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Original Research

Effect of Complex Technological Means on Biodegradation of Oil Products and Succession of Microorganisms in Polluted Soil

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Abstract

The impact of various technological means (addition of NPK, sewage sludge, and biopreparation) on oil product degradation was studied *ex situ* in biodegradation sites. The degradation degree of oil products depended on pollutant concentration and addition of fertilizers. The succession of microorganisms during biodegradation process in polluted soil was ascertained. The addition of NPK stimulated the growth of ammonifying bacteria and fungi, while sewage sludge promoted the growth of ammonificators and yeasts but suppressed fungi. The increase of OO bacteria positively correlated with the abundance of heterotrophic bacteria, while the amounts of OO yeast and fungi correlated negatively. It was demonstrated that sewage sludge is a suitable organic fertilizer for polluted soil bioremediation and, moreover, in this way the problem of this industrial waste utilization could be solved as well.

Keywords: polluted soil, oil products, biodegradation, microorganism succession

Introduction

Recently, attention toward the preservation of the environment has increased, including attention devoted to remediation of oil-polluted soil. Pollutant degradation must cause minimal environmental alterations; therefore, biological means that are effective, inexpensive and environmentally friendly are used more and more extensively. Bioremediation methods based on the occurrence of microorganisms and their ability to break down the complex hazardous waste are usually used for the detoxification of pollutants [1, 2].

Heterotrophic microorganisms are able to mineralize organic matter and thus supply the microorganisms decomposing xenobiotics with nutrients. Therefore, one of the ways of the control of oil biodegradation processes is an activation of microorganism communities by creation of optimal conditions for their development. The most widely used bioremediation procedure is biostimulation of indigenous microorganism growth and their ability to degrade hydrocarbons by the addition of inorganic nutrients, such as nitrogen and phosphorus [3], although there are reports that excess nutrient concentrations decrease the amount of oil oxidizing microorganisms (OOM). This is a point of consideration for soil bioremediation practice [4].

It has been determined that long-action organic fertilizers more efficiently affect the oil product degradation than the addition of mineral fertilizers [5]. Since oil products increase the C:N ratio, high amounts of organic fertilizers (biohumus, peat, active sludge) are added in order to balance it to a level favorable for microorganism development (C:N 10:1).

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The sewage sludge is successfully applied as an organic amendment in the composting of oil-polluted soil. It serves as a source of nutrients and microorganisms, and positively affects physical and chemical properties of the soil [6, 7].

Another bioremediation method is bioaugmentation – the inoculation of the contaminated soil with active hydrocarbon degraders [3]. Inoculation of biodegradative bacterial strains into soil result in enhanced biodegradation activity as well as changes in the structure of the microorganism community [8, 9]. However, there are reports that while increasing indigenous biodegradation, the inoculum might have been replaced, and the oil amount decreases in soil during the short period, and, to a lesser extent [3, 10]. Researchers of the public agency "Soil Remediation Technologies" (SRT) select eurytopic strains of microorganisms that are most tolerant to fluctuations of environmental factors and used for the creation of biopreparations [11, 12].

The efficiency of the above-mentioned soil bioremediation means greatly depends on physico-chemical properties of soil, origin and concentration of decomposed pollutants, indigenous microorganisms, and meteorological conditions. One of the bioremediation measures is polluted soil excavation, and taking it away to special sites for pollutant removal *ex situ* [4]. Under temperate climate conditions of Lithuania the degradation of pollutants is observed only for a short time, so it is important to use various means for acceleration of this process. We hypothesized that the complex application of biostimulation and bioaugmentation means will be favourable for microorganism development in oil-polluted soil, and will shorten the period of pollutant degradation. The relevancy of the problem demands a complex investigation of biodegradation processes and improvement of bioremediation technologies.

The objective of the study was to investigate the influence of various technological means (the addition of NPK, sewage sludge, and "Devoroil") to oil product biodegradation and microorganism succession during polluted soil cleaning process *ex situ*. To clarify the issue we had to ascertain:

1) the differences in biodegradation of oil products in different variants formed *ex situ*
2) the effect of NPK, sewage sludge, and biopreparation "Devoroil" on the acceleration of the biodegradation process for oil products
3) the succession of various microorganism groups in soil during the process of oil product biodegradation

Material and Methods

Three variants of the *ex situ* bioremediation were arranged at the biodegradation site of Klaipėda (Lithuania), department of public agency SRT in 2009. The substrata of the variants consisted of soil polluted with toxic oil products and sewage sludge of a chemical factory producing polyethylene terephthalate granules (PET). The substrata were bulked in 0.4-0.5 m layers. The substratum composition of the cleaning variants was:

variant 1 – first-season cleaning polluted soil – 1,690 t + 291 t of sludge (brought on May 29, 2009), ratio 1:0.17; oil product amount – 20566 mg/kg;

variant 2 – first-season cleaning polluted soil – 1,670 t + 100 t of sludge (brought in 2008 preparing for remediation) + 387 t of sludge (brought on May 29, 2009), ratio 1:0.3; oil product amount – 22,290 mg/kg;

variant 3 – second-season cleaning polluted soil – 2,190 t + 100 t of sludge (brought in 2008 during the remediation) + 377 t of sludge (brought on May 29, 2009), ratio 1 0.2; oil product amount – 27,237 mg/kg.

The substrata at the sites were tilled (aerated) twice a week. Soil humidity was constantly controlled and moisturized up to 65-70% of water-holding capacity.

During the experiment average temperature was close to normal: in April – 8.3, May 11.3, June – 15.0, July – 18.7, August – 18.2, and September – 15.4ºC. The rate of precipitation was the following: in April – 3, May 3, June – 52, July – 73, August – 77, and September – 62 mm. It should be noted that in April only 10% of average precipitation was observed. In July-August the rate of precipitation was higher than average and, furthermore, heavy rainfall took place in July.

The chemical analysis of sludge and of cleaned substratum was carried out using standard methods at the Centre of Agrochemical Studies (a branch of the Lithuanian Agrarian and Forest Science Centre).

The amount of oil products in soil was estimated gravimetrically four times in the course of bioremediation. The analysis of C_6-C_{10} (benzine) fraction of oil products was performed by the EPA 5021:1996 method. The analysis of C_{10}-C_{28} (diesel fuel) and C_{28}-C_{40} (long chain hydrocarbons) fractions of oil products were performed following ISO 16703:2004 methods at the Analytic Laboratory "Grota."

The cleaned substrata were fed with NPK mineral fertilizers once on the 27-28 of April. The fertilizers used were: ammonia niter (NH_4NO_3, 34% N) – 342 g/t, powdery superphosphate ($CaH_2PO_4 \times 2CaSO_4$, 23% P_2O_5) – 42 g/t, potassium chloride (KCl, 59% K_2O) – 172 g/t.

Biopreparation "Devoroil" [13], which consist of oil-oxidizing microorganisms *Rhodococcus erythropolis*, *Rh. maris*, *Pseudomonas stutzeri*, and *Yarrowia lipolytica* strain was used for the bioremediation. Biopreparation was used in suspension, titre – 5×10^{10}-1×10^{11} cells/mL. It was sprayed out with a high-pressure pump of a sanitation machine – 3 L/m^2. The biopreparation suspension was injected into cleaned substrata on the 7-10 May, 1-5 June, and 3 July, 2009.

The soil samples for the microbiological analysis were taken on 28 April before the addition of mineral fertilizers and "Devoroil." Afterward, soil samples from all cleaned variants were collected monthly: on 15 May, 9 June, and 15 July (5-10 days after biopreparation insertion) and at the end of remediation on 24 September.

The cultivable microorganisms were isolated and enumerated for the serial dilution plate technique by sowing 1 ml of soil suspension in five replications on the sur-

Table 1. Chemical rates of sewage sludge used for biodegradation.

Investigated rates	Rate value	Investigation methods
Dry substance amount, %	5.9	LST EN ISO 12880:2002
Antimony (Sb), mg/kg	13.6	LST ISO 11047:2004
Cobalt (Co), mg/kg)	11.9	
Zinc (Zn), mg/kg	30.0	
Cadmium (Cd), mg/kg	<0.13	
Plumbum (Pb), mg/kg	<7.37	
Copper (Cu), mg/kg	11.1	
Nickel (Ni), mg/kg	3.7	
Total chromium (Cr), mg/kg	5.7	
Total nitrogen (N), g/kg	101.544	LST EN 13342:2002
Total phosphorus (P), g/kg	8.773	LAND 78-2006
Organic substances. %	93.5	LST ISO 10390:2003
pH	7.07	LST EN ISO 12879:2002

face of appropriate standard media for different microorganism groups. Nutrient agar (Oxoid) was used for ammonifying bacteria; starch-ammonia agar: $(NH_4)_2SO_4$ – 2, K_2HPO_4 – 1, $MgSO_4$ – 1, NaCl – 1, $CaCO_3$ – 3, starch – 10, agar – 20 (g/l) – for mineral nitrogen assimilating bacteria. Sabouraud CAF agar (Liofilchem, Italy) was used for yeast isolation. Yeast identification was performed by routine methods following certain identification systems [14, 15]. Malt agar with chloramphenicol (250 mg/L for bacteria growth inhibition) was used for fungi isolation. Czapek, potato-dextrose (Liofilchem, Italy), Sabouraud, and Czapek yeast extract agar were used additionally for pure fungal cultures and identification. Fungal species were identified with reference to their cultural and morphological properties using various manuals [16-19].

A new medium, GVT-A, which was offered by the public agency SRT, containing 1% of oil as a sole carbon source was used for isolation of oil-oxiding microorganisms (OOM). Bacteria, fungi, and yeasts grow well on this medium [20].

The microorganism amount in soil samples was expressed as an amount of colony-forming units per 1g of dry substratum (cfu/g d. s.) [21].

Statistical analysis of the data was performed with the Statistica 6.0 program. Correlation coefficient (*r*) was used for calculation of the relations between the amount of oil products and abundance of microorganisms during the cleaning process. Data were analyzed by multiple range analysis (LSD, $P \leq 0.05$).

Table 2. Chemical rates of cleaned substratum the Klaipėda Department of the Public Agency SRT site.

Investigated rates	Rate value	Investigation methods
Total nitrogen (N), %	0.23	ISO 11261-1995
Total phosphorus (P), %	0.049	SVP D-2:2005
Organic carbon (C), %	3.71	ISO 10694-1995
Calcium (Ca), %	5.36	ISO 22036:2008
Magnesium (Mg), %	1.62	
Carbonates (CO_3), %	14.03	ISO 10693-1995
Cadmium (Cd), mg/kg	0.097	ISO 11466-1995
Chromium (Cr), mg/kg	17.6	ISO 22036:2008
Nickel (Ni), mg/kg	7.37	
Plumbum (Pb), mg/kg	11.8	
Copper (Cu), mg/kg	35.0	
Zinc (Zn), mg/kg	72.1	
Antimony (Sb), mg/kg	<0.9	SVP D-3:2005
Mercury (Hg), mg/kg	0.029	
Ammonium nitrogen ($N\text{-}NH_4$), mg/l	35.1	LST EN ISO 11732-2005

Results

The samples of sewage sludge for chemical analysis were taken before the experiment. The metal amounts in this sludge did not exceed the setting concentration, except for antimony (Table 1).

The samples of cleaned substratum for chemical analysis were taken on 10 June, 2009 after the addition of sewage sludge. The generalized results of the analysis are presented in Table 2.

The ratio of C:N:P in dry substance was 100:6.2:1.3. The ratio of 100:10:1 is considered to be optimal for remediation. The amounts of heavy metals did not exceed the maximum permissible concentration (MPC) in soil.

At the beginning of the polluted substrata remediation (in April) the greatest amount of oil products (27,237 mg/kg d. s.) was detected in the soil that had been cleaned during the second year (variant 3, which was not completely cleaned in 2008). The amount of oil products in the substratum of experiment variant 2 was higher than in variant 1 (22,290 mg/kg and 20,566 mg/kg kg d. s., respectively) (Fig. 1).

It was determined that intensive degradation of oil products proceeded during the first months of biocleaning. In June the total amount of oil products in soil samples of variants 1-3 decreased by 69.7, 75.5, and 54.8%, respectively. In September the least amount of oil pollutants (34,00 mg/kg d. s.) was estimated in substratum, where the sludge

had been added in two stages: in autumn and repeatedly in spring, and where the ratio of polluted soil and sludge was the highest (variant 2). Nevertheless, at the end of the biocleaning season, in the soil of variant 3 oil products were decomposed most evidently (from 27,237 to 4,571 mg/kg d. s.), compared with the initial amount.

The investigation of fractional composition of oil pollution was performed in June-July (Fig. 2). The changes in total oil product amount showed that at this time the intensive degradation of oil products has already happened.

The hydrocarbons of diesel C_{10}-C_{28} and long-chain hydrocarbons C_{28}-C_{40} rank remained and dominated in cleaned soil. Less than 1 mg/kg d. s. of petrol rank hydrocarbons was estimated.

In June the highest amount of diesel and long-chain hydrocarbons was detected in the soil that had been cleaned during the second year (variant 3) – 6,370 mg/kg d. s. The amount of these fractions in variants 1 and 2 were markedly lower (2,560 and 2,070 mg/kg d. s., respectively). In July the alterations were distinct only in the substrate of variant 2, where the amounts of both diesel and long-chain hydrocarbons decreased.

The number and succession of cultivable microorganisms during the biocleaning process of polluted soil was monitored. At the beginning of the bioremediation the amount of ammonifying bacteria in the substrata prepared for biocleaning was rather low. Only in the substratum of variant 3 did their number reach 106×10^6 cfu/g d. s. (Fig. 3). At the same time, quite a few ammonificators were recorded in other variants: in freshly overspread soil (variant 1) – 32×10^6, and in variant 2 (soil spread the previous year) – 16.5×10^6 cfu/g d.s.

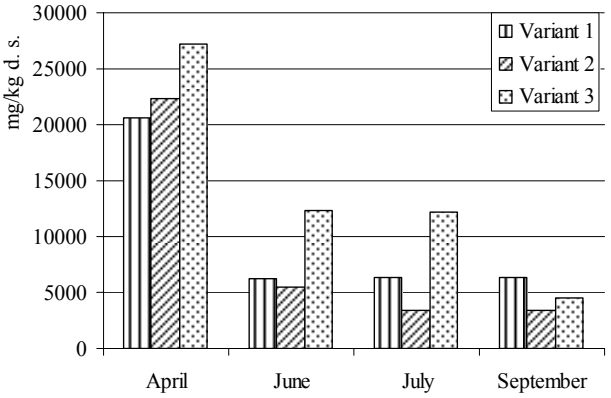

Fig. 1. Alteration of oil product amount in the variants of cleaned substrata in April-September.

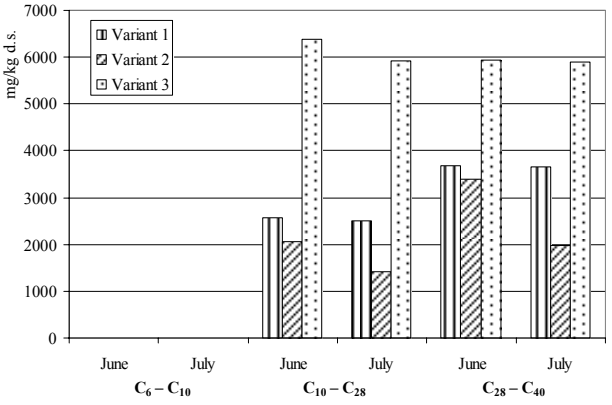

Fig. 2. Fractional composition of oil pollutants in the samples of substrata in June and July.

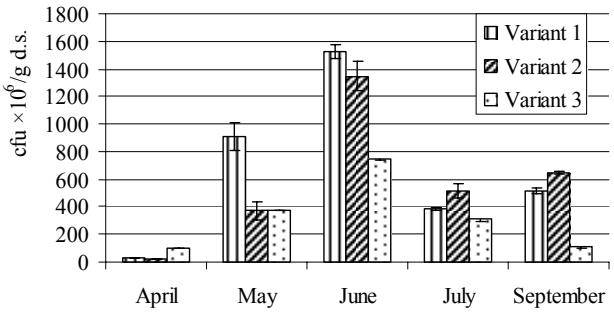

Fig. 3. Amount of ammonifying bacteria in variants of cleaned substrata.

In May, after the addition of NPK and biopreparation, the amount of the ammonificators increased in every variant of the experiment ($371\text{-}914\times10^6$ cfu/g d. s.), especially in variant 1 (28 times).

The addition of the sewage sludge positively influenced the development of the ammonifying bacteria in June, when their amount increased from 745 to $1{,}530\times10^6$ cfu/g d. s. From May the number of these bacteria in variant 2 increased 3.6 times, in variants 1 and 3 – about 2 times.

Since July the decrease in the amount of ammonifying bacteria was noticed. However, until the end of the experiment their highest amount was found in the substratum of the variant 2 (513×10^6 cfu/g d. s. in July and 645×10^6 cfu/g d.s. in September). Lower amounts of the bacteria were estimated in the substratum of variant 1 (385 and 516×10^6 cfu/g d. s., respectively), and the least amount in the substratum that had been cleaned for two years (311 in July and 114×10^6 cfu/g d. s. in September).

At the beginning of the research the amount of the mineral nitrogen assimilating bacteria in cleaned substrata was lower than the amount of ammonificators, but later their amount increased (Fig. 4). The number ranged from 22.9 to 48.9 cfu/g d. s. in April and May.

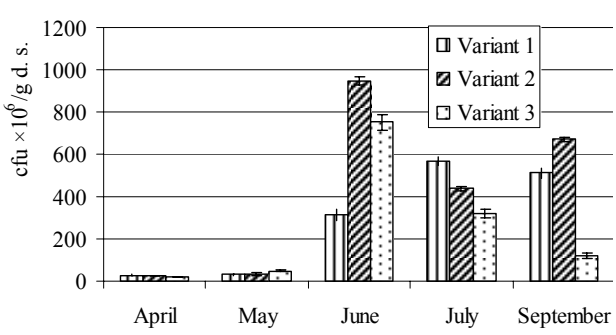

Fig. 4. Amount of mineral nitrogen-assimilating bacteria in variants of cleaned substrata.

In June the quantity of the mineral nitrogen-assimilating bacteria increased by more than 10 times in cleaned substrata. In variant 1 the amount of these bacteria reached 313×10^6 cfu/g d. s. and in variant 2 – up to 949×10^6 cfu/g d. s. In these variants the high rates of the studied bacteria group remained in July and September (ranging from 437 to 671×10^6 cfu/g d. s.).

Moreover, the mineral nitrogen-assimilating bacteria from the genus *Streptomyces* were isolated on starch-ammonia agar medium. In the oil-product polluted substrata their numbers were low as compared with other microorganisms ($0.06\text{-}0.91\times10^6$ cfu/g d. s.). They were slightly more abundant during the first part of the experiment after the addition of nutrients and biopreparation. In May the quantity of the *Streptomyces* genus bacteria became similar in all variants and reached $0.56\text{-}0.63\times10^6$ cfu/g d. s. In July they were found only in substrata of variants 1 and 3 (0.2 and 0.06×10^6 cfu/g d. s., respectively), and in September about 0.4×10^6 cfu/g d. s. of the bacteria were detected only in variant 3.

The ratio M/H between the number of ammonifying (M) and mineral nitrogen assimilating (H) bacteria varied in all variants during the experiment (Fig. 5). When it started in May, the part of the ammonifying bacteria made up 87-96% of the total bacteria number. The addition of the biopreparation and sewage sludge stimulated their intensive growth. In June the percentage of ammonificators remained high in variant 1, but in variants 2 and 3 the development of mineral N-assimilating bacteria increased and reached 41-50% of the total bacteria amount.

The amount of yeasts varied in the studied samples, depending on the substratum composition and cleaning duration (Fig. 6). It started to increase after NPK addition in May. A high amount of the *Rhodotorula* genus yeasts was isolated from variant 3 at this time. However, the highest amount of the yeasts was estimated in the summer period (in June) in variant 2, and it reached 11.7×10^5 cfu/g d. s. The least amount of the yeasts during the whole experiment was ascertained in the variant containing the substratum of the previous year (variant 3). During summer, which is the most favorable for yeast development, their amount reached only 1.2×10^5 cfu/g d. s.

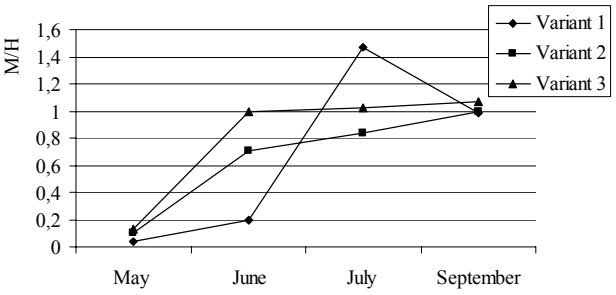

Fig. 5. Dynamics of mineralization-humification coefficient in variants of cleaned substrata.

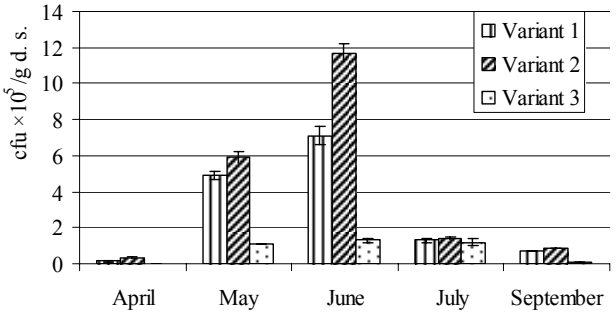

Fig. 6. Alteration of yeast amount in variants of cleaned substrata.

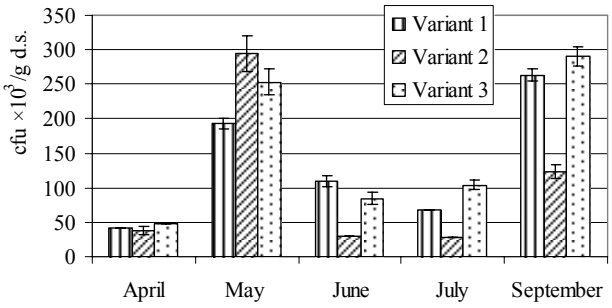

Fig. 7. Alteration of fungal amount in variants of cleaned substrata.

Quite a limited occurrence of fungi was estimated in the beginning of the investigations – in April. The quantity of their cfu ranged from 37.9 to 42.7×10^3/g d. s. (Fig. 7).

In May, after the addition of nutrients (NPK) and when the air temperature rose, the abundance of fungi significantly increased. In June (after effluent sludge addition at the end of May) the decrease of fungi amount was recorded in all variants, especially in variants 2 and 3 (up to 30.2 and 83.9×10^3 cfu/g d. s., respectively). In autumn (i.e. in September) the fungal occurrence repeatedly increased in all studied variants.

The diversity of fungal species in the studied substratum was not high. Fungal species of the *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Fusarium*, *Paecilomyces*, *Humicola*, *Chrysosporium*, *Circinella*, and *Trichoderma* genera were isolated (Table 3).

A new medium GVT-A with 1% of oil as a sole carbon source was used for OOM isolation. From 1.4 to 5×10^6 cfu/g d. s. of the oil-oxidizing bacteria (OOB) were isolated from the cleaned substrata at the beginning of the experiment (Table 4). In May the amount of OOB decreased and was more abundant only in variant 3. In June the amount of bacteria increased after the second addition of biopreparation, especially in variant 2 (to 11×10^6 cfu /g d. s.). The highest amount of OOB remained until the end in this variant.

At the beginning of the cleaning process the oil oxidizing yeasts (OOY) were detected only in variant 1. Their amount increased after the addition of the biopreparation, which included OOY strains. Total OOY amount reached 71×10^3 cfu /g d. s. there. After a month the yeast amount decreased to 24×10^6 cfu/g d. s. in this variant, although in other variants it remained similar to the previous month. The OO yeasts developed more intensively during the second part of the cleaning process, in July and September.

Table 3. Species diversity and occurrence of fungi and yeast-like fungi in variants of examined substrata.

Fungi and yeast-like fungi species	Date of substratum sampling				
	April	May	June	July	September
Aspergillus flavus Link		2		1	
A. terreus Thom		2			
A. fumigatus Fresen.				1, 2, 3	1
A. niger Tiegh.	2, 3	1, 2, 3	1, 3		1, 2, 3
Chrysosporium merdarium (Ehrenb.) J.W. Carmich.				2, 3	1
Circinella circinans (Bainier) Milko					3
Cladosporium resinae (Lindau) G.A. de Vries				3	
Fusarium oxysporum Schltdl.		2, 3			
Geotrichum fermentans (Diddens et Lodder) Arx	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
Humicola fuscoatra Traaen				3	
Yarrowia lipolytica (Wickerham, Kurtzman et Herman) van der Walt et von Arx	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
Mucor circinelloides Tiegh. f. *circinelloides*				1	2, 3
M. hiemalis Wehmer f. *hiemalis*	1, 2, 3	2, 3	1, 2	1, 2	1
Paecilomyces farinosus (Holmsk. et Gray) A.H.S. Br. et G. Sm.					2
P. janczewskii K. M. Zalesski		2	2	1	2, 3
P. simplicissimum (Oudem.) Thom		1, 2, 3	2, 3		1, 2 3
Penicillium sp.		3		2	2
Rhizopus sp.		3			2
Rhodotorula mulcilaginosa (Jörgensen) F. C. Harrison	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
Trichoderma longibrachiatum Bissett		1, 2, 3	2		2

Legend: 1, 2, 3 – variants

In April the highest amount (48-56×10³ cfu/g d. s.) of the fungi growing on medium with oil products was estimated in variants 2 and 3. In May their quantity decreased in variant 3, but quite markedly increased in variants 1 and 2. In July the oil-oxidizing fungi were abundant only in variant 3 (about 72×10³ cfu/g d. s.), but in September their quantity noticeably increased in variant 1 as well.

Discussion

The significant decrease of oil products in cleaned soil was estimated already a month after the beginning of the biodegradation process. The distinct reduction of oil pollutant was determined by both OOM action and the addition of sewage sludge, which mechanically reduced oil pollutant amount in substratum bulk. The least amount of oil pollutants was found in the substratum, with the highest ratio of polluted soil and sludge (variant 2). In the soil that had been cleaned during the second year (variant 3), where in June the largest amount of pollution remained, the cleaning processes proceeded even in July after the addition of the biopreparation for the third time. At the end of biocleaning season, in this soil oil products were degraded mostly as compare with the initial amount – 83.2%. Whereas in variants 1 and 2, where lower residual oil amounts had been estimated already in May, the processes of oil pollutant biodegradation practically stopped at the middle of the cleaning season. We suppose that the interruption of the biodegradation processes in the mentioned variants could be influenced by the shortage of organic substances – too low a ratio of polluted soil and sludge (about 1:0.17 and 1:0.3, respectively). Considerably better results of oil removal were obtained when the ratio of contaminated soil and sewage sludge was of 1:0.5 [7]. Supplementary addition of sludge and NPK could stimulate the action of OOM.

Precipitation excess in July-August must be mentioned among other factors negatively affecting the process of the degradation of oil products by worsening the aeration conditions in the soil. Furthermore, in July a part of nutrients and OOM of the biopreparation might have been leached from substrates because of frequent strong rainfalls. It is reported that insufficient amount of mineral macro ele-

Table 4. The number of oil-oxidizing microorganisms in cleaned substrata.

Date	Variant	Bacteria (cfu×10^6)	Yaests (cfu×10^3)	Fungi (cfu×10^3)
April	1	5.0±0.2	5.34±0	10.68±0
	2	2.50±0	0	56.82±16.1
	3	1.36±0.08	0	48.31±0
May	1	2.02±0.99	5.20±0	73.22±0
	2	0.77±0.03	15.96±7.5	132.98±22.6
	3	6.66±0.02	70.9±9.1	12.89±0
June	1	5.75±0.55	7.45±0	67.06±10.5
	2	10.94±1.07	15.13±0	30.26±21.4
	3	3.2±0	23.66±11.2	31.55±0
July	1	8.24±0.8	25.7±1.2	9.09±3.9
	2	12.7±0.6	28.25±1.4	19.1±8.3
	3	2.95±0.2	30.55±2.4	71.8±12.0
September	1	0.72±0.1	47.0±1.2	90.87±8.2
	2	4.05±0.2	30.0±1.7	32.26±16.1
	3	2.83±0.3	45.0±2.1	74.86±7.6

ments becomes a reason for a decrease of OOM activity, and this determines the break of bioremediation [3].

The pollution of substrata by certain oil fractions and their destruction tendencies correlated with the decrease of the estimated total oil amount. At the end of the cleaning season oil pollution decreased mostly in the second-year cleaned soil (variant 3), but starting from June the amounts of studied oil fractions did not alternate further. It is presumable that oil pollution degradation of indeterminate origin went on in this substratum.

After intensive degradation of oil product pollutants, diesel and long-chain hydrocarbon fractions remained in cleaned soil. The stabilization of oil fraction degradation at the end of the cleaning season showed that the remaining fractions were recalcitrant and thus not available to microorganisms. In this case, not only the ratio of nutrients and organic substance might be corrected but it would be efficient to enrich the clean soil with selected eurytopic cultures of reducers that would decompose specific pollutants [4]. Nevertheless, at the end of the cleaning season the amount of OOM in oil product-polluted soil was rather high. If possible the soil cleaning should be continued in order to reduce the amount of oil pollution more markedly.

The results of the investigation showed that sewage sludge of chemical industry can be used for the bioremediation of soil polluted with oil product. Thus the problem of industrial waste utilization could be solved as well. For successful biodegradation of pollutants it is necessary to monitor the amounts of heavy metals or other toxic elements in sludge – it should not exceed maximum permissible concentration (MPC). For example, it was found that a 400 mg/ml concentration of chlorides suppressed the activity of microorganisms [22]. In our experiment the amount of antimony in sludge exceeds the MPC. According to Lithuanian hygiene standards [23] the setting concentration of antimony in soil is 1-1.5 mg/kg and MPC – 5-10 mg/kg. The occurrence of high quantities of potentially pathogenic fungi cannot be ignored as well [24, 25].

Special attention is given to the formation of active associations of pollutant-decomposing microorganisms and their community succession under changeable substratum conditions. The criterion of cultivable microorganism (fungi and two different physiological groups of bacteria) abundance was used as a mean of monitoring the biodegradation process. It was ascertained that the technological means used for cleaning of oil product-polluted soil differently affected certain microorganism groups. The addition of nutrients and "Devoroil" stimulated the population of ammonifying and *Streptomyces* genus bacteria, yeasts, and fungi. The addition of sewage sludge most markedly stimulated the development of ammonifying bacteria. At the beginning of bioremediation the amount of ammonifying bacteria in the oil-polluted substrata was rather low. Only in the soil cleaned for the second season (the cleaning over the first season was not successful because of the high amount of oil products – about 50 g/kg) their amount was higher. This shows that ammonifying bacteria survived and started to reproduce intensively in spring. The addition of NPK and the biopreparation influenced the increase of ammonificators, especially in variant 1. The addition of the sewage sludge had a positive effect on the amount of the ammonifying bacteria as well. This might be influenced by bacteria developing in the sludge. A rather pronounced effect of sewage sludge addition, when soil was not rich in microoganisms, was underlined by other researchers [7]. Since July the noticed decrease in the amount of ammonifying bacteria may be due to the reduction of organic components in substrata, because in May and June the climatic conditions were the most favorable for decomposition of organic matter.

Though at the beginning of the research ammonificators prevailed in comparison with the mineral nitrogen assimilating bacteria, from the midsummer the latter amount markedly increased in cleaning substrata (10-26 times). There are reports that mineral nitrogen assimilating bacteria use the metabolism products of ammonifators for their nutrition [5]. The high rates of this physiological group of bacteria remained till the end of the cleaning season, which shows an intensive destruction process of organic substances. The decrease of oil products in substrata was one of the factors that capacitated the intensive development of mineral N assimilating bacteria. During the cleaning process significant negative correlation $r = -0.64$ ($P<0.05$) between the amount of oil products and abundance of mineral nitrogen-assimilating bacteria was determined.

Actinomycetes of the genus *Streptomyces* were found among mineral nitrogen-assimilating bacteria. Their amount in the oil product polluted substrata was rather low in comparison with other microorganisms. Nevertheless,

the *Streptomyces* spp. are rather often found in oil-polluted soils, and their positive potential in bioremediation is described [26-28].

The ratio among physiological groups of various microorganisms can outline the trend of the mineralization-humification process in soil and its ecological state. These processes in natural and conditionally undamaged soils are in balance [29]. The mineralization-immobilization ratio M/H between the number of ammonifying (M) and mineral nitrogen assimilating (H) microorganisms shows that in all variants of substrata, the part of the mineral nitrogen assimilating microorganisms increased from July, and the ratio M/H became close to 1:1. The M/H coefficient more gradually increased in variants 2 and 3, in which the organic fertilizers (sewage sludge) were added twice. Although it is impossible to affirm that the cleaned substrata has become comparable to natural, this ratio shows the activation of oil product degradation.

Yeasts are a common component of the oil-oxidizing microorganism community. They can develop at pH 3.0-6.0 and oxidize oil products at 10-20ºC [30, 31]. The amount of yeasts in cleaned substrata started to increase after NPK addition in May. It is reported that biostimulation enhanced oil biodegradation to a significantly greater degree than inoculation with oil-degrading yeast *Yarrowia lipolytica* [3]. The amount of yeasts directly depended on the added "Devoroil" and sludge. The highest amount of yeasts was isolated from the substratum, where the largest amount of sludge had been added (variant 2). Though summer was favorable for yeast development, the least amount of yeasts was found in the variant that had been cleaned during the second season (variant 3). It shows that not only oil products but other pollutants as well could negatively influence yeasts in this substratum. The decrease of yeast number during the second half of summer could be influenced by the decrease of oil product amount at this time. *Yarrowia lipolytica* (the component of OOM association of "Devoroil") and indigenous *Geotrichum fermentans* and *Rhodotorula mulcilaginosa* yeast strains predominated in oil product-polluted soil.

Fungi are able to adapt and survive under unfavorable conditions. They can actively participate in oil product degradation processes when conditions are less favorable for bacterium development (insufficient humidity, acidic medium) [32]. Fungi made up a small part of microbial communities in the studied soil. The increasing fungi development was observed after the addition of NPK. It is reported that mineral nitrogen added as ammonium salt stimulates fungal growth better than in nitrate form. The amount of fast-growing, abundantly sporulating, and toxic fungi increases enormously. After biodegradation the overabundant occurrence of these fungal species may become a reason for phytotoxicity [5]. After the addition of industrial sludge, a decrease in the amount of fungi was recorded in all variants. There are some reasons for this fungal development suppression. Firstly – industrial waste has altered the pH of substrata to alkaline reaction, and this confined fungal occurrence. Furthermore, a great amount of indigenous bacteria and yeasts adapted to this substratum were brought together with the sludge. The latter competed with the fungi and limited their growth. OOM that were introduced together with "Devoroil" heightened the competition for nourishment. Secondly – a very important reason – meteorological conditions: cold weather and too much rainfall. The aeration conditions worsened in the substrata and this negatively influenced the fungal occurrence.

The increased number of fungi in September may be directly related to the degradation processes of the oil products and sewage sludge. During these processes the amount of yeasts and bacteria decreased, resulting in more favorable conditions for fungal growth.

Fungi from 10 genera were isolated from oil-polluted substrata. Most of the isolated fungi from the genera *Aspergillus*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium*, *Humicola*, and *Rhizopus* are known as oil product decomposers and are able to assimilate and utilize various hydrocarbons [33, 34]. Fungi *Aspergillus niger*, *Penicillium simplicissimum*, *P. nigricans*, *Mucor hiemalis* f. *hiemalis*, and *Trichoderma longibrachiatum* predominated in the studied substrata. It confirmed the statement that in polluted substrata, fungal species that are resistant to environmental conditions, sometimes toxic and even pathogenic usually, begin to develop [18, 35, 36].

At the end of the soil-cleaning process the abundance of eurytopic *Penicillium simplicissimum* fungi increased significantly. Fungi of this species are characteristic of Lithuanian soils. Furthermore, these fungi and above-mentioned yeasts may be used in creation of associations for polluted soil bioremediation.

It is reported that introduced microorganisms actively participate in biodegradation of oil products for a short time; their abundance and activity reached maximum in 5-25 days, and later their amount intensively decreased [37]. Therefore the "Devoroil" was added to polluted soil three times during the cleaning season. The dynamics of the number of OOB coincided with the alteration of the abundance of heterotrophic bacteria isolated on standard media. The increasing amount of OOB was noticed from June after the second addition of the biopreparation. They were more abundant in variant 2, evidently because nutrient environment favorable for their growth was created by the ammonifying bacteria. At the end of the cleaning the amount of OOB decreased, but the residual amount could be important in case the cleaning continues for the next season [8].

The amount of oil-oxidizing yeasts increased after the addition of the biopreparation, which included OOY strains. The proliferation of OO yeasts and fungi at the end of the experiment showed that the OOM of the biopreparation and indigenous microorganism species adapted to substratum survived and developed. The increase of OO yeast and fungi abundance at the end of the season could be related with the decrease of total bacteria amount at this period.

The bioremediation of soil polluted with oil products using sewage sludge of chemical industry solves the problem of utilization of this industrial waste as well. It is necessary to ensure that the amount of heavy metals would not exceed the MPC in sludge.

Bioremediation is a complex process that is being constantly improved on the basis of experimental studies. The results of the investigation showed that the monitoring of oil product destruction as well as microbial community succession during bioremediation is necessary in order to control this process throughout.

References

1. KLEIN J. Possibilities, limits, and future developments of soil bioremediation, in: Rehm H.J., Reed G. (Eds.) Environmental processes II. Soil decontamination. Biotechnology 11b, Wiley-VCH: Weinheim, 465, **2000**.
2. SABATÉ, J., VIÑAS, M., SOLANAS, A.M. Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. Int. Biodeter. Biodegr. **54**, (1), 19, **2004**.
3. MARGESIN R. Potential of cold-adapted microorganisms for bioremediation of oil-polluted Alpine soils. Int. Biodeter. Biodegr. **46**, 3, **2000**.
4. JANKEVIČIUS K., LIUŽINAS R. (Eds.). Important aspects of environmental bioremediation. Apyaušris: Vilnius, pp. 134, **2005**.
5. TERESHCHENKO N.N. Ecologo-biological factors and mechanisms of human-damaged soils remediation. Tomsk University: Tomsk, pp. 42, **2007** [In Russian].
6. VAN GESTEL K., MERGAERT J., COOSEMAN J, RYCKEBOER J. Bioremediation of diesel oil-contaminated soil by composting with biowaste. Environ. Pollut. 125, (3), 361, **2003**.
7. CHUA C.L., ISA, M.H. Bioremediation of oil sludge contaminated soil by co-composting with sewage sludge. J. Sci. Ind. Res. **65**, (4), 364, **2006**.
8. MURYGINA V.P., MARKAROVA M.Y., KALYUZHNYI S.V. Application of biopreparation „Rhoder" for remediation of oil polluted polar marshy wetlands in Komi Republic. Environ. Int. **31**, (2), 163, **2005**.
9. JUHANSON J., TRUU J., HEINARU E., HEINARU A. Temporal dynamics of microbial community in soil during phytoremediation field experiment. J. Environ. Eng. Landsc. Manag. **15**, (4), 213, **2007**.
10. WHITE D., CROSBIE J. D., ATKINSON D., KILHAM K. Effect of an introduced inoculum on soil microbial diversity. FEMS Microbiol. Ecol. **14**, (2), 169, **1994**.
11. DIDŽIAPETRIS A., JANKEVIČIUS K., JANUŠKA V., KRIŠČIŪNAS J., LIUŽINAS R., LUGAUSKAS A., PAŠKEVIČIUS A., REPEČKIENĖ J. 05 25. Strain Candida lipolytica 0.6. 1-5, oxidizing oil and its products. Patent LT 4793 B. **2001** [In Lithuanian].
12. LIUŽINAS R., DIDŽIAPETRIS A., JANKEVIČIUS K., KMITA M., VIZBARIENĖ R., ZVEROČKINIENĖ D., ŠATINSKIENĖ V. Biopreparation for oil- and its products-polluted soil and water cleaning, its production and use. Patent LT 5057 B. **2003** [In Lithuanian].
13. BORZENKOV I.A., BELIAJEV S.S., IBATULLIN R.R., POSPELOV M.E., SVITNEV A.I. Bioremediation technique of soil, natural and waste water, polluted with oil and oil products by applying biopreparations. Patent 2114071 RU. **1998** [In Russian].
14. KREGER-VAN RIJ N.J.W. (Eds.) The Yeasts, a Toxonomic Study, 3rd ed, Elsevier Science Publishers: Amsterdam, pp. 1082, **1984**.
15. KURTZMAN C.P., FELL J.W. The yeasts, a toxonomic study, 4th ed., Elsevier Science Publishers: Amsterdam, pp. 1055, **1998**.
16. SAMSON R.A., FRISVAD J.C. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Stud. Mycol. **49**, 1, **2004**.
17. DOMSH K.H., GAMS W., ANDERSON T.-H. Compendium of soil fungi, 2nd ed. taxonomically revised by W. Gams. IHW-Verlag: Eching, pp. 672, **2007**.
18. SALINA O. Micromycetes of *Trichoderma* sect. *Longibrachiatum* in Lithuania. Botanica Lithuanica, **13**, (4), 261, **2007**.
19. PEČIULYTĖ D., BRIDŽIUVIENĖ D. Fungi of Lithuania. II. *Mortierellales* and *Mucorales*, Vilnius: Botanikos instituto leidykla. 256 p. **2008** [In Lithuanian, summary English].
20. LIUŽINAS R. JANKEVIČIUS K., PAŠKEVIČIUS A., REPEČKIENĖ J. Means of reception of medium for oil oxidizing microorganisms and medium produced by these means. Patent LT 5587 B. **2009** [In Lithuanian].
21. CARTER M.R., GREGORICH E.G. (Eds.) Soil sampling and methods of analysis, 2nd ed., CRC Press and Taylor and Francis Group: Oxford, pp. 1264, **2008**.
22. SKAISGIRIENĖ A., JANUTĖNIENĖ J., VAITIEKŪNAS P. Modelling of chloride influence upon activated sludge community growth. J. Environ. Eng. Landsc. Manag. **17**, (2), 114, **2009**.
23. HN 60:2004. The highest permissive concentrations of dangerous chemical sub stances. Valstybės žinios, pp. 8, **2004** [In Lithuanian].
24. BIEDUNKIEWICZ A., OZIMEK T. Qualitative and Quatative changes of potentially pathogenic fungi in a hydrophyte wastewater treatment plant. Pol. J. Environ. Stud. **18**, (2), 161, **2009**.
25. ULFIG K., PLAZA G., JANDA-ULFIG K., JASTRZĘBSKA S. Examination of keratinolytic and associated non- keratinolytic fungi in sewage sludge. Pol. J. Environ. Stud. **18**, (6), 1163, **2009**.
26. ESSIEN J.P., UDOSEN E.D. Distribution of *actinomycetes* in oil contaminated ultisoils of the Niger delta (Nigeria). J. Environ. Sci. **12,** (3), 296, **2000**.
27. BANIASADI F., SHAHIDI G.H., NIK A.K. *In vitro* petroleum decomposition by *actinomycetes* isolated from petroleum contaminated soils. American-Eurasian J. Agric. Environ. Sci. **6**, (3), 268, **2009**.
28. GURIELIDZE M., BERISHVILI T., CHOLOKAVA N., PATARAYA D., NUTSUBIDZE N. Oil destructing extremophilic *Actinomycetes*, isolated from various types of soil of Georgia. Bull. Georg. Nat. Acad. Sci. Microbiol. **3**, (3), 118, **2009**.
29. PIAULOKAITĖ-MOTUZIENĖ L., LAPINSKAS E., ČIUBERKIENĖ D. Influence of soil acidity and mineral fertilizers on distribution of ammonifying and mineral nitrogen assimilating microorganisms. Žemd.-Agric. **4**, (88), 198, **2004**.
30. MARGESIN R., SCHINNER F. Effect of temperature on oil degradation by a psychrotrophic yeast in liquid culture and in soil. FEMS Microbiol. Ecol. **24**, (3), 243, **1997**.
31. SINGH H. Mycoremediation: fungal Bioremediation. Wiley-Interscience: New York, pp. 592, **2006**.
32. PRENAFETA-BOLDÚ F.X., KHUN A., LUYKX D.M.A.M., ANKE H., VAN GROENESTIJN J.W., BONT J.A. M. Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. Mycol. Res. **105**, (4), 477, **2001**.
33. BRIDŽIUVIENĖ D., LEVINSKAITĖ L., LUGAUSKAS A., PAŠKEVIČIUS A., PEČIULYTĖ D., REPEČKIENĖ J., SALINA O., VARNAITĖ R. Microbiological Damage of Materials. UAB "Valstiečių laikraštis": Vilnius, pp. 467, **1997** [In Lithuanian, summary English].

34. KIREEVA N.A., BAKAEVA M.D., GALIMZIANOVA N.F. Influence of petroleum products on the complex of soil micromycetes. Mycol. Phytopath. **38**, (1), 27, **2004** [In Russian, summary English].
35. LUGAUSKAS A., PAŠKEVIČIUS A., REPEČKIENĖ J. Patogenic and toxic microorganisms in human environment. Aldorija: Vilnius, pp. 434, **2002** [In Lithuanian, summary English].
36. TANG P., MOHAN S., SIGLER L., MAZZULLI T. Allergic Fungal Sinusitis Associated with *Trichoderma longibrachiatum*. J. Clin. Microbiol. **41**, (1), 5333, **2003**.
37. ZHUKOVA O.V., MERKUSHIN O.S., MOROZOV N.V. Influence of active association of hydrocarbon oxidizing microorganisms on the degradation of oil products. In: Fundamental Science and Practice, 2nd Int. Conf. „Problems and perspectives in modern medicine, biology and ecology. Tomsk University: Tomsk, 73, **2010**.