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РЕМЕДИАЦИЯ НЕФТЕЗАГРЯЗНЕННЫХ ГРУНТОВ БИОКОПОЗИЦИЯМИ НА ОСНОВЕ ГУМИНОВЫХ КИСЛОТ

Н.Ю. Гречищева, Е.Д. Дмитриева, К.А. Стародубцева

Наталья Юрьевна Гречищева (ORCID 0000-0001-8860-9133) *, Ксения Александровна Стародубцева (ORCID 0000-0002-5348-6660)

Кафедра промышленной экологии, РГУ нефти и газа (НИУ) им. И.М. Губкина, Ленинский пр-т., 65, Москва, Россия, 119991
E-mail: yanat2@mail.ru *, starodubtsevaksenia@gmail.com

Елена Дмитриевна Дмитриева (ORCID 0000-0001-6408-5873)

Кафедра химии, Естественнонаучный институт, Тульский государственный университет, пр. Ленина, 92, Тула, Россия, 300012
E-mail: dmitrieva_ed@rambler.ru

Кроме традиционных методов, в настоящее время активно разрабатываются новые подходы и технологии, основанные на совместном применении сорбентов и микроорганизмов, позволяющие эффективно бороться с нефтяными загрязнениями. В качестве биотехнологического решения для ремедиации различных нефтезагрязненных грунтов были исследованы биокомпозиции на основе органической матрицы – гуминовых кислот торфа и биологической составляющей – одиночного штамма/или ассоциации микроорганизмов-нефтедеструкторов рода Rhodococcus и Pseudomonas. Оценку эффективности биодеградации нефтяного загрязнения при использовании биокомпозиций проводили на различных типах модельных нефтезагрязненных грунтов с различным уровнем загрязнения: серой лесной почве, черноморской гальке (фракция 20-50 мм), строительном щебне (фракция 10-20 мм). Также использовали натурную загрязненную нефтепродуктами почву и образцы железнодорожного щебня (фракции 5-30 мм и 35-50 мм). Показано, что наибольшей способностью к разложению нефтяных углеводородов всех исследуемых систем обладала биокомпозиция на основе трех штаммов микроорганизмов «ГК R. X5 R. S67 Ps. NF142». Уровень биодеградации нефтяных углеводородов при внесении данной биокомпозиции варьировал от 62 до 82% в зависимости от типа нефтезагрязненного грунта при очень высоком уровне загрязнения. Монобактериальная биокомпозиция «ГК Ps.NF142» показала свою перспективность для ремедиации почвенных экосистем, загрязненных нефтью с высоким и очень высоким уровнем загрязнения. Видимо, повышение уровня биодеградации нефтяного загрязнения грунтов обусловлено комплексным действием гуминовых кислот, состоящим как в стимулирующем действии гуминовых кислот на микроорганизмы – компоненты биокомпозиций и аборигенную микрофлору модельных систем, так и в детоксицирующем действии гуминовых кислот в отношении нефтяных углеводородов.

Ключевые слова: гуминовые кислоты, нефтяное загрязнение, микроорганизмы-нефтедеструкторы, биокомпозиции, биодеградация

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REMEDIATION OF OIL-CONTAMINATED SOILS WITH COMPOSITIONS BASED ON HUMIC ACIDS

N.Yu. Grechishcheva, E.D. Dmitrieva, K.A. Starodubtseva

Natalia Yu. Grechishcheva (ORCID 0000-0001-8860-9133) *, Kseniya A. Starodubtseva (ORCID 0000-0002-5348-6660)

Department of Industrial Ecology, National University of Oil and Gas «Gubkin University», Leninsky ave., 65, Moscow, 119991, Russia
E-mail: yanat2@mail.ru *, starodubtsevaksenia@gmail.com

Elena D. Dmitrieva (ORCID 0000-0001-6408-5873)

Department of Chemistry, The Institute of Natural Sciences, Tula State University, Lenina st., 92, Tula, 300012, Russia
E-mail: dmitrieva_ed@rambler.ru

In addition to conventional methods, nowadays new approaches and technologies based on combined application of sorbents and microorganisms are intensively developed for efficient control of oil contaminations. As a biotechnological solution for remediation of various oil-contaminated soils, biocompositions based on an organic matrix – humic acids of peat, and a biological component – a single strain/or an association of oil degrading microorganisms of the genera Rhodococcus and Pseudomonas, have been studied. The efficiency assessment of oil contamination biodegradation using biocompositions was carried out on different types of model oil-contaminated soils with different levels of contamination: grey forest soil, Black Sea pebbles (fraction 20-50mm), constructional broken stone (fraction 10-20mm). Natural soil contaminated with oil products and samples of railroad broken stone (fractions 5-30mm and 35-50mm) were also used. It has been shown that the biocompositions based on three strains of microorganisms – 'HA R. X5 R. S67 Ps. NF142' – possessed the highest capability of degrading oil hydrocarbons in all systems under study. Biodegradation of oil hydrocarbons upon addition of this biocompositions varied between 62 and 82% depending on the type of oil-contaminated soil, the level of contamination being very high. Monobacterial biocomposition 'HA Ps.NF142' has proven to be promising for remediation of oil-contaminated soil ecosystems with a high and very high level of contamination. The increase of the level of biodegradation of oil contamination of soils appears to be due to the combined action of humic acids comprising both the stimulating effect of humic acids on microorganisms – the components of biocompositions and indigenous microflora of model systems, and the detoxifying effect of humic acids in respect of oil hydrocarbons.

Key words: humic acids, oil contamination, oil degrading microorganisms, biocompositions, biodegradation

INTRODUCTION

Oil and products of its refinery belong to the most wide-spread class of contaminants of soil and aqueous environments. This is connected with enormous volumes of oil production and refinery, which are associated with serious ecological risks because of the hazard of oil entering environmental compartments as a result of accidents at drill wells and oil transportation facilities. The issue of contamination of soil ecosystems with oil and oil products receives particular attention as the processes of soil self-recovery and self-cleaning cannot anymore cope with incoming contaminants moving and accumulating therein from the air and water medium. Hence, the demand in soil cleaning technologies is increasing.

In the general case, oil migration through the soil profile takes place via two paths: frontal percolation and gravitational passage through migration channels. It has been shown that when oil moves vertically along the soil profile, high-molecular oil components concentrate in the humus horizon. These are mostly low-molecular petroleum paraffin, naphthenic and aromatic hydrocarbons that penetrate lower levels [1]. The rate of oil degradation, soil self-cleaning activity or persistence of contaminants therein varies from landscape to landscape. In natural environments, the gravel and pebble soil is the hardest to be successfully cleaned due to penetration of oil and oil products into gaps between stones and sinking into the strata of oil or sand. The typical technique for cleaning such soil consists in mechanical cleaning using mobile vacuum units providing powerful purging and high-pressure water flushing, which is obviously a rather expensive implementation. Moreover, excavation of soil causes distortion of the morphological structure of the oil-contaminated area under treatment and disturbance of the flow of surface and ground water. The passive 'natural' cleaning following the mechanical cleaning is based on the action of sorbents of natural or synthetic origin and frequently leads to residual oil layers and spots of oil products characterized by low viscosity, which, depending on the situation, require additional treatment. Besides, the sorbents of synthetic origin consumed need to be properly disposed of. In case of isolation and treatment of oil-contaminated soils with sorbents outside the contaminated area, cleaning is quite effective and expeditious as well as safer for the ground water, flora and fauna, but still expensive. Bioremediation using microorganisms providing natural decomposition of oil and oil products largely depends on the location of gravel, stones and pebbles. Coastlines can be washed with water containing indigenous oil degrading bacteria, soil fills are harder to yield to the action of local bacteria because of nutrient limitations, but both types of gravel and pebble soil location are heavily limited by the time of remediation and depend on the climate and seasonal weather factors. In such cases, bioaugmentation makes no sense because of the serious problem of bacteria adaptation to specific environment conditions and frequent possibility of formation of biologically inert residual oil products. In view of the above, bioremediation cannot be considered a rigorous and ultimate method of cleaning fills from oil products. The process requires alignment of a mechanism of combined action with sorbents and/or preliminary mechanical cleaning, which, in turn, increases the cost and time of remediation.

Nowadays new approaches and technologies based on combined application of sorbents and microorganisms are intensively developed for efficient control of oil contaminations. In the present work, as a biotechnological solution for remediation of different oil-contaminated soils, biocompositions based on the active matrix of the organic matter of soils – humic substances, and associations of oil degrading microorganisms of the genera *Rhodococcus* and *Pseudomonas*, have been used. Such biocompositions have proven to be effective bio-degrading and detoxifying agents for oil and oil products in aqueous and soil media [2, 3]. Humic substances, being natural detoxicants, are capable of abating the ecological load of an oil contamination on the environment. Humic substances feature high reactivity to petroleum hydrocarbons thanks to the hydrophobic aromatic backbone in their structure [4]. The stimulating action of humic compounds on the growth and development of living organisms including indigenous microflora assists greater resistance of biota to adverse environmental factors and a higher rate of biodegradation of oil biodegradation [5-8].

In view of the foregoing, the purpose of the work was to study the influence of different biocompositions based on humic substances and associations of strains of oil degrading microorganisms on the efficacy of biodegradation of oil contamination of different types of soils.

MATERIALS AND METHODS

As the organic matrix in the biocompositions under study, humic acids (HA) of reed lowland peat (RLP) isolated by the standard method were used [9, 10]. The resultant humic acid preparation was described by potentiometric titration and gel exclusion chromatography.

RLP humic acids are enriched with carboxy and phenol groups, contain four fractions that differ in their molecular weight and relative content of elements manifesting an increased stimulating effect in respect of oil destructing microorganism strains, being independent biologically active compounds themselves [2, 10-13].

The biological component of biocompositions is destructing microorganisms - (*Rhodococcus erythropolis S67 (R.S67)*, *Rhodococcus erythropolis X5(R.X5)*, *Pseudomonas NF142 (Ps.NF142)*), which are capable of oxidizing a wide range of petroleum hydrocarbons thanks to their enzyme systems [14]. The microorganisms were supplied by the Plasmids Biology Laboratory, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, the Russian Academy of Sciences, Pushchino, and All-Russia Collection of Microorganisms.

The solution of humic acids in concentration of 50 mg/L was prepared by dissolving the appropriate weighted quantity of the preparation in 0.1 M alkali and subsequent dilution to the volume using distilled water. pH of the resultant solutions was adjusted to neutral reaction with the help of nitric acid [15]. Microorganisms were cultured in full LB medium (Luria-Bertani medium) for 24 h to obtain the inoculum [15].

Biocompositions were produced as follows: to the humic acid solution, suspension of a single strain of oil degrading microorganisms/or an association of strains (two strains 1:1; three strains 1:1:1 in concentration of 10^5-10^6 CFU/ml) were added in the relation of HA: microorganisms = 3:5 (by volume) [15].

The degrading capability of biocompositions in respect of oil and oil products was investigated using different types of model oil-contaminated soils: grey forest soil, Black Sea pebbles (fraction 20-50 mm), constructional broken stone (fraction 10-20mm). Natural soil contaminated with oil products, which was received from Lukoil AZS gasoline service station, and samples of railroad broken stone (fractions 5-30 mm and 35-50mm) from the territory of an iron and steel plant were also used. As the model contamination, oil from the Moscow Refinery, diesel fuel (DF) from Lukoil AZS, and used synthetic motor oil (USMO) in quantities corresponding to low – 2000 mg/kg (II), moderate – 2300 mg/kg (III), high – 4000 mg/kg (IV), and very high – 8000 mg/kg (V) level of soil contamination with oil products [16].

The degree of oil degradation (X) was assessed as a relative reduction of the content of oil products in soil samples in the presence of biocompositions (C) compared to the concentration of oil products in the control sample (C_0):

$$X = \frac{C_0 - C}{C_0} \cdot 100\%$$

It was previously shown [2] that the independent introduction of both RLP humic acids and petrodestructor microorganisms into oil-contaminated systems caused an increase in the biodegradation of petroleum hydrocarbons, the degree of which was significantly lower compared to the presence of the corresponding biocompositions. It was shown that, on average, the level of biodegradation of petroleum hydrocarbons of various model systems was in the range of 47±3% and 37±4% in the presence of RLP humic acids and microorganisms-oil destructors, respectively. In this regard, in this work, studies have been conducted on the in-depth study of the effect of only biocompositions on the degree of biodegradation of oil pollution.

The experiment design included the following variants: Soil + oil (control); Soil + biocompositions. The soil to biocomposition ratio was equal to 10:1. The experiment was carried out in the setting of daily watering and continuous light; respective agents were added to experimental variants every 3 days. Six serieses of experiments were performed. The mass concentration of oil products in soils was determined by the gravimetric method [17]. The duration of the experiment was 7 days to 14 days.

RESULTS AND DISCUSSION

Selection of the optimum technique for detoxification of oil-contaminated soils requires fast evaluation of the degree and type of contamination, depending on which there are three main stages that are considered in cleaning technologies: a critical stage, a design stage, and an add-on stage. Each of these stages envisages emergency response to an oil spill incident, but it often takes time to choose the method of detoxification and, not least importantly, the method of treatment and disposal of collected oil waste and/or residual oil refinery products. These phases are quite labor-intensive and expensive. Hence, in planning the experiment of evaluating the developed compositions' performance, several levels of soil contamination up to the highest (more than 5000 mg/kg) were used stage-by-stage.

The findings of experiments studying the influence of the biocomposition based on *Pseudomonas sp. NF142* strain on the level of contamination of grey forest soil samples with model toxicants are given in Table 1.

Table 1

Dynamics of biodegradation (%) of oil contamination of grey forest soil in the presence of the biocomposition based on *Pseudomonas sp. NF142* microorganisms depending on the content of toxicants over the experiment period

***Таблица 1.* Динамика биодеградации (%) нефтяного загрязнения серой лесной почвы в присутствии биокомпозиции на основе микроорганизмов *Pseudomonas sp. NF142* в зависимости от уровня содержания токсикантов за время проведения эксперимента**

Toxicant	Oil Contamination Level*					
	II			III		
	3 days	5 days	7 days	3 days	5 days	7 days
Oil	51±3	69±3	98±1	44±3	61±3	89±1
DF	49±1	70±1	94±2	42±1	60±1	84±2
USMO	54±3	70±3	91±1	40±1	58±2	81±1
Toxicant	Oil Contamination Level*					
	IV			V		
	3 days	5 days	7 days	3 days	5 days	7 days
Oil	42±1	59±2	86±1	39±1	55±3	83±2
DF	36±1	54±2	78±1	30±1	49±2	80±2
USMO	40±1	54±3	80±1	35±2	49±3	75±2

Note: *The content of petroleum hydrocarbons in the control samples corresponded to the level of initial contamination during the whole period of experiment

Примечание: *Содержание нефтяных углеводородов в контрольных образцах соответствовало уровню исходного загрязнения на всем протяжении эксперимента

The findings evidence significant decrease in the level of oil contamination of grey forest soil in model experiments after application of the biocomposition based on *Pseudomonas sp. NF142* strain. Decreased contamination level was observed for all model petroleum hydrocarbons as early as experimental day 3 with a change of 50 to 30% depending on the contamination level. Maximal biodegradation of oil and oil products by the biocomposition was noted by the end of the experiment. The greatest degradation was observed for the low-level oil contamination of soil and amounted to 91±1, 94±2, and 98±1% for USMO, DF, and oil, respectively; while for the maximal content of toxicants, the respective values were equal to 75±2, 80±2, and 83±2%.

The findings of the experiments investigating the influence of biocompositions on the degree of degradation of different levels of oil contamination of the gravel and pebble soil and constructional broken stone are shown in tables 2 and 3, respectively. In this instance, both earlier developed biocompositions [2, 15], and new biocompositions based on *Pseudomonas sp. NF142* strain were studied.

Table 2

Dynamics of biodegradation (%) of oil contamination of Black Sea pebbles depending on the level of initial contamination in the presence of biocompositions

Таблица 2. **Динамика биодеградации (%) нефтяного загрязнения гальки черноморской в зависимости от уровня исходного загрязнения в присутствии биокомпозиций**

Biocom-position	Oil Contamination Level*					
	II			III		
	3 days	5 days	7 days	3 days	5 days	7 days
HA *R.X5*	20±3	45±4	62±2	26±4	42±4	52±3
HA *R.S67*	23±1	48±2	65±5	36±3	40±3	56±4
HA *R.X5 R.S67*	35±3	51±3	76±3	48±2	60±3	66±5
HA *Ps.NF142*	30±3	45±3	74±4	36±3	48±3	58±3
HA *R.X5 R.S67 Ps.NF142*	50±3	68±5	88±5	56±3	68±1	84±5
Biocom-position	Oil Contamination Level*					
	IV			V		
	3 days	5 days	7 days	3 days	5 days	7 days
HA *R.X5*	23±5	25±5	43±3	20±4	38±4	50±3
HA *R.S67*	25±3	25±1	40±2	20±3	38±1	50±4
HA *R.X5 R.S67*	38±4	40±4	63±1	20±5	44±3	75±2
HA *Ps.NF142*	30±1	43±2	63±3	20±1	38±3	63±3
HA *R.X5 R.S67 Ps.NF142*	40±1	64±2	83±3	33±2	61±2	85±3

Note: *The content of petroleum hydrocarbons in the control samples corresponded to the level of initial contamination during the whole period of experiment

Примечание: *Содержание нефтяных углеводородов в контрольных образцах соответствовало уровню исходного загрязнения на всем протяжении эксперимента

As one can see from the data presented, all biocompositions under study have demonstrated high biodegrading efficacy in respect of oil contamination of pebble soil: the content of petroleum hydrocarbons in pebble samples decreased by 50 to 88 % by the end of experiment depending on the initial level of contamination. In this instance, the maximal degrading activity was manifested by the biocomposition based on the association of three strains: *R.X5, R.S67,* and *Ps.NF142*. Introduction of this biocomposition into contaminated pebble samples reduced the content of petroleum hydrocarbons by more than 80% by the end of experiment for all levels of oil contamination. Biocompositions consisting of one strain of microorganisms only: either *Rhodococcus* or *Pseudomonas,* turned out to be somewhat less effective.

Similar findings on the biodegradation of petroleum hydrocarbons were obtained for contaminated constructional broken stone. The highest biodegrading activity was exhibited by the biocomposition based on association of three strains of microorganisms. However, in contrast to the previous experiment, the corresponding biodegradation level values were a bit lower, which might be apparently explained by high porosity of broken stone structure and lower accessibility of petroleum hydrocarbons for microorganisms (Table 3).

Table 3

Dynamics of biodegradation of oil contamination of constructional broken stone (fraction 5-3mm) depending on the level of initial contamination in the presence of different biocompositions

Таблица 3. **Динамика биодеградации нефтяного загрязнения строительного щебня (фракция 5-3 мм) в зависимости от уровня исходного загрязнения в присутствии различных биокомпозиций**

Biocom-position	Oil Contamination Level*					
	II			III		
	3 days	5 days	7 days	3 days	5 days	7 days
HA *R.X5*	19±3	38±4	45±1	27±1	38±3	52±3
HA *R.S67*	20±2	44±5	50±3	27±3	47±1	56±4
HA *R.X5 R.S67*	29±3	51±2	58±5	37±5	53±3	66±1
HA *Ps.NF142*	25±3	43±4	50±3	35±4	50±2	58±2
HA *R.X5 R.S67 Ps.NF142*	45±2	55±3	73±2	47±1	57±5	74±3
Biocom-position	Oil Contamination Level*					
	IV			V		
	3 days	5 days	7 days	3 days	5 days	7 days
HA *R.X5*	30±5	32±5	48±1	8±3	14±4	29±4
HA *R.S67*	32±3	36±1	52±3	9±4	15±4	31±3
HA *R.X5 R.S67*	52±4	58±4	62±3	7±3	13±2	43±2
HA *Ps.NF142*	28±1	38±2	52±2	9±2	15±1	51±3
HA *R.X5 R.S67 Ps.NF142*	52±1	64±2	74±1	13±4	23±5	64±2

Note: *The content of petroleum hydrocarbons in the control samples corresponded to the level of initial contamination during the whole period of experiment

Примечание: *Содержание нефтяных углеводородов в контрольных образцах соответствовало уровню исходного загрязнения на всем протяжении эксперимента

The remediation efficacy of biocompositions was additionally analyzed on natural soil samples: the oil-contaminated soil of LUKOIL AZS gasoline service station and broken stone from an iron and steel plant (Tables 4 and 5, respectively). The initial content of oil products in the soils under study equaled to 13000±3000 and 14000±3000 mg/kg, which was typical for contamination level V (very high).

Table 4

Biodegradation (%) of petroleum hydrocarbons in the samples of contaminated soil from LUKOIL AZS gasoline service station in the presence of different biocompositions

***Таблица 4.* Биодеградация (%) нефтяных углеводородов в образцах загрязненной почвы с автозаправочной станции «АЗС ЛУКОЙЛ» в присутствии различных биокомпозиций**

Biocomposition	5 days	7 days	14 days
HA *R. X5*	30 ± 1	36 ± 1	68 ± 2
HA *R. S67*	32 ± 3	38 ± 3	69 ± 3
HA *R. X5 R. S67*	40 ± 2	52 ± 2	83 ± 3
HA *Ps. NF142*	32 ± 1	42 ± 1	52 ± 2
HA *R. X5 R. S67 Ps. NF142*	44 ± 3	58 ± 2	92 ± 3

Note: The content of petroleum hydrocarbons in the control samples corresponded to the level of initial contamination during the whole period of experiment

Примечание: Содержание нефтяных углеводородов в контрольных образцах соответствовало уровню исходного загрязнения на всем протяжении эксперимента

As one can see from Table 4, the greatest effectiveness in respect of oil contamination of natural soil was exhibited by the biocomposition based on the association of three strains of microorganisms: the content of petroleum hydrocarbons in the soil decreased by 92±3% by the end of the experiment. Application of the biocomposition based on two strains of microorganisms - HA *R. X5 R. S67* – was less effective. The content of petroleum hydrocarbons in the soil decreased by 83±3% by the end of the experiment.

It is known [18-21] that *Rhodococcus* efficiently degrades light petroleum hydrocarbons – n-alkanes with varying length of their carbon chain while *Pseudomonas* degrades hydrocarbons contained both in the benzene and benzene alcohol fractions, including mono- and polyaromatic hydrocarbons. Since only aromatic hydrocarbons are involved in the formation of analytical signal in the fluorometric assay, the findings illustrate bacteria's selective capability of biodegrading particular petroleum fractions and this should be taken into consideration while selecting degrading microorganisms for biocompositions. Hence, it seems reasonable to use associations based both on *Rhodococcus* strain and *Pseudomonas* strain.

It should be noted that the preliminary experiments for the evaluation of biodegrading activity of biocompositions in respect of natural oil contamination of the iron and steel plant's broken stone have not produced the expected result. In case of application of the biocomposition based on the association of *R. X5 R. S67* and *Ps. NF142* strains, on day 7 the biodegradation amounted to 26±1% for broken stone fraction 5-30 mm and 29±2% for fraction 35-50 mm. The findings seem to be explained by both the high initial content of oil products in the samples of broken stone and high porosity of the latter. Taking the above into consideration, further experiments were carried out using a double dose of the suspension of microorganisms in preparing a biocomposition, the HA: microorganisms ratio being = = 7:3 (by volume). The results of the remedial efforts are given in Table 5.

Table 5

Biodegradation (%) of petroleum hydrocarbons in the samples of natural contaminated broke stone of varying fractional make-up from the motive-power depot of the iron and steel plant in the presence of biocompositions

***Таблица 5.* Биодеградация (%) нефтяных углеводородов в образцах натурного загрязненного щебня различного фракционного состава локомотивного депо металлургического предприятия в присутствии биокомпозиций**

Biocom-position	Fraction 5-30 mm			Fraction 35-50 mm		
	3 days	5 days	7 days	3 days	5 days	7 days
HA *R. X5*	12±5	23±2	44±1	14±2	21±2	45±3
HA *R. S67*	12±3	24±3	46±3	14±4	24±4	45±1
HA *R. X5 R. S67*	16±4	29±1	53±2	20±3	27±2	49±2
HA *Ps. NF142*	13±1	24±2	44±2	14±1	19±1	43±2
HA *R. X5 R. S67 Ps. NF142*	19±1	28±1	56±1	27±2	47±2	77±2

Note: The content of petroleum hydrocarbons in the control samples corresponded to the level of initial contamination during the whole period of experiment

Примечание: Содержание нефтяных углеводородов в контрольных образцах соответствовало уровню исходного загрязнения на всем протяжении эксперимента

As one can see from Table 5, all biocompositions are characterized by quite high degrading efficacy in respect of petroleum hydrocarbons: within 7 days the content of oil products in the samples decreased by 50% on average. A better efficacy was demonstrated by the biocomposition based on three strains of microorganisms – HA *R. X5 R. S67 Ps. NF142*»: by the end of the experiment, biodegradation reached 77±2% for the coarse broken stone fraction featuring a very high level of contamination.

CONCLUSION

Thus, the experiments performed have delivered data characterizing the biodegrading capability of biocompositions in respect of oil contamination in different soils. Biocompositions are potent biopreparations rendering a comprehensive effect, which can be used *in situ* in the biotechnological remediation of soil media characterized by various levels of contamination and high toxicity. It has been shown that among all the systems studied, the biocomposition based on three strains of microorganisms - *HA R. X5 R. S67 Ps. NF142* – possessed the highest capability of degrading petroleum hydrocarbons. Upon addition of this biocomposition, biodegradation of petroleum hydrocarbons varied between 62 and 82% depending on the type of oil-contaminated soil, the level of contamination being very high. Monobacterial biocomposition *'HA Ps.NF142'* has been shown to be promising for remediation of oil-contaminated soil ecosystems with a high and very high level of contamination. The enhanced biodegradation of oil contamination of soils is explained by the comprehensive action of humic acids including both a stimulating effect of humic acids on the biocomposition component microorganisms and indigenous soil microflora and the detoxifying effect rendered by HA on oil contaminations of soils.

DECLARATIONS

The authors declare the absence a conflict of interest warranting disclosure in this article.

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