-microbiology-book.pdf (wordpress.com)
 15. Head, J., Jones M., Larter, S. 2003. Biological activity in the deep subsurface and the origin of heavy oil. *Nature*, 426, 344–352. doi: 10.1038/nature02134
 16. Jang, K., Cho, S., Seok, S., Kong, W., Kim, G., Sung, J. 2009. Screening of biodegradable function of indigenous lingo-degrading mushroom using dyes. *Mycobiology*, 4, 53–61. doi: 10.4489/MYCO.2009.37.1.053
 17. Margesin, R., Labbe, D., Schinner, F., Greer, C., Whyte, L. 2003. Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Appl. Environ. Microbiol.* 69 (6), 3085–3092. doi: 10.1128/AEM.69.6.3085-3092.2003
 18. Mishra, S., Jyoti, J., Kuhad, R.C., Lal, B. 2001. In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Curr. Microbiol.*, 43, 328–335. doi: 10.1007/s002840010311
 19. Persianova, I.P. 2010. Microbiology of canning and microbiological control of canning production - Odessa: Izd. “Vneshreklamservis”. https://www.studmed.ru/persianova-i-p-gerasimenko-l-n-stoyanova-l-a-mikrobiologiya-konservirovaniya-pischevyh-produktov_431bd04f3fb.html
 20. Prykhodko, M. 2015. Remediation of lands contaminated with oil and oil products. *Constructive geography and geoecology. Scientific notes.*, 1, 176-180. <http://geography.tnpu.edu.ua/wp-content/uploads/2016/11/291.pdf>.
 21. Public Health England. 2015. Identification of Bacillus species, UK Standards for Microbiology Investigations, 9(3). www.gov.uk/uk-standards-formicrobiology-investigations-smi-quality-and-consistency-in-clinical-laboratories
 22. Ron, E., Rosenberg, E. 2014. Enhanced bioremediation of oil spills in the sea. *Curr. Opin. Biotechnol.* 27, 191–194. doi: 10.1016/j.copbio.2014.02.004
 23. Sharma, S. 2012. Bioremediation: features, strategies and applications. *Asian J. Pharmacy Life Sci.*, 2(2), 202–213. Microsoft Word - shilpi_et_al after correction (psu.edu)
 24. Varjani, S.J. 2017. Microbial degradation of petroleum hydrocarbons. *Bioresour. Technol.*, 223, 277–286. doi: 10.1016/j.biortech.2016.10.037
 25. Varjani, S., Rana, D., Jain, A., Bateja, S., Upasani, V. 2015. Synergistic ex situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India. *Int. Biodeterior. Biodegrad.*, 103, 116–124. <https://doi.org/10.1016/j.ibiod.2015.03.030>
 26. Varjani, S., Upasani, V. 2017. A new look on factor affecting microbial degradation of petroleum hydrocarbon pollutants. *Int. Biodeterior. Biodegrad.*, 120, 71–83 <https://doi.org/10.1016/j.ibiod.2017.02.006>
 27. Verma, S., Bhargava, R., Pruthi, V. 2006. Oily sludge degradation by bacteria from Ankleshwar, India. *Int. Biodeterior. Biodegrad.*, 57, 207–213. doi: 10.1016/j.ibiod.2006.02.004
 28. Yadav, K.K., Singh, J.K., Gupta, N., Kumar, V. 2017. A review of nanobioremediation technologies for environmental cleanups: A novel biological approach. *J. Mater. Environ. Sci.*, 8(2), 740–757. 78-JMES-2831-Yadav.pdf (jmaterenvironsci.com)
 29. Yang, Y., Javed, H., Zhang, D., Li, D., Kamath, R., McVey, K., Sra, K., Alvarez, P. 2017. Merits and limitation of TiO₂ based photocatalytic pretreatment of soils impacted by crude oil for expediting bioremediation. *Front. Chem. Sci. Eng.*, 11(3), 387–394.
 30. Yang, C., Kublik, A., Weidauer, C., Seiwert, B., Adrian, L. 2015. Reductive dehalogenation of oligocyclic phenolic bromoaromatics by *Dehalococcoides mccartyi* strain CBDB1. *Environ. Sci. Technol.*, 49(14), 8497–8505. <https://doi.org/10.1021/acs.est.5b01401>
 31. Yeung, A. 2010. Remediation technologies for contaminated sites, in: Chen, Y., Zhan, L., Tang, X. (Eds.), *Advances in Environmental Geotechnics*. Zhejiang University Press, Hangzhou, 328–369. doi: 10.1007/978-3-642-04460-1_25