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AEROBIC PROCESS FOR *IN SITU* BIOREMEDIATION OF PETROLEUM-DERIVED CONTAMINATION OF SOIL: A FIELD STUDY BASED ON LABORATORY MICROCOSM TESTS

TLENOWA BIOREMEDIACJA METODĄ *IN SITU* ROPOPOCHODNYCH SKAŻEŃ GRUNTU: PROCES W WARUNKACH POLOWYCH NA PODSTAWIE OPRACOWAŃ LABORATORYJNYCH TESTÓW UKŁADÓW MODELOWYCH

Abstract: The on-site ground-water recultivation project was established in April 2007 and lasted till August ’07. The work was carried out in the area of a fuel station of the chemical industry-production plant, after dismantling and scrapping of three corroded and leaking oil-storage tanks, each of 50 m^3 capacity. Geochemical analyses revealed that the area of approximately 150 m^2 was affected by a significant pollution of ground with migrating oily products whose concentration exceeded permissible standard levels. The average content of high-boiling (T_b > 105 ^oC) organic compounds was 3 655 mg · kg^{-1} and the hydrocarbon contamination reached the level of 5.5 m of underground water. Possible pollutant migration with the aqueous phase caused high risk of affecting the nearby river that served as a drinking-water resource.

The aim of the study was to optimize the *in situ* cleanup biotechnology to enable pollution biodegradation within one season of 2007. The treatment was based on biological activities of soil-derived microorganisms. The occurrence of soil autochthonous bacteria was established as $0.8 \cdot 10^6$ cells · g^{-1}.

Tests carried out in microcosm models revealed that contaminant bioremediation was effective only in the presence of oxygen that proved to be a limiting factor for indigenous bacteria proliferation. Then, the additional soil inoculation with specialized, biochemically active microbial consortia enabled to significantly accelerate kinetics of organic compounds removal.

In a field study, an active aeration system was constructed to provide growing microbial biomass with the oxygen. Next, the area became bioaugmented with the active community by applying biomass at initial density of $1.5 \cdot 10^5$ cells per g of soil. The pollution level and cell population dynamics were monitored in soil samples collected at several distinct levels of the first geotechnical layer, ie from 0 to 120 cm. The content of high-boiling organic substances as well as the cell frequency were analyzed with standard procedures.

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The final biodegradation yields of 92.3 %, 68.1 %, 84.3 % and 93.9 % were obtained within 16 weeks for layers 0–30, 30–60, 60–90 and 90–120 cm, respectively. The observed diversity of the resultant effect was due to heterogeneous geochemical structure of the analyzed soil profile. The highest drop in contamination content correlated with a dramatic increase of soil microflora population up to $7.1 \cdot 10^7$ cells $\cdot$ g^{-1}. The method of biological treatment, elaborated and implemented in the study, led to a decrease of pollutant concentration to the limits acceptable for industrial group "C" areas within one bioremediation season.

Keywords: *in situ* bioremediation, petroleum-derived contaminants, biorecultivation of soil, bioaugmentation, autochthonous microorganisms, microbial consortia

The environmental release of petroleum-derived products bears high ecological risk since most of these substances are known to be hazardous agents [1], toxic to a variety of organisms and detrimental to human health. Oily wastes negatively affect physical, chemical and biological characteristics of soil: they lead to dramatic changes in quantity and chemical content of organic substances as well as they disturb the ratio of carbon to nitrogen and phosphorus. Furthermore, these pollutants hamper free gas exchange between soil and the atmosphere, lower soil permeability and water capacity, alter the acidity, ion exchange, and colloidal parameters. As a result, the natural soil live forms become inhibited or extinct. For the above reasons there is a strong need for reclamation of the ground-water environment and full restoration of its original conditions [2–4].

Processes of natural attenuation of hazardous organic waste contamination may be very slow and in some most severe cases can last up to hundreds of years. This process involves both, physicochemical spontaneous breakdown reactions, and bioremediation based on enzymatic action of indigenous microorganisms [5].

So far, a number of technologies have been elaborated and implemented on the industrial scale to substantially improve the rate of pollution removal, with the aim at shortening recultivation period to the order of months. Some of the approaches require the contaminated ground to be removed from its original site (the *ex situ*/off site treatment), whereas the others can be applied directly at areas of pollution release (the *in situ*/on site treatment). Among the most popular physical and chemical processing-based methods are: thermal desorption, air sparging, soil vapor extraction, dual-phase extraction, soil washing, and UV oxidation. On the other hand, several combined technologies employ biological activities of metabolically potent organisms. These are: landfarming, bioventing, biopile formation, bioslurry/bioreactor systems, biosparging, and *in-situ* groundwater bioremediation. The detailed information on the usage conditions and environmental applicability of these techniques can be found in the Guide Manual of the U.S. Environmental Protection Agency [6].

In recent years, there has been a growing interest in employing biological methods, especially bioremediation, which have proved to be efficient and economically favorable. There are numerous strains isolated from contaminated sites that had evolved unique capabilities of biodegrading a variety of recalcitrant xenobiotics [7–10]. Biological breakdown of organics is complex and requires multistage enzymatic action involving either aerobic or anaerobic metabolism [9]. Under aerobic conditions, the oxygen serves as the final electron acceptor and a maximum energetic yield is achieved, which enables biodegradation process to be relatively the most efficient and the shortest [11].

Increasing usage of bioremediation techniques for treatment of environmental pollution results from the following advantageous characteristics, that is:

a) the direct contaminant (xenobiotic) degradation often serves microorganisms as an energy-yielding process and leads to H_2O and CO_2 generation as the final metabolism products,

b) there is no need for contaminant transfer between different media which is typical of several alternative, physical-chemical approaches,

c) relatively low biorecultivation costs.

In addition, when the *in situ* biotechnologies are used, no effect of deterioration and landscape devastation is observed.

Apart from biostimulation of soil autochthonous microflora to accelerate proliferation and metabolism of the contaminant xenobiotics, it is often recommended to bioaugment the cleanup process by introducing additional cultures of specially-selected and adapted, active microorganisms [3, 4, 6, 9]. Such an approach becomes crucial for the cases of sites polluted with toxic waste where no indigenous bacteria can be found or they occur at densities too low to trigger efficient biodegradation.

The aim of this work was to optimize the *in situ* bioremediation of soil polluted with petroleum chemicals based on the results of laboratory microcosm tests. The project was carried out under fully aerobic conditions and was bioaugmented with specialized bacterial suspensions, pre-grown in the presence of hydrocarbon xenobiotics.

Materials and methods

Description of the contaminated site

The ground-recultivation project launched early 2007 was a part of a large-scale modernization plan of a fuel station located in the area of a chemical-industry production plant. According to the Polish regulations [12], the ground-water environment of this site was qualified to the group “C” areas, described as industrial regions, for which the permissible pollution limits are given by the relevant state Methodological Directive [13]. The elevated contamination with petroleum-derived hydrocarbons (the average concentration of high-boiling, $T_b > 105\ ^{o}C$, organic compounds was 3 655 $mg \cdot kg^{-1}$) was reported to affect approximately a 150 m^2 area adjacent to two leaking, corroded, 50 m^3 oil-storage tanks. The tanks were then dismantled and scrapped. Based on hydrogeological and geochemical analyses, significant ground pollution with migrating oily products was established to reach the underground water table at 5.5 m depth. The ground structure was described as very heterogeneous, consisting of Quaternary combined with Tertiary formations containing sand, silt, and a large portion of gravel together with the presence of gluey inclusions. Such morphology facilitated further migration of organic fraction with groundwater. Thus, a high risk was posed to the nearby river as the pollution might reach a drinking water resource.

Approximately 4 weeks before the first inoculation with bacterial consortium (see below), a system of active soil aeration was constructed to stimulate oxidation reactions

within soil microflora. It consisted of a network of pipes of 3 cm diameter, connected to an air compressor and penetrating the ground to be reclaimed.

Bacterial consortium used for bioaugmentation

Bioremediation was bioaugmented using the aerobic bacterial biocenosis developed in Biochemistry Department of University of Agriculture in Krakow. All the constituent microorganisms were environmental, autochthonous strains, selected and isolated over years from sites heavily polluted with organic compounds. After integration period, the strains were cultured altogether and were constantly subjected to the selective and adaptational pressure by incubating with sublethal concentrations of organic xenobiotics. The resultant microbial community was highly biodiverse and consisted of a number of bacterial species belonging to various genera. It was then applied as a soil *inoculum* at initial cell density of approx. 10^9 CFU · cm^{-3} (CFU – *colony forming units*). The details of the construction and growing conditions of the consortium are given elsewhere [14].

Laboratory test of soil bioremediation

In a laboratory pot test three identical microcosm systems were constructed, each containing 2 kg of averaged soil collected at the polluted site. One of the containers – the control – was hermetically sealed so that no exogenous oxygen could penetrate into the soil. This sample was not inoculated with any bacterial suspensions, and thus the observed microorganism occurrence was due to indigenous bacteria only. The other two soil samples were supplemented with the mineral salts mix containing (final contents per kg of soil): 23 mmol $(NH_4)_2SO_4$, 1 mmol $MgSO_4 \cdot 7H_2O$, 7 mmol KH_2PO_4, and 1 mmol $CaCl_2 \cdot 5H_2O$. These systems remained unsealed and were systematically, thoroughly mechanically mixed to allow free oxygen penetration. Soil in the third container was additionally inoculated with 10 cm^3 of the pre-grown microbial consortium suspension at the cell density of $0.9 \cdot 10^9$ CFU · cm^{-3}. In all the model systems the soil moisture was kept at approximately 50 % of the maximum sorption capacity. Bioremediation test was conducted for 16 weeks in a dark place at room temperature.

Sample collection in a field study

In a field project, bioremediation process was monitored in the first geotechnical layer whose thickness was estimated as 120 cm. For sample collection, a special tubular soil auger of 5 cm diameter was used. The samples were collected at four independent sites and the material obtained from the respective layers 0–30 cm, 30–60 cm, 60–90 cm, and 90–120 cm was averaged and subjected to further analyses.

Extraction of organic material from soil and analyses of contamination levels

The organic content of soil samples was extracted using petroleum ether. The samples were ground up and averaged mechanically and divided into 10 g specimens. Each one was then acidified with 1 cm^3 18 % HCl, treated with 12.5 g of anhydrous magnesium sulfate, mixed thoroughly and placed in an extraction thimble made of a Whatman filter paper. Extractions, carried out in duplicates, were conducted for approximately 6 h (40–60 runs) with 200 cm^3 of ether in a 65 cm^3 Soxhlet apparatus. After evaporating the excess solvent at 85 ^oC, the flasks containing extracted material were dried at 105 ^oC for 1.5 h, then cooled in a vacuum desiccator and weighed. Soil dry weight was established with a standard method and finally, the total content of high-boiling organic substances was expressed as a % of soil d.w.

Determination of microbial population in soil

Soil bacterial cell population was determined in aqueous microorganism suspensions obtained by suspending 4 g soil samples in 40 cm^3 of distilled, sterile water, and then by vigorously mixing for 2 h at room temperature in 300 cm^3 flasks on rotary shakers (300 rpm). Microbial cell density in the resultant water phase was determined with a Koch plating method by spreading defined volumes of appropriate culture decimal dilutions onto agar-solidified optimal media (2.5 % enriched agar, BTL, Poland), incubating 3 days at 37 ^oC and then by counting CFU. Before making any dilutions, in order to obtain homogeneous cell suspensions, 1 cm^3 of the original bacterial sample was placed in an Eppendorf tube and sonicated under mild conditions for 10 min with a laboratory ultrasound washer (UN-2 Unitra/Unima, Poland).

Petroleum ether, fractions boiling at 40–60 ^oC, was from POCh, Poland. All other chemicals were of analytical grade. Whenever required, fully sterile conditions were applied.

Results and discussion

A laboratory-scale bioremediation experiment (Fig. 1) was carried out in a set of comparative soil microcosm systems to evaluate the importance of oxygen penetration and to verify the process-stimulating role of bioaugmentation with bacterial cultures. Based on the obtained results it can be clearly seen that the lack of any gas exchange totally inhibited bioremediation (a sealed container, Fig. 1A, squares) by hampering proliferation and metabolic activity of bacteria. In Fig. 1B (squares) a gradual decline in autochthonous cell population is shown. This result also reveals that anaerobic processes were not triggered, which suggests that the indigenous soil microflora consisted of aerobic microorganisms, only. On the contrary, free atmospheric oxygen penetration enabled efficient biodegradation of hydrocarbon contaminants. This was observed in both cases studied, that is in bioremediation test guided exclusively by autochthons (Fig. 1, circles) as well as after bioaugmentation with a pre-grown

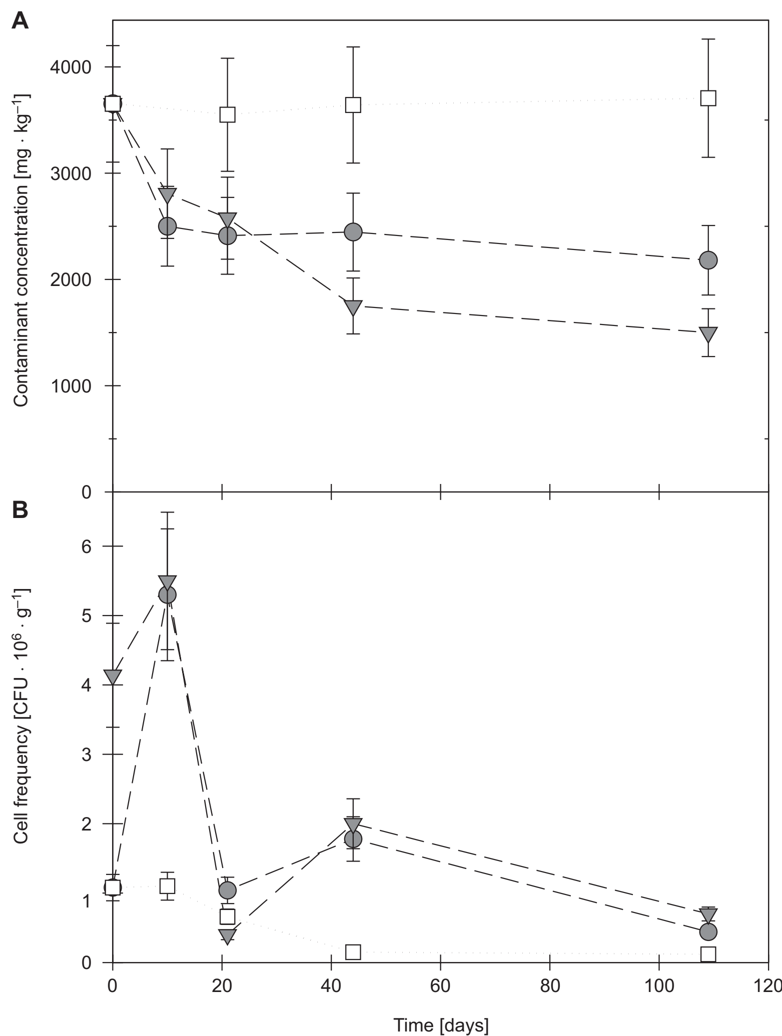

Fig. 1. A microcosm study of organic contaminant bioremediation in soil; squares – a control experiment, lack of oxygen penetration; circles – free gas exchange between air and soil; triangles – soil inoculated with a microbial community to augment xenobiotic biodegradation, free gas exchange between air and soil; (A) kinetics of soil contamination removal; (B) cell population dynamics in the tested soil samples

specialized community (Fig. 1, triangles). Moreover, in the latter case the contaminant removal rate was significantly higher (biodegradation yield of about 59 % as compared with 40 % for the case of uninoculated soil). Such a result proves that bioaugmentation took the advantage of the concerted action of both, indigenous and exogenously-added bacteria. In accordance with the observed biodegradation kinetics, Fig. 1B reveals significant growth of cell population in aerated samples relative to control. This increase of the cell density was especially visible within the first stage of bioremediation (up to 2

weeks) in which the process was launched. During this phase the most accessible fraction of organic carbon was seemingly metabolized, which enabled microbial proliferation.

Based on the laboratory test, a field bioremediation project was established in April 2007. Preliminary monitoring of the site showed that the soil contained autochthonous bacteria at the density of $0.8 \cdot 10^6$ cells $\cdot$ g^{-1}. This original cell population is represented as white bars at time zero in the right-hand panel of Fig. 2. After 4 weeks of active soil venting, cell frequencies in all the tested layers increased by more than one order of

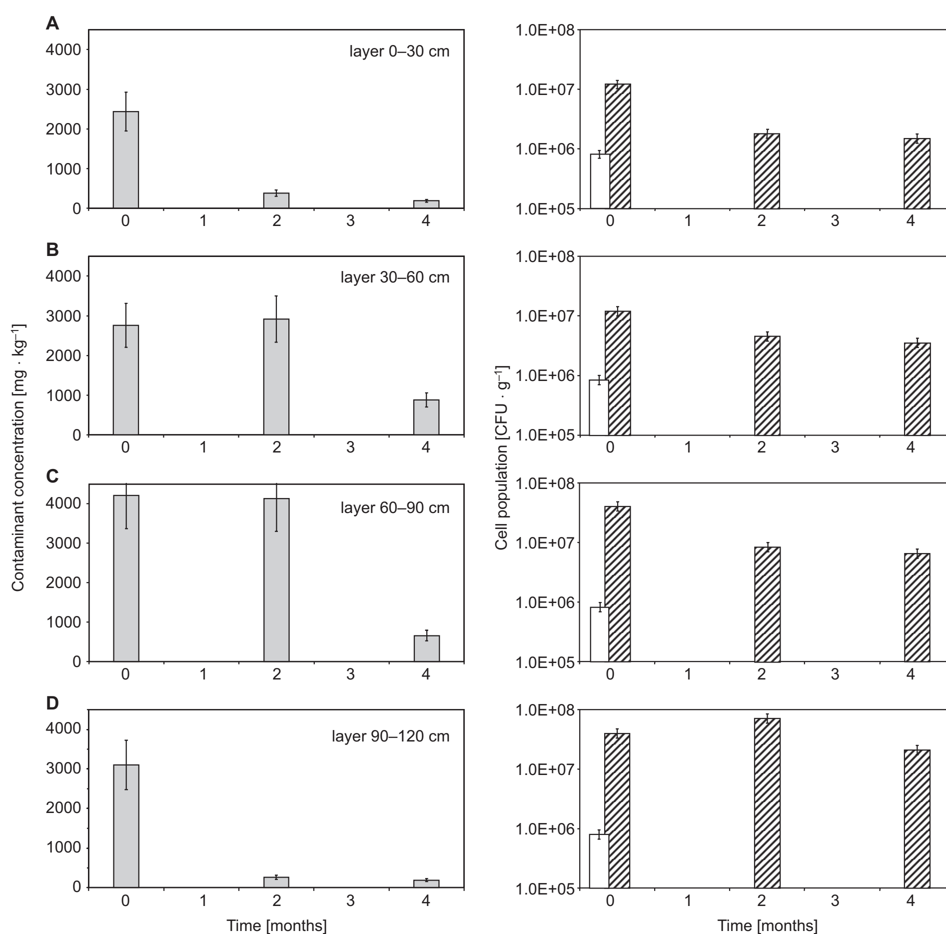

Fig. 2. An *in situ* field project monitoring of soil bioremediation in the area polluted with oil-derived substances; sections (A), (B), (C) and (D) represent the results obtained for different levels of the first geotechnical layer: 0–30 cm, 30–60 cm, 60–90 cm, and 90–120 cm, respectively; the left-hand panel presents biodegradation kinetics, whereas the right-hand one – cell population dynamics during treatment; white bars represent averaged frequency of indigenous bacteria identified in the untreated soil; grey bars at time 0 show autochthonous cell population after 4-week extensive venting of the polluted site, directly before the inoculation with bacterial consortia

magnitude (Fig. 2, the respective grey bars at time zero). This, again, proved the necessity of providing bacteria with the oxygen as a limiting factor for proliferation.

Next, the polluted area became bioaugmented with the active bacterial cultures pre-grown under controlled selective conditions to the logarithmic phase (cell density of $1.2 \cdot 10^9$ CFU $\cdot$ cm^{-3}). The aqueous microbial suspension of 20 dm^3 volume was inoculated twice by introducing directly to the soil; first, at the beginning of the process, and second, in August'07. The latter action was done at the end stage of bioremediation project, and provided the reclamation site with fresh strains to enable more efficient metabolism of the remaining, less-accessible carbon fraction.

Bioremediation project monitoring results are presented for each examined soil layer in Fig. 2A–D. The left-hand panel of Fig. 2 shows the content of high-boiling organic pollutants during the course of the study, whereas the right-hand one represents the cell population dynamics. Upon inoculation no significant increase of the total cell population was observed, which can be explained by substantial dilution of the microbial suspension used for bioaugmenting. However, judging by the degradation results, the microbial process was triggered and its kinetics were satisfactory. During the course of the cleanup project, a slight tendency to decline the cell population was observed, which correlated with the decay of the pollutants that served as carbon sources required for cell growth and energy supply.

It should be noted that for individual layers different contamination removal kinetics and different final bioremediation yields were obtained. In two cases, ie for the layers 30–60 cm and 60–90 cm, bioremediation was not launched within the first 3 months (Fig. 2B and C). We suggest that the above observations were caused mainly by a highly heterogeneous geochemical structure of the reclaimed ground, which led to variable substrate accessibilities and moisture levels in particular soil regions. The latter fact might, in turn, effect in different oxygen solubility and thus could influence the level of oxidation reactions.

The final results of the field bioremediation study are summarized in Table 1.

Table 1

Summary of bioremediation results obtained in the *in situ* field study

Depth of a ground layer [cm]	Concentration of high-boiling organic contaminants		Biodegradation yield [%]
	at the start of the process	at the end of the process (4 months)	
	[mg $\cdot$ kg^{-1} d.m.]		
0–30	2436 ± 487	188 ± 38	92.3
30–60	2762 ± 552	882 ± 176	68.1
60–90	4205 ± 841	660 ± 132	84.3
90–120	3102 ± 620	190 ± 38	93.9

The resultant average biodegradation yield, as achieved within 4 months, was 85 %. For all the soil layers monitored, the resultant organic contaminant concentrations were

acceptable in terms of the limits defined as permissible ones for industrial areas of type "C" [12].

Conclusions

1. Laboratory microcosm tests revealed that the process of oil-derived contamination removal from polluted soil was oxygen-limited. Bioaugmentation of the indigenous soil microflora with specialized bacterial consortia stimulated aerobic degradation kinetics significantly.

2. The four-month *in situ* biorecultivation project of the ground-water environment polluted with oily xenobiotics was satisfactory in terms of fulfilling permissible levels defined for type "C" industrial areas. The applied biotechnology which proved effective involved active venting, to stimulate bacterial proliferation and metabolism together with inoculation with pre-grown microbial communities, to enhance pollutant biodegradation potential.

Acknowledgements

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Abstrakt: Prace rekultywacyjne środowiska gruntowo-wodnego prowadzono w okresie kwiecień – sierpień 2007 r. na terenie przebudowywanej stacji paliw w obrębie kompleksu zakładów produkcyjnych przemysłu chemicznego. Po usunięciu starych, skorodowanych i przeciekających zbiorników paliwa o poj. 50 m^3 każdy dokonano geochemicznego rozpoznania stanu środowiska i wykazano znaczące skażenie gruntu migrującymi substancjami ropopochodnymi. Ponadnormatywne poziomy zanieczyszczeń węglowodorowych stwierdzono na obszarze ok. 150 m^2, sięgające w głąb gruntu aż do poziomu lustra wody podziemnej na głębokości ok. 5,5 m. Średnia zawartość wysokowrzących związków organicznych ($T_{wrz.}$ > 105 °C) wynosiła 3 655 mg · kg^{-1}. Dodatkowo, powstało zagrożenie dalszej migracji skażeń wraz z wodą gruntową do pobliskiej rzeki stanowiącej ujęcie wody pitnej.

Celem pracy była optymalizacja proponowanej biotechnologii oczyszczania ziemi *in situ*, wykorzystującej aktywność biologiczną drobnoustrojów glebowych tak, aby umożliwić biodegradację skażeń w ciągu jednego sezonu. W ziemi stwierdzono występowanie autochtonicznej mikroflory glebowej o liczebności 0,8 · 10^6 komórek · g^{-1} gruntu. Testy prowadzone w układach modelowych (ang. *microcosms*) wykazały, że procesy bioremediacji zanieczyszczeń przebiegały wyłącznie w obecności tlenu, umożliwiającego proliferację bakterii autochtonicznych (40 % spadek poziomu skażeń w ciągu 16 tygodni). Dodatkowe zaszczepienie gruntu specjalistycznym konsorcjum aktywnych biochemicznie drobnoustrojów (biopreparatem) pozwoliło przyśpieszyć kinetykę rozkładu skażeń organicznych (wzrost wydajności do 59 %).

W pracach polowych skonstruowano system aktywnego napowietrzania zapewniający dostępność tlenu dla rozwijających się autochtonów, po czym grunt suplementowano aktywnymi drobnoustrojami w ilości ok. 1,5 · 10^5 komórek · g^{-1}. W próbkach ziemi, pochodzących z poszczególnych poziomów pierwszej warstwy geotechnicznej do głębokości 120 cm prowadzono monitoring poziomu skażeń oraz dynamiki rozwoju populacji drobnoustrojów. Oznaczanie zawartości substancji ropopochodnych prowadzono według standardowej procedury oznaczania wysokowrzących substancji organicznych w glebie. Liczebność mikroorganizmów glebowych określano standardową, płytkową metodą Kocha.

Dla warstw 0–30 cm, 30–60 cm, 60–90 cm oraz 90–120 cm uzyskano końcową efektywność biodegradacji wynoszącą, w ciągu 16 tygodni, odpowiednio: 92,3 %, 68,1 %, 84,3 % oraz 93,9 %. Zróżnicowanie końcowego wyniku wiązało się z heterogeniczną strukturą geochemiczną analizowanego profilu glebowego. Najsilniejszy obserwowany spadek zanieczyszczeń korelował z gwałtownym rozwojem mikroflory glebowej (do 7,1 · 10^7 komórek · g^{-1}). Opracowana i zastosowana metoda biorekultywacji pozwoliła obniżyć koncentrację skażeń w sezonie 2007 do poziomu akceptowalnego dla obszarów przemysłowych grupy C.

Słowa kluczowe: bioremediacja *in situ*, zanieczyszczenia ropopochodne, biorekultywacja ziemi, bioaugmentacja, drobnoustroje autochtoniczne, biopreparaty