PROBLEMS IN CLEANUP PROCEDURES OF SOILS CONTAMINATED BY MAZUT

PROBLĒMAS AR MAZUTU PIESĀRŅOTU GRUNŠU ATVESEĻOŠANĀ

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Key words: persistent organic substances, mazut, bioremediation, rapeseed oil, crude glycerine, microorganisms

Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute the largest group of environmental contaminants released in the environment [Zongqiang, 2005]. The major sources of PAHs in soil are petroleum products such as mazut, coal tar and creosote [Pinto, 2000]. Mazut contains a very high level of polycyclic hydrocarbons and other classes of persistent hydrocarbons. The incomplete or very long-term biodegradation of mazut may be due to a number of different reasons: mazut is a mixture of compounds that differ in their extent of biochemical degradability and the soil that it has contaminated may not contain the appropriate microorganisms for breaking down the mazut under given environmental conditions.

It is not possible to separate the processes taking place in soil from the processes taking place in water, since both processes are very closely related in the biodegradation processes of soil polluted by mazut. Bioremediation is commonly believed to be a sequential process, whereby a contaminant must be first desorbed and then degraded [Hatzinger, 1995]. The rate of bioremediation is limited by many biotic and abiotic factors. The abiotic factors are temperature, oxygen concentration, the presence of nutrients and others. The main biotic factor that limits biodegradation is the low number of indigenous oil-degrading bacteria in the soil.

Bioremediation methods for cleaning mazut-contaminated soil seem to be more effective and cost-efficient, but slower, than physical or chemical methods. One way to improve biodegradation processes is by applying different utilizable wetting, dispersing, and emulsifying and/or solubilizing agents to increase the bioavailability of mazut. Some principles for selecting surfactant combinations for the stimulation of biodegradation are described by a number of authors [Sobisch, 1994; Sobisch, 2000; Zheng and Obbard, 2000; Mulligan, 2001]. Nowadays, great interest is being accorded to different microbial biosurfactants because of their biological activities, emulsifying properties and well-documented practical test or laboratory results [Mulligan, 2004; Lang, 2002].

Mazut has the highest PAH content of any oil product. It is also an interesting biodegradation research object because of the fact that it contains various PAHs.

The distribution of mazut in soil is so non-homogenous that in one case, no significant reduction in PAHs could be detected after fungal treatment, they were detected as individual substances [Mahro, 1994]. Soil as a matrix for investigations is very sophisticated and complex. This is the main reason that there is no research has been found that fundamentally describes the mechanisms responsible for PAHs' strong sorbtive binding with the organic and inorganic compounds of soil.

The complexity of the biodegradation of oil products and refined products is increased by the phenomenon of cometabolism. Some of the more recalcitrant hydrocarbon compounds may fail to support growth but may undergo limited transformations as a side-effect (cometabolic effect) of the microbial growth on other, more easily utilizable carbon-sources. The easily degradable carbon-substrates may be other hydrocarbons or organic fertilizers. A number of natural products or modified natural products have been studied for use in oil-spill cleanups: liposomes (composed of soy phosphatides) [Barenholz, 2003] and even chemicals as acetone and ethanol [Lee, 2002]. Literature describes research on the use of biodiesel for collecting different types of oil products and further research into biodegradation [Miller, 1997; Mudge, 2000; Taylor, 2001]. Biodiesel has been approbated as an effective agent in different cleanup activities, even in the cleaning of oiled shores [Von Wedel, 1997; Pereira, 2003; Fernández-Álvarez, 2006; Fernández-Álvarez, 2000].

One biotechnological strategy is to optimize the parameter which may give the highest input in the use of biodegradation technologies – that is to increase the biological diversity of soil. It has to be admitted that attempts to model natural processes for the investigation of biodegradation in biodegradation research are considerably more effective than individual PAH studies in laboratory conditions.

In our studies, we attempted to establish the most promising carbon source that could be used as an easily acceptable additional carbon source to enhance the fungal degradation of mazut and how this source affects the biological diversity of indigenous microorganisms in soil.

In our research of biodegradation, we used the patented microscopic fungus *Cylindrocarpon* sp. [Latvian patent LV 13049], isolated from soils polluted by heavy oil products available in the area of old gas depositories.

This paper gives an overview of the results obtained by focusing on the selection of agricultural products and residues that are inexpensive and locally available and that could be used for improving the growth and/or activity of the microscopic fungus *Cylidrocarpon* sp. and/or indigenous microbial populations.

Experimental part

Materials and methods

Soil characterization. The soil used in this study was a mazut-polluted soil. The average mazut content was 7750 ppm of dry soil (total extractable constituents) and 4900 ppm of polar substances. Before treatment, the soil was crushed, sieved and homogenized and organic material was added. The spiked soil samples were homogenized through vigorous mixing. Mazut in hexane was spiked to soil samples to yield 7750 mg/kg of dry soil.

Suspensions of the microscopic fungus *Cylindrocarpon* sp. were prepared in water at concentrations of 10^8 colony forming units (CFU)/ml. Soil samples were inoculated with $\sim 1\times10^7$ CFU/g. During the bioremediation process, the soil material was periodically moistened, homogenized and aerated by mixing. Soil samples were taken randomly for investigation.

The soil used for the experiments was sand and for improving the growth and/or activity of indigenous microorganisms, commercially available soil was mixed in the ratio relationship (1:1). It was initially predicted that microorganisms would be used at background concentrations for the bioremediation processes.

Mazut characteristics using IR and GC infrared spectroscopy. The concentration of oil products was determined by the infrared (IR) spectrophotometer SPECORD–75, Jena Zeis, double beam, recording, interface–SMI, calculating program COATCH. Calculation: measuring the absorbency of samples and standards by constructing a straight base line over the scan range and measuring absorbency at the peak maximum at 2930 cm^{-1}. The infrared method validation was performed according to a document developed for chemical testing laboratories [Kalnina, 2000]. It is more economical if the majority of biodegradation experiments are based on the IR spectroscopy method. Detailed information on the validation test of the method can be found in Kalnina, 2003. Duplicate samples were taken from each processed soil sample.

Extraction. The 5-g soil samples were extracted by ultrasonic extraction for 3 min in 30 ml CCl_4. The extract was filtered on a glass-sinter filter.

Cleanup. Cleanup was made using a chromatographic column with Al_2O_3 (neutral, mesh size 50-200 µm). The spectra were recorded both before and after cleanup. All used chemicals were analytical grade. Solvent CCl_4 spectroscopic grade.

Gas Chromatograph analyses. The collected extracts from soil samples were subjected to GC-analysis using a DANI Mono-column system, capillary column 10m* 0.32 mm OV-1, film thickness 0.25 µm. Inlet pressure 4 psi, nitrogen. Temperature program 35 °C for 3 min, 60-270 at 20 °C/min, 270 °C infinite. Injector and detector at 290 °C. Splitless injection, split time 30 sec. Injection volume 2 µl. SIMDIS calibration mixture C15-C36. Gas chromatograph analysis used for the description of the chemical structure of mazut. The lack of compounds with retention times <20 min indicates that no volatile contaminants remained in the bioremediated soil.

Elementary content of mazut: C(%) = 87.2, H(%)=11.7, S(%) = 0.5, (O+N)(%) = 0.6.

Determination of bacteria and fungi. The number of microorganisms was determined by the soil suspension dilution method [Pepper et al., 1982] using selective media – agarized malt extract (30 g/l, pH = 5.4±0.2) for fungi and agarized peptone yeast extract [Merck,] for bacteria. CFUs were expressed per gram of wet soil. The genera of filamentous fungi were determined according to their morphological characteristics and light microscopy results using keys [Barnett, 1957; Kiffer and Morelet, 2000].

Chemicals: NH_4NO_3 (ACROS)

Agricultural products. Nitrogen was supplemented to aqueous solutions in the form of NH_4Cl to give a final C:N:P ratio of 100:10:1. The phosphorous content in the soil was sufficient to ensure the above-mentioned proportions. The incubation temperature was 10 °C and 29 °C.
The following biodiesel production raw material and byproducts were used as agrochemicals: rapeseed oil (RO) and crude glycerine samples from production of rapeseed ethyl esters (REE-22) and rapeseed methyl esters (RME-5).

Results and discussions

The most commonly cited drawback of bioremediation compared to other remediation options is the relatively longer time scale required for treatment, due to slower kinetics. In the case of mazut, the rate of *in situ* metabolism is limited mainly by two factors (discussed in the introduction) and the generally high partitioning to the solid phase, as well as the low solubility of these compounds, resulting in low bioavailability to the organism(s) responsible for biodegradation. It is generally accepted that this limited bioavailability is the most important factor involved in the slow degradation of PAHs. Furthermore, bioavailability appears to decrease with aging time, in part due to alteration and the tighter binding of the PAHs to soil.

During the preliminary studies, different agricultural products for further experiments were selected. After a screening procedure, only more promising edible oil (rapeseed oil), crude glycerine RG-REE–22 and crude glycerine RG-RME–5 were selected for additional investigations.

Preliminary experimental results in our laboratory investigations showed that in the presence of crude glycerine obtained in production of RME-5 and REE-22, the amount of naturally occurring *Mucor* spp. (typical fungi for Mazut) increased 8-10 times and in the presence of rapeseed oil, more than 100 times. The growth of *Cylindrocarpon* sp. was suppressed both by crude glycerine (from RME-5) and in the presence of rapeseed oil. This was in good correlation with another scientific investigation in that field [Pizzul, 2007].

The further goal was to continue experiments in conditions that are maximally close to natural. A mazut mixture was prepared using a sand matrix. This made it possible to assess the impact of other microorganisms existing in the soil on the endurance of *Cylindrocarpon* sp. in competitive soil conditions.

The next series of experiments was started with mazut-polluted soil. This allowed us to assess the impact of injected additional carbon sources on the endurance of *Cylindrocarpon* sp. in commercially available soil. Samples were inoculated with ~1×10^7 CFU of *Cylindrocarpon* sp. per g of soil. Regardless of the fact that mazut had been lying in the natural environment for a long time, no cultivable microorganisms were detected in the mazut itself.

When implementing the cleanup of contamination at the northern latitudes where Latvia is located (i.e. at the Nordic chemosphere) and where climate conditions are such that optimal biodegradation processes can take place for only a few months per year, it is important to optimize biodegradation processes in temperatures existing at the beginning and at the end of the summer season, in order to extend technological processes in natural conditions. This means that the biological and ecological features of *Cylindrocarpon* sp. have to be taken into account in order to develop new methods for soil decontamination. Experiments were held at temperatures of 10 °C and 29 °C.

When experiments are held in optimal conditions (29 °C) [Kalnina et al., 2003] (Fig. 1), the impact of temperature is a considerable prevailing factor and there is no significant difference in the impact of various easily usable additional carbon sources, when the ratio of biodegradation is valuated as a reference function determining the total sum of non-polar fractions of mazut.

The obtained results showed that the biological activity of soil increased significantly at the higher experiment temperature of 29 ^{0}C. The soil used in the experiment initially contained bacteria 1×10^6 CFU/g and microscopic fungi 8×10^3 CFU/g. The number of bacteria at the end of the experiment in all analyzed samples at 29 ^{0}C was significantly bigger (from 8×10^8 to 6×10^9 CFU/g) as compared to soil at the beginning of the experiment. The added microscopic fungus *Cylindrocarpon* sp. survived and dominated (3×10^6 CFU/g) in samples (among other microscopic fungi), where glycerine from REE-22 was added as an additional source of carbon (Table 1), but in all other samples which were tested at temperatures of 10 ^{0}C it was in undetectable, low concentrations.

Biodegradation processes at a temperature of 10 °C showed a significant difference between easy-to-use carbon sources used in our experiments (Fig. 2). The speed of biodegradation (with the biodegradation of mazut being calculated as a decrease in the concentration of non-polar compounds) at the beginning (up to the 58th day) was bigger, then it decreased. The average speed of biodegradation in all samples was actually the same. The speed of biodegradation at a temperature of 10 °C as compared to a temperature of 29 °C was considerably lower, even in same time periods. Microbiological results also showed a ratio between the number of soil bacteria and microscopic fungi – samples with a bigger number of microscopic fungi also had a bigger number of bacteria, and microorganisms work as destructors (break complicated chemical compounds into simpler ones, turn them into biomass, thus obtaining raw material and energy for their own development).

The theoretical explanation of inhibition after 58 days is the formation and accumulation of toxic intermediates (mainly different hydroxylated forms of PAHs) [Klein, 2000]. Low initial counts of bacteria and fungi were found in the soil at the beginning of the experiment (Table 1). The microbiological degradation of mazut is a dynamic process and the diversity of microscopic fungi determined at the end of the experiment allows one to anticipate that the process of biodegradation may continue further and does not contradict the results of research done by other authors. At a temperature of 10 °C, even after 343 days, the concentration decrease of non-polar hydrocarbons continued.

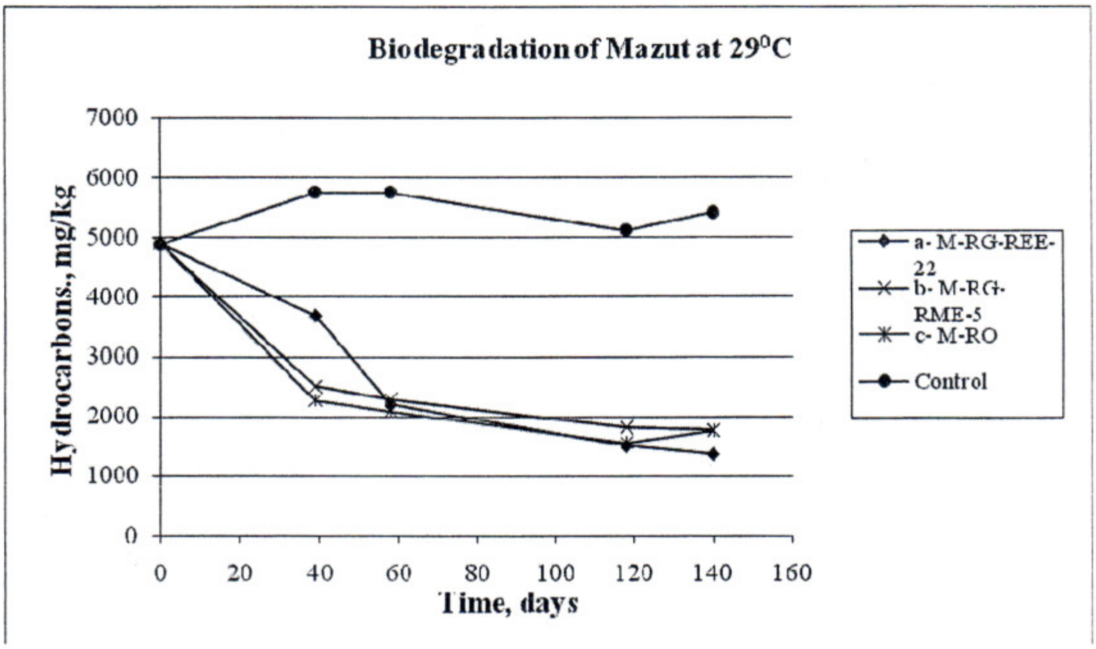

Fig.1. Biodegradation dynamics of mazut at 29 °C. The concentration of hydrocarbons is expressed as non-polar compounds of mazut.
a- (M-RG-REE-22) – Mazut-Crude glycerine from REE-22 (1000 mg/kg);
b- (M-RG-RME-5) - Mazut -Crude glycerine from RME-5 (1000 mg/kg);
c- (M-RO) – Mazut-Rapeseed oil (1000 mg/kg);

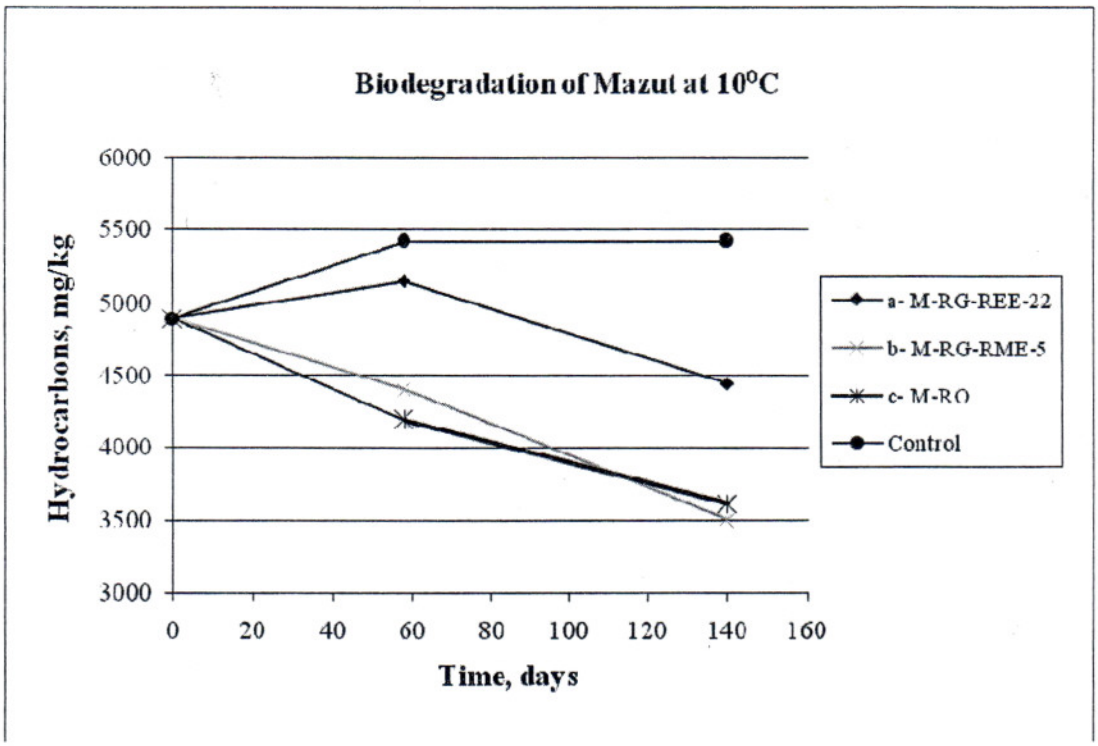

Fig.2. Biodegradation dynamics of mazut at 10 °C. The concentration of hydrocarbons is expressed as non-polar compounds of mazut.

Table1.

Changes in microflora during the soil bioremediation experiment (140 days)

Description of the experiment		**Fungi, CFU/g**					**Bacteria, CFU/g**
Soil sample	**Tempera-ture, °C**	**Total fungi**	***Cylindro-carpon* sp.**	***Mucor* spp.**	***Rhizo-pus* spp.**	***Penicil-lium* spp.**	
M-RG-REE-22[a]	10	9×10^3	-*	-*	3×10^3	6×10^3	8×10^7
M-RG-REE-22[a]	29	3×10^6	3×10^6	-*	-*	-*	6×10^9
M-RG-RME-5[b]	10	1×10^5	-*	1×10^5	-*	-*	3×10^9
M-RG-RME-5[b]	29	3×10^6	-*	2×10^4	1×10^6	1×10^6	3×10^9
M-RO[c]	10	3×10^4	-*	3×10^4	-*	-*	6×10^8
M-RO[c]	29	1×10^5	-*	2×10^2	1×10^5	1×10^4	8×10^8
Control (soil), 0 day	-	8×10^3	-*		7×10^3	1×10^3	1×10^6
Mazut	-	-*	-*	-*	-*	-*	-*

-* concentration of bacteria or fungi $<10^2$ CFU/g.

Conclusions

The following key conclusions can be made from our experimental results:
Temperature at other optimal biodegradation conditions is the main influencing factor in the biodegradation of mazut. The obtained results show that at a temperature of 29 °C, after 140 days an average of 80 % of non-polar hydrocarbons (initial concentration average 5000 mg/kg, remaining concentration average 1000 mg/kg) had degraded and that further biodegradation did not occur. Due to additional easy-to-use carbon sources, the biological activity of soil increased. The number of microscopic fungi and the number of bacteria in samples was bigger than in control test samples of soil. The microscopic fungus *Cylindrocarpon* sp. can survive at a temperature of 29 °C and can compete with such local microscopic fungi as *Mucor*, *Rhizopus* and *Penicillium* spp. and dominates at 29 °C, when glycerol from REE is used as an additional, easy-to-use source of carbon. A correlation between the total number of microorganisms and a decrease of mazut concentration was not observed.

The results indicate that a minor portion of residual contamination will remain in polluted soil even after a good biodegradation study.
At the temperature of 10 °C, biodegradation activity was increased by adding additional nutrition – rapeseed oil. The adding of *Cylindrocarpon* sp. did not provide any significant improvement. The obtained results would allow for an extension of the cleanup season in Latvia, because the usage of additional, easy-to-use carbon sources at 10 °C is more effective and the results vary among this easy-to-use carbon sources.

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Kalniņa D., Stikāns I., Nikolajeva V., Jure M., Bērziņa-Cimdiņa L. Problēmas ar mazutu piesārņotu grunšu atveseļošanā.

Mazuts ir naftas produkts, kas satur visaugstāko poliaromātisko un citu noturīgo ogļūdeņražu daudzumu. Šādu savienojumu klātbūtne mazutā, to zemā šķīdība un izteiktā spēja sorbēties uz augsnes organiskajām un neorganiskajām daļiņām ir galvenais apstāklis, kas nosaka to zemo biodegradācijas spēju.

Noturīgie organiskie savienojumi, kuri atrodas mazuta sastāvā, ir ar ļoti daudzveidīgām struktūrām un var stipri atšķirties pēc to biodegradācijas spējas un augsnēs var nebūt piemēroti mikroorganismiem, kuri noteiktos apstākļos var strādāt kā mazuta destruktori.

Darbā tika veikti pētījumi, lai izmantotu kā papildus viegli pieejama oglekļa avotus biodīzeļa ražošanas izejvielu un blakusproduktu – rapšu eļļu un jēlglicerīnu. Izmantojot šos oglekļa avotus, notikusi augsnes bioloģiskās aktivitātes palielināšanās. Gan mikroskopisko sēņu skaits, gan baktēriju skaits paraugos bija lielāks nekā kontroles paraugu augsnē.

Mikroskopiskā sēne Cylindrocarpon sp. ir dzīvotspējīga 29 °C un iztur konkurenci ar vietējām augsnes mikroskopiskajām sēnēm Mucor, Rhizopus un Penicilium spp. un dominē 29 °C, ja kā papildus oglekļa avots tiek izmantots REE sintēzē iegūts jēlglicerīns..

Iegūtie rezultāti norāda, ka papildus viegli izmantojamie oglekļa avoti 10 °C temperatūrā ir efektīvi, atšķirīgi un to izmantošana var pagarināt augsnes attīrīšanas sezonu.

Kalnina D., Stikans I., Nikolajeva V., Jure M., Berziņa-Cimdiņa L. Problems in cleanup procedures of soils contaminated by mazut.

Mazut contains a very high level of polycyclic hydrocarbons and other classes of persistent hydrocarbons. The very-low solubility in water and a high adsorbtion ability to soil's organic and inorganic fractions, are the main reasons for the hindrance to biodegradation of mazut.

The occurrence of incomplete or very long term biodegradation of mazut may be due to a number of different reasons as well as: mazut is a mixture of compounds that differ in their extent of biochemical degradability and soil does not contain the appropriate microorganisms being able to attack the mazut under the given environmental conditions.

During the studies a research was done with the purpose to use easy to access carbon sources – raw material and byproduct of biodiesel production: rapeseed oil and crude glycerine. When using additional easy to access carbon sources, increase of biological activity of soil occurred. The number of microscopic fungi as well as the number of bacteria in samples was bigger than in test samples of soil. Microscopic fungus Cylindrocarpon sp. can survive at temperature 29 °C and can compete with local microscopic fungi Mucor, Rhizopu and Penicillium spp. and dominates at 29 °C, when crude glycerine from REE synthesis is used as an additional easy to use source of carbon. The obtained results allow extend the cleanup season, because the usage of additional easy to use carbon sources at 10 °C is more effective and different between themselves.

Калниня Д., Стиканс И., Николаева В., Юре М., Берзиня-Цимдиня Л. Проблемы очищения грунта загрязненного мазутом.

Мазут – это нефтяной продукт с самым высоким содержанием полициклических и других углеводородов. Присутствие таких соединений в мазуте, их низкая растворимость и выраженная способность сорбироваться на органических и неорганических частицах почвы, это главное обстоятельство низкой биодеградации.

Устойчивые органические соединения, которые находятся в составе мазута, очень разнообразны и могут сильно различаться по способности биодеградации между собой, а также в почвах могут не

существовать микроорганизмы, способные в определенных обстоятельствах работать как деструкторы мазута.

В работе исследованы легко добываемые источники углерода, которые являются сырьем и побочным продуктом производства биодизеля – рапсовое масло и сырой глицерин.

Найдено, что при использовании легко усваиваемых дополнительных источников углерода произошло повышение биологической активности почвы.

Микроскопический гриб Cylidrocarpon sp. устойчив при температуре 29 °C и способен конкурировать с местными микроскопическими грибами Mucor, Rhizopus и Penicillium spp. Cylidrocarpon sp. доминирует при 29 °C, если как источник углерода использован сырой глицерин образующийся при производстве этилового эфира рапсового масла (REE).

Полученные результаты свидетельствуют, что легко усваиваемые дополнительные источники углерода при температуре 10 °C эффективны и существенно различаются и могут продлить сезон очистки загрязненных почв.