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BIODEGRADATION OF LUBRICANT OIL IN SOIL

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BIODEGRADACJA PRZEPRACOWANEGO OLEJU SMAROWEGO W GLEBIE PIASZCZYSTEJ

Przepracowane oleje smarowe należą do zanieczyszczeń niebezpiecznych z uwagi na ich toksyczność i małą podatność na biodegradację. W procesie bioremediacji gleby piaszczystej zanieczyszczonej przepracowanym olejem Mobil 1 (0,5 g/100 g suchej masy gleby) zastosowano wyizolowane szczepy bakterii aktywne w rozkładzie zanieczyszczenia. Mikroorganizmy wprowadzono do gleby w postaci wolnych komórek i immobilizatu w wiórach dębowych, uzupełniono zawartość soli biogennych i wody oraz zapewniono warunki tlenowe procesu. Gleba była ponadto okresowo doszczepiana aktywnymi szczepami bakterii. Po pięciu miesiącach trwania badań uzyskano eliminację zanieczyszczenia na poziomie 93% w reaktorze zawierającym immobilizowaną biomasę. Dla porównania, w reaktorze kontrolnym, w którym nie stosowano żadnych zabiegów biotechnologicznych, eliminacja zanieczyszczenia wynosiła 47%. Zawartość bakterii w trzech reaktorach była zbliżona w ciągu pierwszych trzech miesięcy trwania badań, jednak aktywność enzymatyczna biomasy w reaktorze kontrolnym przez cały czas utrzymywała się na bardzo niskim poziomie. Było to prawdopodobnie przyczyną niskiej efektywności biodegradacji zanieczyszczeń w tej glebie.

Summary

Exploited lubricants are dangerous contaminants because of their toxicity and low biodegradability. In this study, microbial culture active in exploited lubricant oil Mobil 1 was isolated and inoculated to sandy soil containing 0.5 g of contaminant per 100 g of dry soil. Microorganisms were used as free cells and immobilizate on wood chips, soil was also properly supplied with water and nutrients. The bioaugumantation seems to enhance biodegradation process. After 5 months, 93% of non-polar compounds were eliminated from soil containing immobilized biomass. Comparatively, in non-treated soil (control system) the contaminant elimination was at the level of 47%. Bacterial number in treated and non-treated soil was similar for about 3 months; however enzymatic activity (dehydrogenases and hydrolases) in control soil was much lower. Finally, after 5 months of treatment the content of bacteria active in contaminant decomposition in inoculated soil was 100-fold higher than in control system. Presumably, the main reason of low remediation results in non-treated soil seems to be low enzymatic activity of the biomass.

INTRODUCTION

Contamination of soil by petroleum products is a widespread environmental problem. Exploited lubricants are potentially classified as dangerous contaminants. Lubricants are

elaborated with base oil (85%) and additives (15%). Base oil is a complex mixture of hydrocarbons such as alkanes, aromatics and cyclic alkanes. Additives consist of viscosators (4–6%), and improvers (9–11%) such as dispersants, detergents, antioxidants, anti-foaming agents and friction modifiers. The hydrocarbons composition and physical properties of engine oils change as a consequence of its use at high temperatures and pressure conditions. Used lubricant oils contain 10 to 100-fold higher amounts of PAHs (polycyclic aromatic hydrocarbons) than new products and some heavy metals derived from engine material [14]. Selection of an effective procedure for soil remediation is an important goal in environmental engineering. Physicochemical treatment, especially incineration, is cost no-effective procedure and may cause loss of soil structure. Therefore bioremediation has become one of the most promising strategies of environmental restoration. Biological methods are based on applying microorganisms in order to reduce the concentration and toxicity of pollutants. Many soil microorganisms have the ability to degrade petroleum hydrocarbons [3]. Selecting the most appropriate strategy for treatment should be guided by considering three basic principles: (1) the amenability of the pollutant to biological transformation, (2) the bioavailability of the contaminant to microorganisms, (3) the optimization of hydrocarbons biodegradation. The biodegradability of lubricant oil is mainly determined by chemical nature of its components. Hydrocarbon constituents differ in their susceptibility to microbial attack. The n-alkanes and low molecular weight aromatics are the most sensitive, followed by the branched alkanes, cyclic alkanes, alkyl-substituted aromatics and PAHs. The low water solubility of petroleum hydrocarbon components is a limiting factor of microbial transformation. Surfactants have been mostly used as mediators for increasing the concentration of hydrocarbons in water phase and improving the bioavailability to microbial cells [1, 16].

In some cases, the biodegradability of petroleum hydrocarbons may be enhanced by introduction of active strains of microorganisms into soil. Unfortunately, there are many limitations to the use of bioaugmentation – the most important is the inability to support the growth and activity of introduced microorganisms in soil because they compete with the indigenous microflora, have poor survival ability and may be grazed by protozoa. A promising approach in this regard might be the development of cells immobilization techniques. It was proved that addition of immobilized cells to soil appeared to remove phenantrene more effectively than by means of non-immobilized cells, even when the latter were used at 100-fold higher biomass concentrations. Presumably, the higher degradation activity and survival ability of microorganisms is due to their cells immobilization. [12]. Weir *et al.* [17] proved nutrient-enhanced survival and phenantrene mineralization in soil by bacterial cells immobilized in Ca-alginate containing skim milk and granular clay. Sztompka [15] stated that petroleum hydrocarbons degradation in soil may be significantly enhanced by the application of active bacteria immobilized in wood chips and repeatedly inoculations of bacterial suspension. The application of immobilized biomass caused double increase in elimination rate of the 1% engine oil in comparison with free cells system (66–160 mg of hydrocarbons/kg dry soil/day).

The objective of the study was to isolate the appropriate microbial culture for exploited lubricant oil Mobil 1 degradation and to demonstrate the process enhancing role of the biomass immobilization. During the experiment the impact of contaminant on soil biological activity was demonstrated.

MATERIALS AND METHODS

Microorganisms

Isolation of microbial strains capable of using lubricant oil hydrocarbons as a sole source of carbon and energy.

The source of strains active in contaminant decomposition was soil taken to experiment, compost and soil strongly contaminated with petroleum derivative products. Strains were selected in series of 100 cm^3 batch cultures performed in Erlenmeyer flasks on rotary shaker with a basal mineral medium containing (mg/dm^3): NH_4Cl – 955, $MgSO_4$ – 2.7, $NaHCO_3$ – 75, K_2HPO_4 – 544, KH_2PO_4 – 213, Na_2HPO_4 – 441, $CaCl_2$ – 6.9, Fe(III)NaEDTA – 0.085, trace element solution – 0.1 cm^3, vitamins solution – 1 cm^3. Lubricant oil and yeast extract were used as carbon and energy source. Primary cultures were inoculated with soil or compost; next cultures were inoculated from previous ones. Every series was incubated at room temperature for 48 h at 120 rpm. Addition of yeast extract to the culture medium was diminished up to the moment when lubricant oil was a sole source of carbon. Afterwards, strains of microorganisms able to grow on solid mineral medium with lubricant oil were used as the inoculum for experimental reactors.

Experimental system

Experimental sandy soil with a filtration coefficient 10^{-4}–10^{-5} m/s was brought from field and loaded into three bioreactors (23 cm of height and 25 cm in diameter). Soil was contaminated with exploited lubricant oil Mobil 1 (synthetic oil, after driving for a distance of 20000 km) to the mass content of 0.5%. Oil was added to soil as a water emulsion. Two reactors were inoculated with bacterial strains active in contaminant degradation. The biomass was applied either as free cells suspension or as an immobilizate in oak wood chips. Immobilization carrier was prepared by keeping in alkaline solution (pH 9–10) for 24 h, rinsing with distilled water and dried at 105°C. The culture for immobilization was prepared on magnetic stirrer in 5 dm^3 vessels on mineral medium supplemented with peptone (0.1%) and lubricant oil (0.5%). Microbial adsorption to the carrier was carried out for 24 h. Wood chips, after separation from the medium, were immediately mixed with soil (the volumetric ratio 1:9, respectively). Additionally, soil was supplied with mineral nutrients for maintaining the proper C:N and C:P ratios (NH_4NO_3 and K_2HPO_4 in amounts of 0.35 and 0.09 g/100 g dry soil, respectively). Water content in soil was held at the level of 60–80% WHC_{max}. Oxygen supply was achieved by manual mixing of reactors content twice a week. Once a week active biomass suspension (100 cm^3, about 10^8 CFU/cm^3) was sprayed to soil in order to keep a proper microbial count.

The third reactor was used as a control. It was not supplied with bacteria and nutrients, soil was not mixed, and only sometimes it was sprayed with water to simulate raining in natural conditions.

Samples were taken every two weeks for biological control and physico-chemical evaluations; contaminant content in soil was determined three times in the course of experiment – in time 0, 73 and 162 days.

Table 1. Experimental conditions applied to different reactors

Content	Reactor		
	R1	R2	Control
Soil	8 kg of sandy soil		
Contamination	exploited lubricant oil Mobil 1 of 0.5% concentration		
Nutrients	NH_4NO_3 and K_2HPO_4 for keeping the ratio C:N = 10 and C:P = 70		–
Inoculum	bacterial suspension	bacterial immobilizate in wood chips	–
Reinoculations	+	+	–
Mixing	+	+	–
Moisture	60–80% WHC_{max}		without treatment

The samples were collected during mixing the whole content of treated soil. In a control reactor soil was taken in four points – two situated at the higher layer and two at the lower layer (the representative sample was made by mixing these four samples).

Analytical procedure of petroleum hydrocarbons estimation was modified according to Polish standards [8, 9]. It concerns gravimetric evaluation of substances extracted with petroleum ether. Every sample was analyzed as crude extract (total content of extractable organic substances) and extract after separation from polar extractable organic material (non-polar extractable organic substances). The separation was performed on Florisil (magnesium silicate) column. The column of 8 mm diameter was filled up with 10 g of Florisil (Fluca AG, 60–100 mesh, conditioned in 250°C for 2 hours). In every elution 50 cm^3 of petroleum ether was used.

Non-polar organic substances extracted with petroleum ether (not adsorbed on Florisil column) are mainly mineral oils and other hydrocarbons.

Polar organic substances extracted with petroleum ether (adsorbed on Florisil column) are lipids, fatty acids, phenols, humic acids and products of partial oxidation of hydrocarbons.

50 g of soil was mixed with water-free $MgSO_4$ and crushed in mortar. This mixture was transferred to Soxhlet apparatus and extracted with petroleum ether for 4 h at the rate of 20 cycles per hour. The extract was then mixed with anhydrous Na_2SO_4. One half of extract was immediately evaporated, dried and weighed; the other half was transferred to Florisil column. Polar extractable organic substances were adsorbed in column material. Purified extract, containing non-polar organic substances, was evaporated, dried and weighed in a similar way as the first half. Polar extractable organic substances were calculated as a result of subtraction from total extractable organic substances content the non-polar extractable organic substances value. The presented results are arithmetic means of three replicates.

A number of bacteria were determined by plating on nutrient agar and mineral medium with lubricant oil as a sole source of carbon. Soil suspensions were prepared by shaking 10 g of soil with 90 cm^3 of sterile sodium pyrophosphate (0.1% solution) for 30 min at 20°C and

120 rpm. A volume of 0.1 cm^3 of appropriate dilutions of the soil suspensions was plated by spreading onto plate's surface. Plates were incubated at 26°C. Heterotrophic bacteria colony forming units were counted after two days, hydrocarbons-degrading colonies were determined after 7 days.

Dehydrogenase activity was assayed by measuring of triphenyl formazan (TF) formation as a product of triphenyl tetrazolium chloride (TTC) reduction occurring in the respiration chain metabolism. A modified method was elaborated according to [10]. Samples were prepared by shaking 10 g of soil in 90 cm^3 of distilled water. Aliquots of 5 cm^3 were used for the analysis. All reagents were added according to Polish standard [10]. Samples were incubated for 48 h at a rotary shaker (120 rpm) at 26°C. Enzymatic reaction was stopped by adding a drop of concentrated H_2SO_4, then TF was extracted with butanol and evaluated spectrophotometrically at 490 nm. Specific enzyme activity was expressed in mmol TF per kg protein per second.

Protein concentration was assayed by Lowry method. Protein was determined spectrophotometrically (at 750 nm) in cell-free extracts obtained after disintegration of cells by ultrasonic waves.

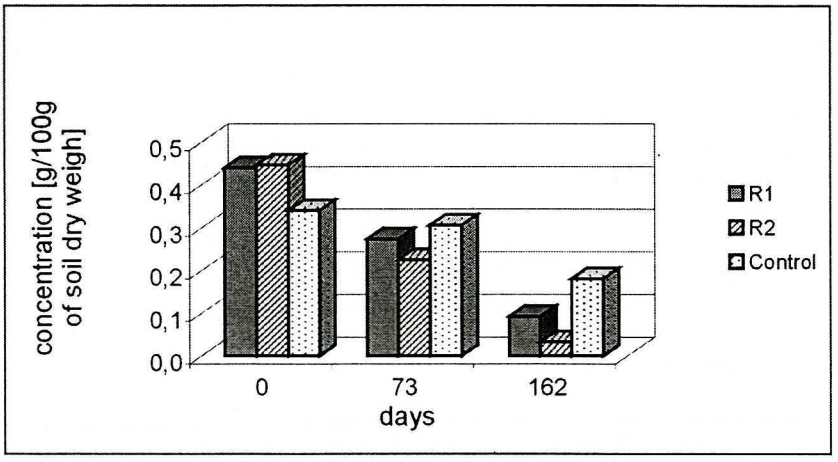
The hydrolase activity was measured by means of modified fluoresceine diacetate (FDA) assay, according to Schnürer and Rosswall [13]. Hydrolytic degradation of FDA was performed in shaking cultures (120 rpm, 26°C) containing 10 g of soil, 50 cm^3 of phosphate buffer (pH 7.6) and fluoresceine diacetate in concentration of 20 $\mu g/cm^3$. After 3 h addition acetone in a volumetric ratio 1:1 stopped the reaction. Fluoresceine concentration was measured in spectrophotometer at 490 nm. Specific enzyme activity was expressed as mg fluoresceine per kg protein per second.

The organics content in soil was determined according to [11]. Soil samples were dried at the temperature of 105°C and then incinerated at 550°C. The organic matter content was determined as the weight difference between dried and incinerated sample.

RESULTS

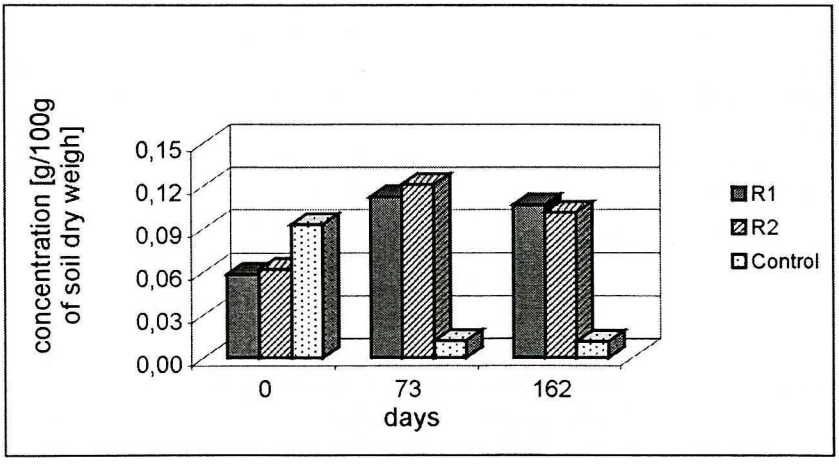
Hydrocarbons content in soil

Non-polar extractable organic compounds amount is representative for contaminant hydrocarbons remaining in soil. Analysis revealed an important difference between treated reactors and control system. In 162 days of experiment the best biodegradation efficiency was observed in soil containing immobilized biomass – 93%, towards 79% in reactor supplied with free bacteria. In control soil this level was only about 47%. It means the decrease from 0.45 to 0.03 g/100 g of dry soil in reactor with immobilized biomass. Within 73 days of the process about 50% of non-polar extractable organic compounds were eliminated in soil with immobilization applied (Fig. 1). Polar extractable organic compounds content increased in treated reactors from about 0.06 to 0.1 g/100 g of dry soil. This was the result of appearance of polar by-products of organic substances oxidation. Comparatively, in control soil the considerable decrease of polar compounds content was observed (in 72 days from 0.09 to 0.01 g/100 g of dry soil). Presumably it was the result of lubricant oil additive compounds mineralization (Fig. 2). Statistical data of results are shown in Table 2.



R1 – free bacteria R2 – immobilized bacteria

Fig. 1. The content of non-polar organic compounds extracted with petroleum ether



R1 – free bacteria R2 – immobilized bacteria

Fig. 2. The content of polar organic compounds extracted with petroleum ether

There are relatively few published studies on the biodegradation of mineral base oils used in lubricants production. The primary biodegradability of 32 oils using the CEC L-33-A-93 test revealed percentages ranged between 15% and 75%. Biodegradability values were dependent on chemical composition and viscosity of oils [5].

Interesting results were obtained during the remediation of sandy soil from mineral base oils in 3% concentration. In 8 weeks these products showed very low degradation level – about 13%, regardless of their degradability in aquatic systems [6].

Battersby and Morgan [2] determined the biodegradability of five mineral base oils using test CEC L-33-A-93, modified to soil conditions. Around 50% of each oil was biodegraded for over 6 weeks of incubation. But then the contaminant removal rate was

much lower – in 5 months it did not exceed 75%. Only fraction of oil that is both available and degradable can be removed biologically, some oil components are persistent to biodegradation.

Table 2. The content of non-polar and polar organic compounds extracted by petroleum ether, statistical data of results

Reactor	Day of experiment	Mean (X_E) concentration value (g/100 g dry soil)	Standard deviation (S)	Coefficient of variation (%)
Non-polar organic compounds				
R1	0	0.4411	0.0154	3.5
	73	0.2750	0.0127	4.1
	162	0.0929	0.0035	3.8
R2	0	0.4476	0.0166	3.7
	73	0.2258	0.0090	4.0
	162	0.0334	0.0010	3.1
C	0	0.3412	0.0150	4.4
	73	0.3067	0.0129	4.2
	162	0.1820	0.0069	3.8
Polar organic compounds				
R1	0	0.0585	0.0024	4.1
	73	0.1125	0.0037	3.3
	162	0.1070	0.0045	4.2
R2	0	0.0618	0.0027	4.3
	73	0.1213	0.0035	2.9
	162	0.1017	0.0032	3.2
C	0	0.0931	0.0033	3.6
	73	0.0119	0.0005	3.9
	162	0.0113	0.0003	3.0

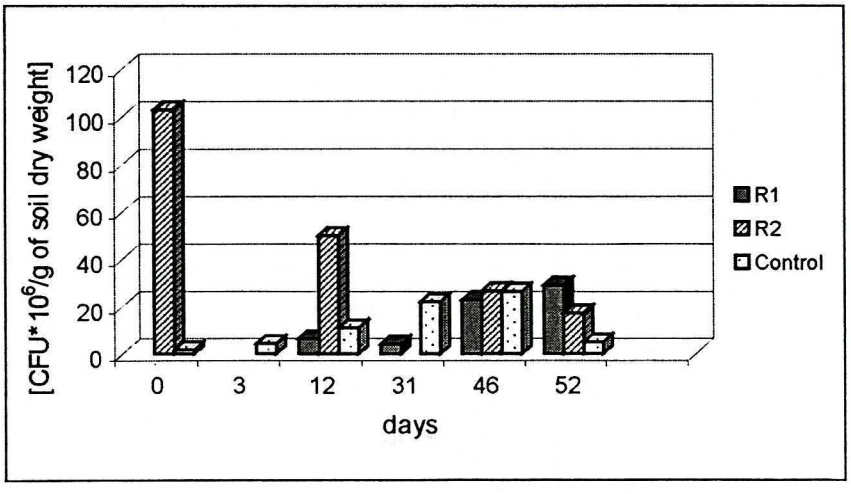
Our research revealed that in 5 months 93% of exploited lubricant oil hydrocarbons were eliminated from soil containing immobilized active bacteria.

Generally, lubricants containing mineral base oils are of relatively low biodegradability in soil. Much better biodegradation efficiency was demonstrated during soil remediation from other petroleum derivative products. Some bioremediation field experiments were

carried out in Poland – at the former Soviet army military bases and areas contaminated by industry and transport. In the case of contaminants such as petrol, crude oil and fuel oil the removal range was 80% to 99% within 22–74 days (the level of contamination was up to 3%) [16]. The duration of the process was longer in soil containing the aircraft fuel and oil (0.55 g/100 g dry soil). In 2.5 months 98% of contaminant elimination was achieved [4].

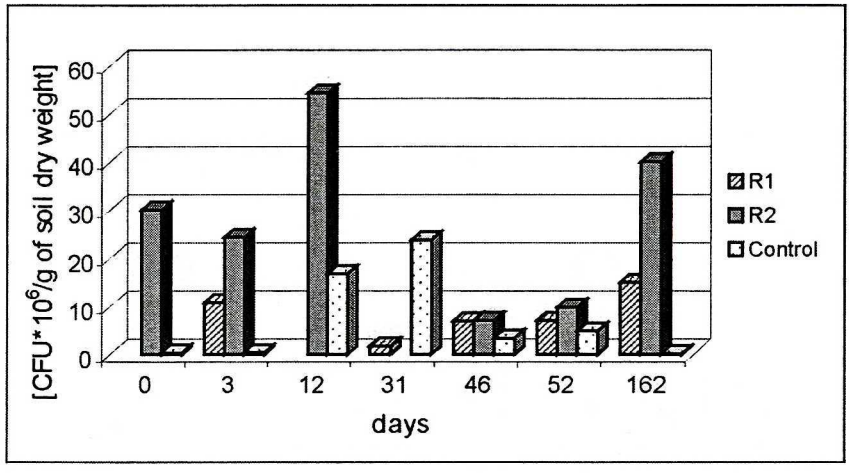
Bacteria enumeration

A bacterial count in all reactors was variable during the time of experiment. The highest bacterial content was in a reactor with immobilized biomass – at the range of 7.1 to $54.3 \cdot 10^6$ CFU/g of dry soil for cells active in contaminant degradation. In remaining reactors – R1 and control, active cells content was from 1.8 to $15.0 \cdot 10^6$ CFU/g of dry soil and $0.18–23.8 \cdot 10^6$ CFU/g of dry soil, respectively. It should be pointed out that the number of bacteria active in contaminant degradation increased quickly and sometimes was even equal to the total heterotrophs counts (Figs 3 and 4). This phenomenon shows the adaptation of autochthonic bacteria to hydrocarbons decomposition. In control soil, containing only the indigenous microflora, a great difference (one order of magnitude) between the total bacterial count and the number of cells active in contaminant decomposition was observed at the beginning of experiment. However, just after 17 days these numbers were comparable. On the 46th and 52nd day of experiment an active bacteria count significantly decreased in all reactors. However, on the 162nd day this number increased one order of magnitude in treated soil, while in control soil significant decrease – from 4.9 to $0.2 \cdot 10^6$ CFU/g of dry soil was observed. Soil reinoculations in treated reactors did not bring the effect of bacterial number increase.



R1 – free bacteria R2 – immobilized bacteria

Fig. 3. Total number of bacteria



R1 – free bacteria R2 – immobilized bacteria

Fig. 4. Number of bacteria active in contaminant biodegradation

Enzymatic activity

An analysis of fluoresceine diacetate hydrolase activity of soil microflora revealed the highest values in reactor with immobilized biomass and its systematic increase till 34th day (from about 0.5 to 1.74 mg fluoresceine per kg protein/s). In reactor R1 and control this activity was nearly at the same level – about 0.3 mg fluoresceine per kg protein/s (Fig. 5). However, significant decrease in hydrolytic activity was observed in all reactors on the 46th and 59th day of experiment – the activity values were from about 0.03 mg fluoresceine/kg protein/s in reactor R2 to 0 in control system. The possible reason of this phenomenon may be the reduction of the hydrocarbons bioavailability. Presumably, polar by-products of metabolic reactions increased the sorption capacity of soil. The decrease of bacteria number active in contaminant decomposition may also confirm this statement. On the 162nd day of experiment a significant increase of activity in treated soil was observed again – to the level of 1.3 mg fluoresceine/kg protein/s.

Dehydrogenases activity estimations revealed a significant difference between control and treated reactors (Figs 6 and 7). In control system activity varied between 0 and about 0.5 mmol TF/kg protein/s, whereas in treated soil activity values reached 15 mmol TF/kg proteins/s. Until the 59th day of experiment the highest activity was in samples for endogenous respiration. In these samples also the phenomenon of decreasing activity on the 46th and 59th day was observed.

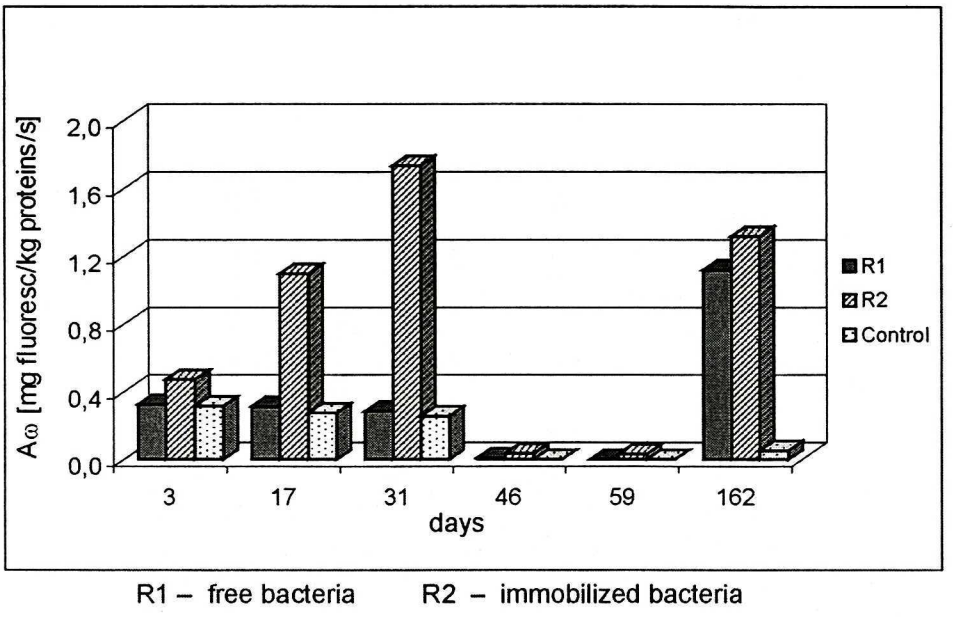


Fig. 5. The hydrolytic activity of soil microflora

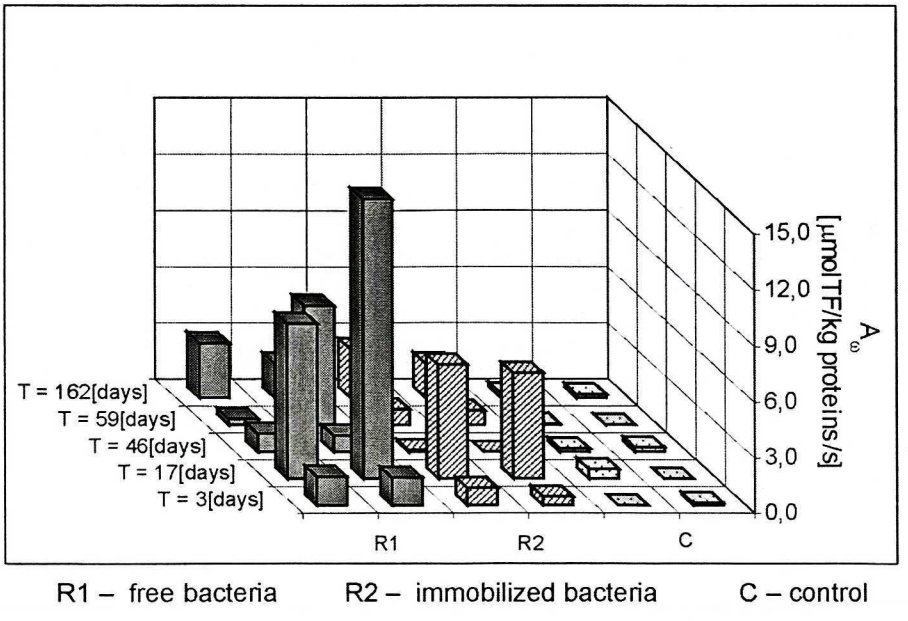
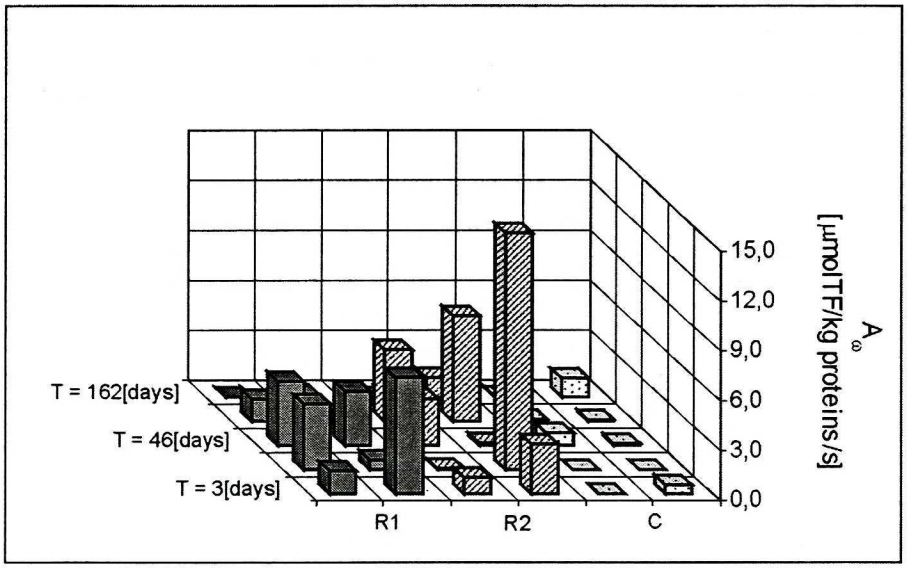


Fig. 6. The dehydrogenase activity of soil microflora – test with glucose

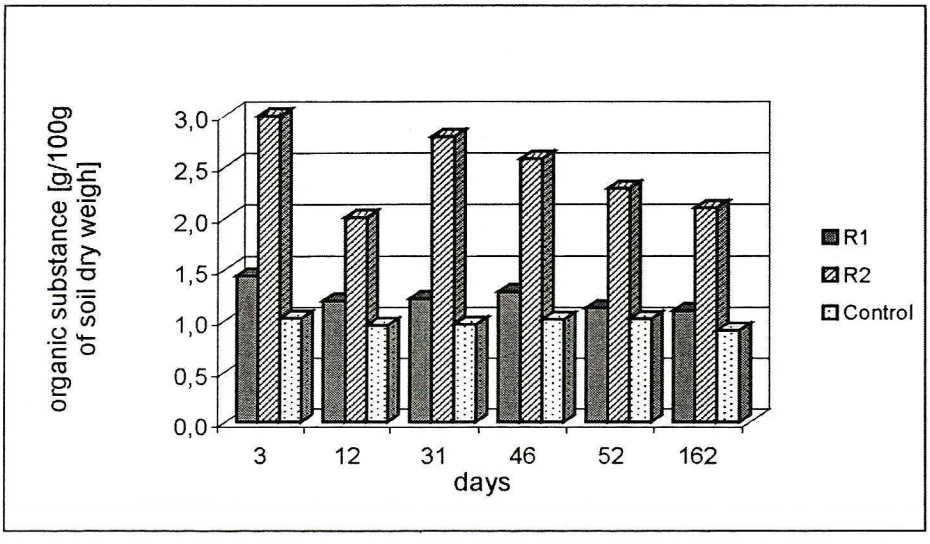


R1 – free bacteria R2 – immobilized bacteria C – control

Fig. 7. The dehydrogenase activity of soil microflora – endogenous respiration test

Organics determination

Organics content evaluation revealed no significant changes in every investigated soil during the time of experiment (Fig. 8). Because of the fact that treated soil was regularly supplied with the biomass, it is obvious that organic compounds mineralization occurred.



R1 – free bacteria R2 – immobilized bacteria

Fig. 8. Organics content in soil

Microbial selection in experimental reactors

In soil of treated reactors four bacterial and four yeast strains active in contaminant decomposition were selected during the process. There were Gram-positive and Gram-negative rods and rod and oval-shaped yeasts. Higher diversity of strains was observed in immobilizate containing reactor.

CONCLUSIONS

Basing on the findings of this study the following conclusions may be drawn:

1. Biological decomposition of exploited lubricant oil is possible in optimal conditions in sandy soil supplied with active biomass.
2. Biomass immobilization is an important enhancing factor in the process.
3. Soil remediation from exploited lubricant oil is a rather long-lasting process – 93% elimination of hydrocarbons at contamination level of 0.5 g/100 g of dry soil was achieved in 5 months.
4. Soil remediation potential may be low, even if the viable bacteria numbers are relatively high. The reason of this phenomenon is a low metabolic activity of soil biomass.

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