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Experimental Investigation of a Pilot-Scale Concerning Ex-Situ Bioremediation of Petroleum Hydrocarbons Contaminated Soils

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Abstract: The soil samples were taken from the site of a former oil products depot from an industrial area (Romania). The soil samples taken were analyzed from a physical and chemical point of view: texture, pH, soil micronutrient content, metals concentration and petroleum hydrocarbon concentration (PHCs). The soil contaminated with total petroleum hydrocarbon (TPH (4280 mg kg^{-1}) was disposed in the form of a pile (L × W × H: 3000 × 1400 × 500 mm). Experiments on a pilot-scale were conducted over 12 weeks at constant pH (7.5–8), temperature (22–32 °C), nutrient contents C/N/P ratio 100/10/1, soil aeration time (8 h/day) and moisture (30%). Samples were taken every two weeks for the monitoring of the TPH and the microorganisms content. During the experiment, microorganisms were added (*Pseudomonas* and *Bacillus*) every two weeks. Results of the analyses regarding the concentration of PHCs were revealed a linear decrease of the concentration of PHCs after only two weeks of treatment. This decrease in concentration was also achieved in the following weeks. Following the analysis performed on the model at the pilot scale regarding the depollution process, it can be concluded that a soil contaminated with petroleum hydrocarbons can be efficiently depolluted by performing an aeration of 8 h/day, adding microorganisms *Pseudomonas* and *Bacillus* to ensure the conditions for increasing in the total number of germs (colony forming units–CFU) from 151 × 10^5 to 213 × 10^7 CFU g^{-1} soil, after 12 weeks of soil treatment—the depollution efficiency achieved is 83%.

Keywords: aeration; bioremediation; moisture; microorganism; petroleum hydrocarbons; soil

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1. Introduction

Currently, petroleum (oil) is the main energy source in the world [1]. Saturated hydrocarbons are the main components of natural gases and oil [2]. Petroleum hydrocarbons are natural chemical substances used by humans for many activities, being a complex mixture of a wide range of chemicals found in crude oil and refined products [1,3].

The development of the oil industry is sometimes accompanied by the appearance of certain side effects, as it pollutes the environment and affects the health of the human population [4]. Environmental pollution with PHC through leaks and spills taking place during production, storage and transport of oil causes water and soil pollution, affecting the safety of ecosystems and human health, thus becoming a global environmental problem [5,6]. The most disastrous effects occur when crude oil doses exceed 200 mg kg^{-1} of soil [7].

Soil pollution caused by petroleum and its derivatives is one of the most widespread environmental problems [8]. Much of this pollution has resulted from the increased activities associated with petroleum exploration, transport and processing. In addition, the lack of waste oil recycling and the disposal of hazardous oil wastes into landfills without sufficient management has further increased the number of contaminated sites [9].

Soil pollution by petroleum hydrocarbons and aromatic hydrocarbons/polyaromatic is one of the most obvious and important environmental problems faced by Romania.

Counties where the soil is affected by oil pollution are: Bacau, Bihor, Covasna, Gorj, Prahova and Timis. Hydrocarbons soil pollution can be produced accidentally, and just in 2011 in Romania, there were 45 accidental pollutions with oil products and polluting substances of organic origin [10,11]. Out of a total of 1393 contaminated or potentially contaminated sites existing in Romania, about 860 are due to the activity of extraction, processing, transport or storage of hydrocarbons [12].

Studies have shown that bioremediation is a safer and less expensive method for removing dangerous contaminants and producing secondary non-toxic substances [13]. Interest for polluted soil and water bioremediation has increased over the past thirty years [14], primarily due to the fact that bioremediation is based on the ability of some microorganisms (bacteria, fungi) to degrade organic matter and allow the acceleration of natural decomposition of organic pollutants [15–17]. The success of bioremediation depends on a series of factors: the affected area, type, amount and concentration of pollutants, soil pH, ambient temperature, soil moisture, amount of nutrients, type and amount of microorganisms and oxygen availability, available time and financial resources [18,19].

The influencing factors depend on the degradability of contaminants and environmental conditions:

- *Concentration of contamination*: In the event of their concentrations being too high, the contaminants will be able to fully develop their possibly existing toxic effects on bacteria, thus preventing their degradability. In the event of the concentrations of contaminants being too low, however, degradation enzymes will not be induced. Bacteria will, first of all, tend to utilizing the substrate degradable more easily and only then to form enzymes for degrading more complicated substrates. However, in a very heterogeneous soil system, degradation will proceed in a way not frequently making these reactions obvious [20];
- *Availability of nutrients*: If the degradation will be limited owing to lacking nutrients, electron acceptors or donators and nutrient salts will have to be added to accelerate the degradation processes. As to nutrient salts, mostly only nitrogen and possibly phosphorus compounds (macronutrients) will have to be added, as other nutrient salts (micronutrients) required for degrading contaminants are frequently available in soil in a sufficient quantity [20];
- *pH:* Microbial degradation processes proceed preferably at a pH of approx. 6–8. For fungi, a pH of approx. 5 is better suited [20];
- *Temperature*: The temperature of the contaminated environment significantly influences the activity of microorganisms. In general, the optimum temperatures conducive to good microbial activity are between 20–37 °C [21];
- *Water content:* A water content of approx. 40–60% of the maximum water capacity of the soil will be optimal for degradation reactions in the unsaturated soil zone. In drier soils, the degradation speed is reduced, in wetter soils water-saturated partial areas (microcompartments) are formed where the supply with oxygen and thus also the degradation will be retarded [20];
- *Redox potential and oxygen content* characterize oxidizing or reducing conditions. The oxygen (O_2) or oxygenated compounds in the soil or in water lead to the acceleration of the biodegradation process [20].

Among the microorganisms used, bacteria occupy the dominant place. In most cases, the native microflora of the polluted area can be the basis of microorganisms needed for bioremediation [21].

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. The main principle of aerobic degradation of hydrocarbons: The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle [22].

For the biodegradation of petroleum hydrocarbons, natural species are used, existing in the soil, of the thype *Arthrobacter*, *Achromobacter*, *Novocardia*, *Pseudomonas*, *Flavo-bacterium*, etc. [21]

Bioremediation is one of the remedial techniques used a lot in countries such as Austria, Belgium, France, USA, with a share between 12 and 35% of the total techniques used [23].

In addition, bioremediation technology is believed to be noninvasive and relatively cost-effective [24]. Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment [25] and is cheaper than other remediation technologies [26].

Bioremediation has several disadvantages: it requires long remediation time, it is climate dependent and its effect is not fully elucidated [27–30].

The biopile method can be used for decontamination of soil polluted with 83% aromatic polycyclic hydrocarbons—nine months of treatment, and for the decontamination of a soil polluted with 60–73% mineral oils after seven months of treatment [31]. To achieve a maximum level of biodegradation by using the biopile method, the aeration and irrigation systems' designs are important [32]. Natural and forced aeration (blowing or extracting air through pipes) can be introduced to improve soil ventilation in order to assure the oxygen supply needed for the bio-reactions taking place in the pile of polluted soil [33].

The efficiency of the biopile method has been demonstrated in several studies, being depolluted soils contaminated with oil: 80–85.2% [34,35]; hydrocarbons: 66–75% [36]; TPH: 75% [37].

Studies have shown that using indigenous microbial strains is preferred in bioremediation processes of soil polluted by total petroleum hydrocarbons [38–40]: efficiency of 55–59% using *Rhodococcus* [41] and 40% using by *Streptomyces* sp. *Hlh1* [42], and adding nutritive substances may increase the efficiency [43,44]: removal of diesel fuel 70% [45] and PHC 60% [46].

A pilot scale bioremediation study of soil contaminated with PHC from a sub-arctic site indicated that aeration and moisture addition were sufficient to obtain efficient biodegradation, while supplementation of nitrogen did not influence the efficiency of biodegradation [47].

Considering the environmental problems created by the pollution of the soils with petroleum hydrocarbons, it is necessary to carry out studies and researches in order to remediation the contaminated soils.

Starting from the results obtained previously through experiments at laboratory level [48], the objective of the study was to determine the yield of the ex situ bioremediation process of soils contaminated with PHC using a pilot scale treatment set that controls soil aeration duration, moisture and microorganism content.

2. Materials and Methods

2.1. Soil Sample Investigation

In order to carry out the pilot scale experiment, soil was collected from the site of a former warehouse of petroleum products from an industrial area (Romania). The taken soil samples were transported into the laboratory where the stones and roots were removed and the soil was mixed to ensure homogeneity of the sample. Soil thus prepared (samples P1, P2 and P3) was quantitatively and qualitatively analyzed:

- The soil's texture was determined using a gravimetric method;
- The soil pH was determined in 1/2.5 (w/v) soil/water extract using a HANNA pH-meter;
- Nitrogen was determined by Kjeldhal [49];
- For determining total potassium and phosphorus content 3 g of soil with 100 µm granulation was used over which was added 7 mL of 12 M HCl and 21 mL of 15.8 M

HNO_3 and the mixture was refluxed for 2 hours, filtered and diluted up to 100 mL with 2% (w/v) HNO_3 [50];

- Mobile phosphorus and potassium were determined by inductively coupled plasma optical emission spectrometer (ICP-OES) after extraction of 5 g soil in 100 mL ammonium acetate–lactate mixture (pH = 3.75) for 4 hours according to Egnèr–Riehm–Domingo method;
- The organic carbon was determined by Walkley–Black method by oxidizing the organic matter from 0.2 g soil with 5–10 ml of 1.6% (w/v) sulfochromic mixture on a hot plate for 20 min. The excess of chromic acid was titrated with 0.2 mol L^{-1} Mohr salt solution in the presence of diphenylamine as an indicator;
- Hydrophysical indices: withering coefficient, field capacity and useful capacity were determined taking into account soil moisture that was determined by the gravimetric method [49,51];
- The PHC content was determined by Fourier Transformed infrared spectroscopy (FTIR) [5]. The dry soil (5–10 g) was subjected to 2 consecutive extractions with 20 mL tetrachlorethylene (TCE) for 30 min/extraction. After extraction, the supernatant was separated from the soil residue. Polar compounds (water, vegetable oils and animal fats) were removed and applied by passing the extract through a 10 cm long and 0.6 cm with column packed with 0.150–0.250 mm grain-size magnesium silicate for column chromatography (Florisil). The purified extract was made up to 50 mL with TCE. The FTIR spectrum of the purified extract was recorded between 3150–2750 cm^{-1} at 4 cm^{-1} resolutions in 10 mm optical path-length quartz cells by a Spectrum BX II (Perkin Elmer) spectrometer equipped with DTGS detector. The measured absorbance at 2925 cm^{-1} attributed to CH_2- group was converted to TPH using the linear regression model. The TPH content of the soil was calculated according to Equation (1) [5]:

$$C = \frac{c \cdot D_f \cdot V}{C_f \cdot w} \quad (1)$$

where:

C is the concentration of TPH in soil (mg kg^{-1});
c—the concentration of TPH in the extract (mg mL^{-1});
D_f—the dilution factor;
C_f—the concentration factor;
V—the volume of the extract (mL);
w—the weight of the sample (kg).

Microorganisms were isolated from the investigated soil and grown on GPS culture media prepared from sodium L-glutamate–10 g L^{-1}, starch soluble–20 g L^{-1}, potassium dehydrogenate phosphate–2 g L^{-1}, magnesium sulfate–0.5 g L^{-1}, phenol red–0.36 g L^{-1} and agar–12 g L^{-1}. The isolated strains were stored and multiplied in a nutrient broth: meat extract–10 g L^{-1}, peptone–10 g L^{-1}; NaCl–5 g L^{-1} [48].

Bacterial strains were incubated and shaken (120 rpm) at 25 °C for 24 h. Inoculation was made with 100 µL of culture from liquid culture medium. The development of microorganisms was observed at 600 nm were using a UV spectrophotometer (Lambda 25, Perkin-Elmer). These cultures were morphologically and tinctorially characterized using the Gram staining technique [48].

The amount of soil microflora in the samples taken from the experimental groups was established by the numerical determination of the microorganisms existent in the PHC polluted soils. For the numerical determination of microorganisms existing in the soil, decimal dilutions (saline solution) were made. Incubation was done at 30 °C for 48–72 h [48].

2.2. Pilot Scale Experimental Investigation

Soil polluted with petroleum hydrocarbons (4000 kg), after sorting and homogenization was added the microorganisms and placed in a pile (L × W × H: 3000 × 1400 × 500 mm) in order to be subjected to the proposed experiments (Figure 1). The pile of soil was placed on an impermeable surface consisting of a concrete platform over which plastic foil was added. At the bottom of the pile was placed a drainage layer made of gravel with a 4–7 mm diameter, which favors the aeration process. In this layer of gravel was introduced a part of the pipe system through which aeration and wetting are carried out.

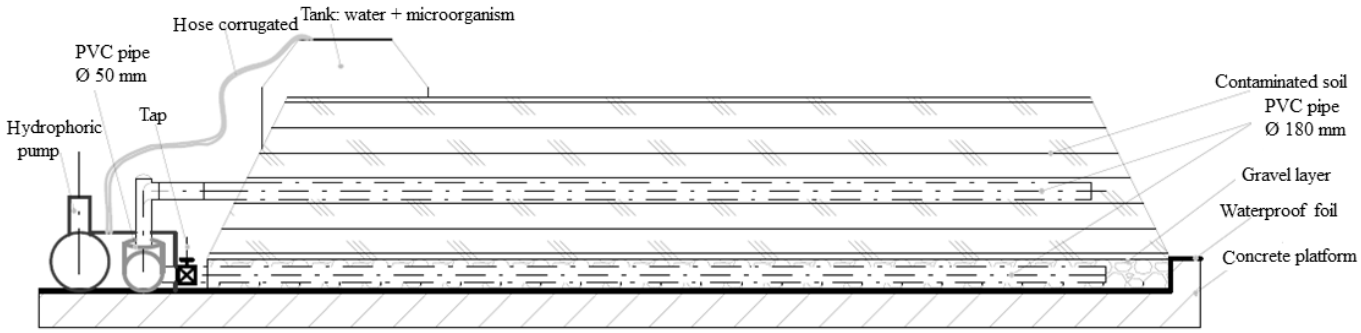


Figure 1. Pilot scale experiment.

The system for introducing water and the nutrients and microorganisms solution (*Pseudomonas* and *Bacillus*) consists of a tank with a capacity of 100 L, a self-priming pump with a flow of 50 L/min, corrugated absorption and discharge hoses and a blower. The discharge hose is connected to the distribution network that consists of an Ø180 mm PVC pipe branched into 5 perforated Ø50 mm PVC pipes placed horizontally in the middle of the pile.

Research carried out at pilot scale level lasted 12 weeks at the following parameters: 30% moisture, a temperature of 20–32 °C, pH of 7.5–8, 8 h/day aeration duration.

Soil moisture was manually achieved by sprinkling through the top of the biopile cell and through the water supply system inside of the pile, aiming for a field capacity moisture of 30%. Moisture was monitored throughout the experiment.

The temperature at which the pilot scale experiment was carried out was between 25–32 °C. Temperature was measured and monitored throughout the experiment with the WTW Multiline IDS-3430 Multiparameter.

Soil pH was monitored weekly using the HI 3512-02 pH meter.

Soil aeration was achieved through the aeration system consisting of a blower and an air distribution network. The air distribution network consists of 5 perforated PVC pipes with a diameter of 50 mm each. These pipes are distributed inside the pile of soil as follows: three pipes are placed horizontally in the gravel layer at the base of the pile and two pipes are placed in the middle of the pile in order to ensure uniform aeration of the soil. Aeration was performed for 8 hours a day throughout the experiment (12 weeks) by means of the blower, having a flow of 10 m^3 min^{-1}.

Quantity of microorganisms. Microorganisms used for bioremediation were isolated from the native micro flora of the polluted soil and grown in the laboratory on culture media.

When making the soil pile for the experiment, after placing each layer of soil with a thickness of 10 cm, a manual sprinkling of the soil was carried out with a solution loaded with microorganisms—a total quantity of 9 L with a concentration of 94 × 10^3 CFU g^{-1}. Throughout the experiment microorganisms were added once every 2 weeks (weeks 2, 4, 6, 8, 10) by means of the perforated pipes inside the pile of soil—the quantity of 9 L with a concentration of 94 × 10^3 CFU g^{-1} and by manual sprinkling through the upper part of the pile of soil.

Soil sampling. Before adding the microorganism loaded solution, soil samples were taken from the pile of soil every 2 weeks (weeks 2, 4, 6, 8, 10, 12) in order to determine the

hydrocarbon concentration (150 g of soil/sample) and to determine the total number of germs (50 g of soil/sample). The determination of the number of microorganisms present in the biopile cell during the experiment can be done according to point 2.2.

Soil samples were taken from 3 points at different depths, according to the diagram in Figure 2, with a total of 9 soil samples being taken every week. These soil samples were coded according to their location, sampling depth and week in which they were taken (Table 1).

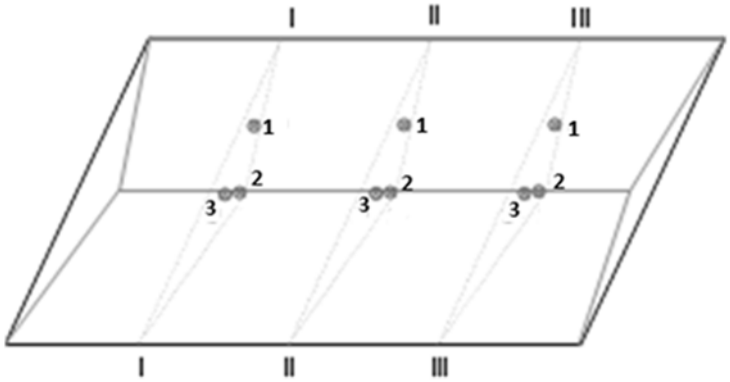


Figure 2. Soil sampling scheme.

Table 1. The code of soil samples according to their sampling location and depth.

The Sampling Section	Sampling Depth	The Code
I-I	Sample 1: 0–5 cm	I.1
	Sample 2: 15–25 cm	I.2
	Sample 3: 25–35 cm	I.3
II-II	Sample 1: 0–5 cm	II.1
	Sample 2: 15–25 cm	II.2
	Sample 3: 25–35 cm	II.3
III-III	Sample 1: 0–5 cm	III.1
	Sample 2: 15–25 cm	III.2
	Sample 3: 25–35 cm	III.3

2.3. The Evaluation of the Effectiveness

The evaluation of the effectiveness of the depollution process was performed by determining the yield for each sample with the following Equation (2) [52]:

$$\eta = \frac{C_i - C_f}{C_i} 100\ [\%] \quad (2)$$

In which:
η is the yield, in %;
C_f—PHC concentration in soil at the end of the treatment time, in mg kg^{-1};
C_i—initial PHC concentration of soil, in mg kg^{-1}.

3. Results and Discussion

3.1. Soil Samples Investigation

The soil was classified as clay medium (LL): 41.6% clay, 17.6% silt, 18.7% fine sand and 22.1% coarse sand according to the USDA classification (United States Department of Agriculture).

The test soil has a weak basic reaction (pH = 7.5 ± 0.5), a content of 2.3% total organic carbon and 0.1% total nitrogen. The investigated soil has a moderate humus content (4%). A high content of potassium (KAL = 272 mg kg^{-1}) and soluble phosphorus (PAL = 19.2 mg kg^{-1}) has been registered.

The concentration of Cu (28.1 mg kg^{-1}), Ni (33.5 mg kg^{-1}) and Pb (76 mg kg^{-1}) exceeds the normal value (20 mg kg^{-1}), according to the Romanian legislation (Order 756/1997) [53], being below the alert threshold. The concentration of Zn (174.6 mg kg^{-1}) exceeds the normal value (100 mg kg^{-1}). The Manganese level found in the analyzed soil (698 mgkg^{-1}) presents lower concentrations compared to the normal value (900 mg kg^{-1}) (Order 756/1997) [53].

The investigated soil has the average field capacity (25.5%) that is used together with the withering coefficient (13%) and the useful capacity (15.5%).

The average concentration of PHC in the tested soil was of 4280 ± 400 mg kg^{-1}. This value was considered the initial content of PHC for the amount of TPH in the pile of soil. The initial concentration of PHC exceeded more than 2 times the intervention threshold for less sensitive soil uses (2000 mg kg^{-1}) established by Romanian legislation (Order no. 756/1997) [53], thus requiring remediation.

In the test soil two bacterial strains with bioremediation potential were isolated using a selective enrichment technique. These cultures are from genus *Pseudomonas* sp. and *Bacillus* sp. (1 mL solution contains 94 × 10^3 CFU).

3.2. Pilot Scale Experiment Investigation

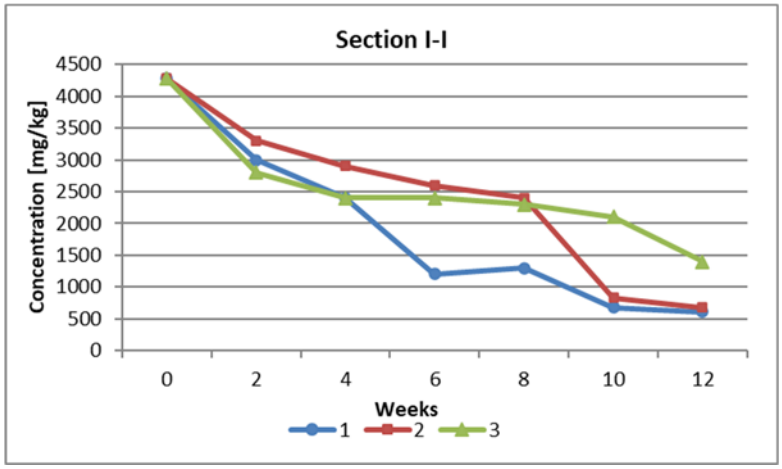
3.2.1. PHC Concentration

Results on the evolution of hydrocarbon concentration in the soil samples taken during experiments were subjected to an analysis in order to highlight the influence of treatment duration and to determine the efficiency of the bioremediation process.

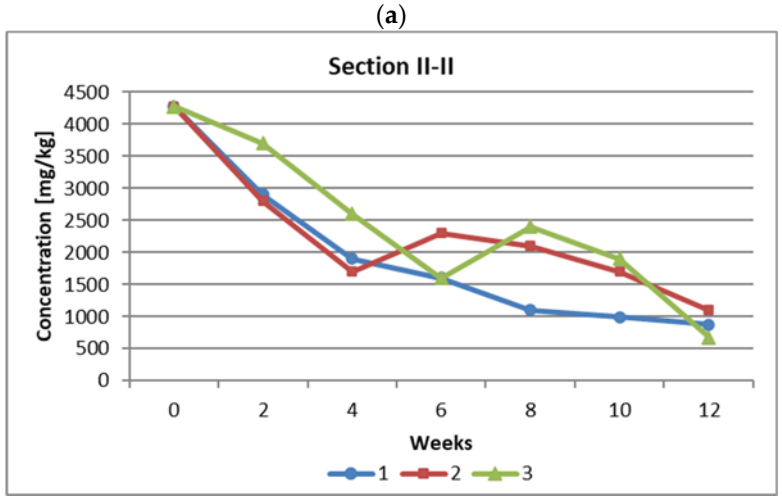
In Figure 3 is presented the evolution of PHC concentration in the nine soil samples taken from the pile of soil throughout the experiment depending on the sampling section (I–I; II–II; III–III) during the experiment (12 weeks).

Analyzing Figure 3, we observe a pronounced and linear decrease of the PHC concentration in the first 4 weeks for all the 3 sections, regardless of the sampling depth (1; 2 and 3). In the case of section I–I (Figure 3a), the PHC concentration from sample 1 continues with a sharp decrease being followed by stability and starting from week 8 the concentrations have a pronounced decrease. In sample 2, we can observe the same tendency starting with week 8 and the PHC concentration by the end of the experiment (680 mg kg^{-1}) is similar to the one in sample 1 (610 mg kg^{-1}), while sample 3 has slightly higher concentrations (1300 mg kg^{-1}).

In section II–II we can observe a continuous and linear decrease for sample 1 throughout the experiment, while samples 2 and 3 show an increase in week 6 and 8, respectively, after which a linear decrease is recorded until the end of the research period, PHC concentrations reaching around 680–1100 mg kg^{-1}.



(**a**)



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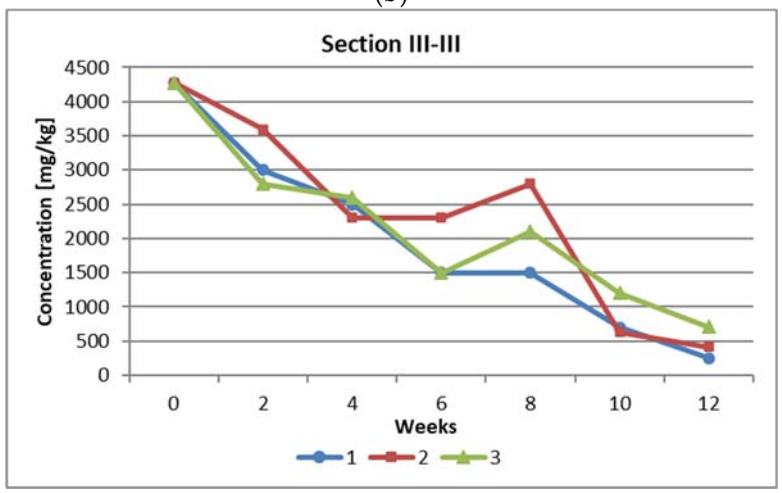

(**c**)

Figure 3. PHC concentration depending on time and section: (**a**) section I-I; (**b**) section II-II; (**c**) section III-III.

Section III–III shows a decrease in the PHC concentration (250–710 mg kg^{-1}). Several stability areas can be observed: sample 3 (weeks 2–4), sample 2 (weeks 4–6) and sample 1 (weeks 6–8), followed by a significant increase (samples 2 and 3) in week 8, except for sample 1, which decreases after the stability period to a value of 250 mg kg^{-1}. After the increase from week 8, samples 2 and 3 show a decrease until the end of the research period (410 mg kg^{-1} and 710 mg kg^{-1}, respectively).

Analyzing Figure 3a, linear decrease can be observed for all nine soil samples. All samples show a pronounced decrease in the PHC concentration after only two weeks of treatment, regardless of sampling point or depth. This decrease of concentration continues, but the amount of TPH extracted from the soil is much lower as of week 4. At the end of the experimentation period (12 weeks), it can be observed that the value of the concentration of pollutant decreased below 1000 mg $kg^{-1,}$ except for samples I.3 and II.2.

Concerning the concentration of PHC (Figure 3a) in the three sampling sections (I, II, III), a linear decrease can be observed throughout the experiment and more pronounced after only 2 weeks. In week 2, the concentrations are close in all the three sampling sections, lower concentrations can be observed for section II in weeks 4 and 8 of experiments, and in weeks 6 and 10 they are higher compared to section I and section III. The value of the PHC concentration decreases, being under 1000 mg kg^{-1} in week 12 in the 3 sections at sampling points, the lowest value being in section III (250 mg kg^{-1}).

Concerning the concentration of PHC (Figure 3b) in the three sampling sections, a linear decrease of the pollutant concentration can be observed throughout the experiment. Section II shows lower values compared to sections I and III in weeks 2, 4 and 8 of the experiments. Towards the end of the testing period (week 10), the PHC concentration in section II is much higher than in section I and in section III, where the concentration is similar. In the last week, there is a stability in section I and a decrease in sections II and III. The lowest concentration is reached in week 12 in section III (250 mg kg^{-1}).

PHC concentration (Figure 3c) is decreasing during the experiment. In week 2, the concentration of pollutant in section II is much higher than in sections I and III. In weeks 4 and 8 in all three sections, the concentration has approximately the same value. Low values can be observed at the end of the experiment in section II (680 mg kg^{-1}) and section III (710 mg kg^{-1}), except for section I (1400 mg kg^{-1}).

Analyzing from the treatment duration point of view, a linear decrease for all the three sampling sections can be observed. All samples show a pronounced decrease in the concentration of PHC after only two weeks of treatment, regardless of the depth at which research is carried out. This decrease in concentration is also achieved in the following weeks, but the amount of TPH extracted from the soil is much lower starting from week 4. At the end of the treatment period, values under 1000 mg kg^{-1} were registered.

Following analyses on the concentration of PHCs, a linear decrease of the PCH concentration was revealed after only two weeks of treatment. This decrease in concentration is also achieved in the following weeks, but the amount of TPH extracted from the soil is much lower starting from week 4 and week 6 of treatment, respectively.

Results obtained at the pilot scale level are similar to those obtained in the preliminary study at laboratory scale level [48], with a pronounced decrease in the first weeks of treatment followed by a much slower decrease in the concentration of PHC.

3.2.2. Quantity of Microorganisms

When the selected microorganisms are added to the soil on the soil to the experiment, an increase in the total number of germs (NTG) is observed independent of the sampling place (Table 2). Concentration of microorganisms in the second week had values between 102×10^5–209×10^5 CFU g^{-1} of soil, followed by an increase in weeks 4 and 6 (106×10^5–298×10^6 CFU g^{-1} of soil). This growth trend is also observed in the following weeks, but after week 8 they are not so significant (127×10^7–238×10^7 CFU g^{-1} of soil).

Analyzing the evolution of the number of microorganisms during the ex situ bioremediation process, a significant increase can be observed in the three sections. This is due to the fact that once every 2 weeks, a new amount of microorganisms was added, but also to the fact that they have the optimal conditions for their development (soil aeration and temperature).

Table 2. Determination of total microflora during the experimental development stage.

Week	Total Number of Germs (NTG)								
	SECTION I–I			SECTION II–II			SECTION III–III		
	1	2	3	1	2	3	1	2	3
0	94×10^3	94×10^3	94×10^3	94×10^3	94×10^3	94×10^3	94×10^3	94×10^3	94×10^3
2	144×10^5	175×10^5	126×10^5	160×10^5	121×10^5	102×10^5	209×10^5	184×10^5	142×10^5
4	125×10^6	127×10^6	106×10^6	158×10^6	176×10^6	85×10^6	183×10^6	162×10^6	199×10^6
6	206×10^6	198×10^6	238×10^6	208×10^6	188×10^6	124×10^6	274×10^6	235×10^6	298×10^6
8	168×10^7	144×10^7	159×10^7	131×10^7	127×10^7	147×10^7	158×10^7	141×10^7	133×10^7
10	170×10^7	130×10^7	144×10^7	165×10^7	165×10^7	207×10^7	171×10^7	162×10^7	153×10^7
12	201×10^7	185×10^7	190×10^7	226×10^7	238×10^7	259×10^7	219×10^7	199×10^7	197×10^7

The increase in the total number of germs is also dependent on the amount of hydrocarbons in the soil, but this microbiological load is not relevant for the bioremediation process due to the fact that it was added in the experimental samples, but its contribution to reducing the amount of pollutant to the end of the experiment, respectively, of the bioremediation process.

3.2.3. The Evaluation of the Effectiveness

Analyzing Figure 4, it can be observed that the depollution efficiency is high, reaching 94% for sample III.1. The lowest efficiency is registered for sample I.3 (67%), and the rest of the samples have efficiency around 80%.

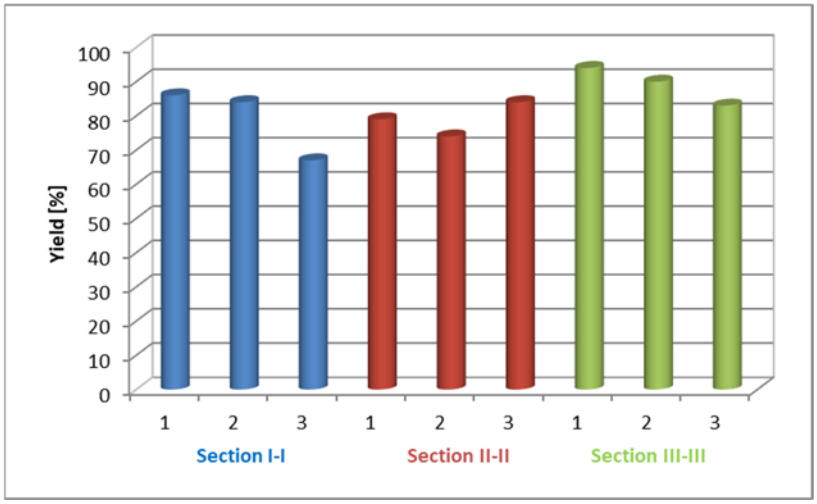

Figure 4. Depollution yield.

Compared to the results obtained in the preliminary study carried out at a laboratory scale level [48], which highlighted the possibility of obtaining high efficiencies (56–76%), the treatment period being 18 weeks on soil containing 7600 ± 400 mg kg^{-1} petroleum hydrocarbons, results of pilot scale experiments carried out under slightly different conditions indicate a reduction in the PHC concentration (64–94%) in only 12 weeks. These results were partially validated by submitting a patent application [54] to the Official Bulletin of Industrial Property (OSIM). It may also be mentioned that the obtained yields are comparable with yields obtained in other research conducted with similar treatment conditions: 80–85.2% [35] and 66–75% [36].

Yields obtained in a relatively short time (3 months) are considerably higher than those obtained by Lecomte (1998): 60–73%—7 months [31], Gogoi et al., (2003): 75%—12 months [37] and Chemlal et al., (2012): 70%—40 days [45].

The results are much better than results obtained by adding microorganisms belonging to genera *Rhodococcus*–55–59% [41]; *Streptomyces* sp. *Hlh1*–40% [1] and *Acinetobacter SZ*–1–34% [46]. The obtained results support the claims of Cerquira et al., (2011) and Suja et al., (2014), according to which indigenous microbial strains are recommended for use in the bioremediation process [38].

4. Conclusions

All samples show a pronounced decrease in the concentration of TPH after only two weeks of treatment, regardless of the sampling point or depth. During the experiment, there was an increase in the number of microorganisms, which led to a significant decrease of TPH. Yields obtained in the pilot scale experiment (64–94%), are comparable or even higher than yields obtained by other researchers.

Following the analysis performed on the model at the pilot scale regarding the depollution process, it can be concluded that a soil contaminated with petroleum hydrocarbons can be efficiently depolluted by performing an aeration and adding indigenes microorganisms *Pseudomonas* and *Bacillus*.

The results obtained from a very large number of experiments, performed using modern research methods, are relevant and represent a scientific contribution to research on ex situ bioremediation of soils polluted with oil hydrocarbons.

The value and accuracy of the obtained results constitute the basis of their application in practice as well as the approach of new research topics.

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References

1. Bastida, F.; Jehmlich, N.; Lima, K.; Morris, B.E.L.; Richnow, H.H.; Hernández, T.; García, C. The ecological and physiological responses of the microbial community from a semiarid soil to hydrocarbon contamination and its bioremediation using compost amendment. *J. Proteom.* **2016**, *135*, 162–169. [CrossRef]
2. Cristorean, C.; Micle, V.; Sur, I.M. A critical analysis of ex-situ bioremediation technologies of hydrocarbon polluted soils. *ECOTERRA* **2016**, *13*, 17–29.
3. Zhou, Q.; Cai, Z.; Zhang, Z.; Liu, W. Ecological Remediation of Hydrocarbon Contaminated Soils with Weed Plant. *J. Resour. Ecol.* **2011**, *2*, 97–105.
4. Adegbola, G.M.; Eniola, K.I.T.; Opasola, O.A. Isolation and identification of indigenous hydrocarbon tolerant bacteria from soil contaminated with used engine oil in Ogbomoso, Nigeria. *Adv. App. Sci. Res.* **2014**, *5*, 420–422.
5. Miclean, M.; Levei, E.; Gog, A.; Ferenczi, L.; Majdik, C.; Puia, C.; Roman, C. Determination of total petroleum hydrocarbons in contaminated soil by FTIR and GC-FID methods. *Studia Univ. Babes-Bolyai Chem.* **2010**, *55*, 83–91.
6. Sihag, S.; Pathak, H.; Jaroli, D.P. Factors Affecting the Rate of Biodegradation of Polyaromatic Hydrocarbons. *Int. J. Pure App. Biosci.* **2014**, *2*, 185–202.

7. Buzmakov, S.; Egorova, D.; Gatina, E. Effects of crude oil contamination on soils of the Ural region. *J. Soils Sediments* **2019**, *19*, 38–48. [CrossRef]
8. Volkering, F.; Breure, A.; Rulkens, W. Microbiological aspects of surfactant use for biological soil remediation. *Biodegradation* **1998**, *8*, 401–417. [CrossRef]
9. Stroud, J.L.; Paton, G.I.; Semple, K.T. Microbe-aliphatic hydrocarbon interactions in soil: Implications for biodegradation and bioremediation. *J. Appl. Microbiol.* **2007**, *102*, 1239–1253. [CrossRef]
10. Dumitru, M.; Manea, A.; Ciovanu, C.; Dumitru, S.; Vrinceanu, N.; Calciu, I.; Tanase, V.; Preda, M.; Risnoveanu, I.; Mocanu, V.; et al. *Soil Quality Monitoring in Romania*; National Research and Development Institute for Soil Science, Agrochemistry and Environment—ICPA: Bucharest, Romania, 2011.
11. Annual Report Status of the Environmental Factors in Romania, 2010, Chapter 4. Land Use. Available online: http://www.anpm.ro/ (accessed on 15 June 2021).
12. Annual Report Status of the Environmental Factors in Romania, 2015, Chapter 4. Land Use. Available online: http://www.anpm.ro (accessed on 15 June 2021).
13. Ward, O.P.; Singh, A.; Van Hamme, J. Accelerated biodegradation of petroleum hydrocarbon waste. *J. Int. Microbiol. Biotechnol.* **2003**, *30*, 260–270. [CrossRef]
14. Rogers, J.E. Soil bioremediation: Review of land treatment and composting options. *Land Contam. Reclam.* **1998**, *6*, 215–222.
15. Sinke, A.; Hecho, I. *Monitored Natural Attenuation: Review of Existing Guidelines and Protocols*; TNO-Nicole Report TNO-MEP R99/313; TNO Institute of Environmental Science, Energy Research and Process Inovatin: Apeldoom, The Netherlands, 1999.
16. Khan, F.I.; Husain, T.; Hejazi, R. An overview and analysis of site remediation technologies. *J. Environ. Manag.* **2004**, *71*, 95–122. [CrossRef] [PubMed]
17. Velea, L.M.; Popovici, D.; Popovici, O.; Podea, Z. Measurement and Control Systems with Programmable SMC-PLC Automation Systems. In Proceedings of the Conference National Symposium of Theoretical Electrical Engineering SNET 09, Bucharest, Romania, 27 November 2009.
18. Megharaj, M.; Ramakrishnan, B.; Venkateswarlu, K.; Sethunathan, N.; Naidu, R. Bioremediation approaches for organic pollutants: A critical perspective. *Environ. Int.* **2011**, *37*, 1362–1375. [CrossRef] [PubMed]
19. Iturbe, R.; López, J. Bioremediation for a Soil Contaminated with Hydrocarbons. *J. Pet. Environ. Biotechnol.* **2015**, *6*, 208.
20. ICSS. *Manual for Biological Remediation Techniques*; International Centre for Soil and Contaminated Sites: Dessau, Germany, 2006.
21. Micle, V.; Neag, G. *Soil and Groundwater Remediation Processes and Equipment*; U.T. Press: Cluj-Napoca, Romania, 2009. (In Romanian)
22. Fritsche, W.; Hofrichter, M. Aerobic degradation by microorganisms. In *Environmental Processes-Soil Decontamination*; Klein, J., Ed.; Wiley-VCH: Weinheim, Germany, 2000; pp. 146–155. [CrossRef]
23. USEPA. *Treatment Technologies for Site Cleanup*; Annual Status Report; United States Environmental Protection Agency: Washington, DC, USA, 2007.
24. April, T.M.; Foght, J.M.; Currah, R.S. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. *Can. J. Microbiol.* **2000**, *46*, 38–49. [CrossRef]
25. Ulrici, W. Contaminant Soil Areas, Different Countries and Contaminant Monitoring of Contaminants. In *Biotechnology: Environmental Processes II*, 2nd ed.; WILEY-VCH Verlag GmbH: Berlin, Germany, 2000; Volume 11b, pp. 5–41.
26. Leahy, J.G.; Colwell, R.R. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* **1990**, *54*, 305–315. [CrossRef]
27. Das, N.; Chandran, P. Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnol. Res. Int.* **2011**, *11*, 1–13. [CrossRef]
28. Agnello, A.C.; Bagard, M.; van Hullebusch, E.D.; Esposito, G.; Huguenot, D. Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. *Sci. Total Environ.* **2016**, *563–564*, 693–703. [CrossRef]
29. Chiu, H.Y.; Verpoort, F.; Liu, J.K.; Chang, Y.M.; Kao, C.M. Using intrinsic bioremediation for petroleum–hydrocarbon contaminated groundwater cleanup and migration containment: Effectiveness and mechanism evaluation. *J. Taiwan Inst. Chem. Eng.* **2017**, *72*, 53–61. [CrossRef]
30. Potra, A.F.; Micle, V.; Băbuț, C.S. Bioremediation of Petroleum Hydrocarbon-Contaminated Soil by Composting Technology. *ProEnviron. ProMediu* **2013**, *6*, 411–415.
31. Lecomte, P. (Ed.) *Les Sites Pollués Traitement des sol Set des Eaux Souterraunes*; Lavoisier: New York, NY, USA, 1998.
32. Urs Nedelcu, A.M.; Micle, V.; Berar Sur, I.M. Study on Mathematical Modelling of the Aeration and Irrigation Processes in Case of Biological Decontamination of Soils through Biopile Method. *ProEnviron. ProMediu* **2011**, *4*, 212–216.
33. Urs, A.M.; Micle, V.; Berar Sur, I.M. Studies on polluted soil aeration systems to improve efficiency of soil remediation using biopile technology. *ProEnviron. ProMediu* **2010**, *3*, 437–441.
34. Iturbe, R.; Flores, C.; Chavez, C.; Bautista, G.; Torres, G. Remediation of contaminated soil using Soil Washing and Biopile methodologies at a field level. *J. Soils Sediments* **2004**, *4*, 115–122. [CrossRef]
35. Iturbe, R.; Flores, C.; Torres, L.G. Operation of a 27 m^3 biopile for the treatment of petroleum-contaminated soil. *Remediat. J.* **2007**, *17*, 97–108. [CrossRef]
36. Seklemova, E.; Pavlova, A.; Kovacheva, K. Biostimulation-Based Bioremediation of Diesel Fuel: Field Demonstration. *Biodegradation* **2011**, *12*, 311–316. [CrossRef] [PubMed]

37. Gogoi, B.K.; Dutta, N.N.; Goswami, P.; Krishna Mohan, T.R. A case study of bioremediation of petroleum-hydrocarbon contaminated soil at a crude oil spill site. *Adv. Environ. Res.* **2003**, *7*, 767–782. [CrossRef]
38. Cerqueira, V.S.; Hollenbach, E.B.; Maboni, F.; Vainstein, M.H.; Camargo, F.A.; Maria do Carmo, R.P.; Bento, F.M. Biodegradation potential of oily sludge by pure and mixed bacterial cultures. *Bioresour. Technol.* **2011**, *102*, 11003–11010. [CrossRef]
39. Suja, F.; Rahim, F.; Taha, M.R.; Hambali, N.; Razali, M.R.; Khalid, A.; Hamzah, A. Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. *Int. Biodeter. Biodegr.* **2014**, *90*, 115–122. [CrossRef]
40. Rodriguez-Campos, J.; Perales-Garcia, A.; Hernandez-Carballo, J.; Martinez-Rabelo, F.; Hernández-Castellanos, B.; Barois, I.; Contreras-Ramos, S.M. Bioremediation of soil contaminated by hydrocarbons with the combination of three technologies: Bioaugmentation, phytoremediation, and vermiremediation. *J. Soils Sediments* **2019**, *19*, 1981–1994. [CrossRef]
41. Puntus, I.F.; Borzova, O.V.; Funtikova, T.V.; Suzina, N.E.; Egozarian, N.S.; Polyvtseva, V.N.; Solyanikova, I.P. Contribution of soil bacteria isolated from different regions into crude oil and oil product degradation. *J. Soils Sediments* **2019**, *19*, 3166–3177. [CrossRef]
42. Baoune, H.; Aparicio, J.D.; Pucci, G.; El Hadj-Khelil, A.O.; Polti, M.A. Bioremediation of petroleum-contaminated soils using *Streptomyces* sp. *Hlh. J. Soils Sediments* **2019**, *19*, 2222–2230. [CrossRef]
43. Baldan, E.; Basaglia, M.; Fontana, F.; Casella, S. Development, assessment and evaluation of a biopile for hydrocarbons soil remediation. *Int. Biodeter. Biodegr.* **2015**, *98*, 66–72. [CrossRef]
44. Ma, J.; Yang, Y.; Dai, X.; Chen, Y.; Deng, H.; Zhou, H.; Yan, G. Effects of adding bulking agent, inorganic nutrient and microbial inocula on biopile treatment for oil-field drilling waste. *Chemosphere* **2016**, *150*, 17–23. [CrossRef]
45. Chemlal, R.; Tassist, A.; Drouiche, M.; Lounici, H.; Drouiche, N.; Mameri, N. Microbiological aspects study of bioremediation of diesel-contaminated soils by biopile technique. *Int. Biodeter. Biodegr.* **2012**, *75*, 201–206. [CrossRef]
46. Wu, M.; Dick, W.A.; Li, W.; Wang, X.; Yang, Q.; Wang, T.; Chen, L. Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil. *Int. Biodeter. Biodegr.* **2016**, *107*, 158–164. [CrossRef]
47. Akbari, A.; Ghoshal, S. Pilot-scale bioremediation of a petroleum hydrocarbon-contaminated clayey soil from a sub-Arctic site. *J. Hazard Matter* **2017**, *280*, 595–602. [CrossRef]
48. Micle, V.; Sur, I.M.; Criste, A.; Senila, M.; Levei, E.; Marinescu, M.; Rogozan, G.C. Lab-scale experimental investigation concerning ex-situ bioremediation of petroleum hydrocarbons contaminated soils. *Soil Sediment Contam. Int. J.* **2018**, *27*, 692–705. [CrossRef]
49. ICPA. *Methodology for Working up Pedological Studies, Part III—Eco-Pedological Indexes*; National Research and Development Institute for Soil Science Agro-Chemistry and Environment: Bucureşti, Romania, 1987. (In Romanian)
50. Senila, M.; Levei, E.A.; Senila, L.R.; Oprea, G.M.; Roman, C.M. Mercury in soil and perennial plants in a miningaffected urban area from Northwestern Romania. *J. Environ. Sci. Health Part A* **2012**, *47*, 614–621. [CrossRef] [PubMed]
51. SR EN ISO 11274:2020. Soil Quality. In *Determination of Water Retention Characteristic*; Laboratory Methods; ASRO: Bucharest, Romania, 2020.
52. Bahemmat, M.; Farahbakhsh, M.; Kianirad, M. Humic substances-enhanced electroremediation of heavy metals contaminated soil. *J. Hazard Mater* **2016**, *312*, 307–318. [CrossRef]
53. Order No. 756 of November 3 1997, Approval of the Regulation on Environmental Pollution Assessment. Official Gazette No 303 bis of 6 November 1997. Available online: http://legislatie.just.ro/Public/DetaliiDocumentAfis/151788 (accessed on 15 June 2021). (In Romanian).
54. Micle, V.; Sur, I.M.; Mitrea, M. Ex-Situ Bioremediation Process of Hydrocarbon Polluted Soils Using Pseudomonas and Bacillus Microorganisms. *Patent RO-BOP15/* **2018**, *34*, 30 May 2018.