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THE USE OF BIOTESTS IN ESTIMATION OF WEATHERED DRILLING WASTE BIOREMEDIATION

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Abstract: Petroleum products are complex mixture of compounds of varied biological properties. They can cause harmful changes in contaminated ecosystems and threaten humans and living organisms as well. Bioremediation (including bioremediation stimulated by biogenic substances and inoculation with biopreparations from autochthonous bacteria and fungi) can result in creation of metabolites of a varied structure and biological activeness, which has been partly recognised. Some of them are more toxic than an initial substrate. Besides, they might have mutagenic features and be responsible for cancer. Estimation of bioremediation effectiveness in waste pits was completed with toxicological monitoring. It was led with the use of living organisms as biomarkers representing all trophic levels of a chosen ecosystem: producers, consumers and reducers. This process enables total estimation of natural environment conditions. The aim of the research was to determine the influence of petroleum contaminants and indirect metabolites (produced during bioremediation) on soil biocenose. The results of biotests (toxicity, phytotoxicity and genotoxity) have been taken into account. The following biotests, prepared and produced by Microbiotest, have been applied: Phytotoxkit™, Ostracodtoxkit F™, acute toxicity tests Microtox® Solid Phase and Ames mutagenicity tests. The obtained results enabled observation of changes in toxic properties during purification of the soil taken from waste pits. In addition, it can be claimed whether the areas are suitable for forest usage.

INTRODUCTION

Drilling wells, done by oil and gas mining industry in Poland, has always resulted in necessity of gathering and re-usage of large amounts of wastes, which have been stored in weathered pits.

Petroleum substances are among main sources of soil/waste contamination stored in weathered pits. They cause degradation of biological life in the area of their storage and are responsible for uselessness of the area. Reclamation of oil-contaminated and degraded areas of the weathered waste pits is a crucial ecological problem, which the Polish oil and gas industry is currently facing.

While selecting an optimum method of purification, an analysis of interdisciplinary laboratory research of groundwater environment and cleaning processes should be taken into account. Recently, an increasing number of research has been devoted to acceleration of hydrocarbons degradation through biotechnological processes, with the use of active bacteria cultures isolated from strongly contaminated natural environment. These

biological methods are applied on a technical scale due to their low costs and high effectiveness [13, 15, 29].

Crude oil is a complex, multi-component system and therefore, the use of mixture of microorganisms with a complex enzymatic system ought to be applied to degradation. The effectiveness of petroleum hydrocarbons biodegradation depends on many factors, including properly numerous and active microflora. However, accumulation of toxic products of microorganisms' metabolism should not take place during bioremediation in the area under purification [16, 21, 27]. Inoculation of bacteria can cause an antagonistic interaction of autochthonous microflora with extraneous cultures unadapted to the given environment. To avoid this, bacteria consortia should be prepared on the basis of autochthonous microorganisms isolated from the purified area [6, 18, 34].

Research on biodegradation with the use of fungi (*Cladosporium sp., Aspergillus sp.*, *Cunninghamella sp.*, *Penicillium sp.*, *Fusarium sp.*, *Cladophialophoria sp., Phanerochaete chrysosporium, Pleurotus ostreatus*, *Trichoderma asperellum*) has been the subject of numerous works [14, 28, 31]. The fungi are crucial in elimination of petroleum hydrocarbons through creation of indirect products of decreased toxicity and increased susceptibility decomposition by bacteria. Deeper purification of contaminated drilling wastes can be obtained in the final stage through application of fungi isolated from the area under purification. The fungi were used in enrichment of a biopreparation based on autochthonous bacteria. The aim of the modified biopreparation was to biodegrade consecutive groups of petroleum pollutants, which were resistant to biological decomposition (BTEX, PAH), and decomposition of long-chain aliphatic hydrocarbons [35].

The purpose of microbiological research is to create a professional biopreparation based on bacteria and fungi taken from areas severely contaminated with petroleum substances. However, the typical microbiological tests have to be broadened by research based on molecular techniques [5, 10, 30]. Alteration of oil contaminants, monitored by the chromatographic analyses, enabled optimisation of bioremediation parameters and estimation of the process effectiveness.

Moreover, it must be said that chemical and microbiological changes can result in creation of metabolites of diversified or poorly recognised biological activeness [2, 21]. Complete elimination of pollutants in bioremediation applied to soils contaminated with petroleum substances is extremely difficult due to the fact that even small amounts of metabolites can be dangerous. Determination of concentration levels of the remaining pollutants is difficult, therefore estimation of contamination is done with the use of toxicological tests, which in addition enable to obtain the image of examined population, including death rate, growth, reproduction and physiological disorders [2, 17, 27].

A bioindication analysis is a part of ecotoxicology, which is an interdisciplinary, intensively developing branch of knowledge nowadays. It includes chemistry, ecology and toxicology aimed at estimation of natural environment condition and human health protection in an indirect way. Bioindication is a method that uses a life form as an indicator because its reaction can serve as a basis to estimate general activeness of a tested system. The reaction includes not only summary activeness of all antropogenous substances and toxins, but also enables to obtain an image of interaction between toxic substances and both abiotic and biotic factors of the environment (pH, water hardness, suspension, etc.) [32].

Selection of research methods is an essential element in toxicological tests. A life form, used in biotests, has to meet strict requirements, such as constant accessibility and

homogeneity. Nowadays, ready-made tests prepared as packets have been introduced. They enable toxicological estimation of samples in a short period of time. Besides, they include cryptobiotic forms of bioindicators coming from standard cultures, they can be stored for a long time and prepared to tests immediately. Cryptobiotic forms of the organisms are the future of bioindication, because possibility of application of these organisms by typical laboratories (including chemical laboratories) enables to treat them as a common chemical reagent [23, 26]. Representatives of three trophic groups can be used as bioindicators. International organisations aiming at standardisation (ISO, OECD, ASTM, DIN) recommend their application.

In order to estimate toxicity level, in bioremediation of soil contaminated with petroleum pollutants the following tests have been applied: Microtox® Solid Phase [1, 2, 4, 7, 12], Ostracodtoxkit F™ [8, 24], Phytotoxkit™ [9, 17, 25] and Ames mutagenity test [11, 18, 19].

The aim of the work was to estimate bioremediation of weathered drilling wastes taken from the area of the waste pit. Toxicological research with the application of the chosen packet of the tests have taken place. The use of life forms as bioindicators belonging to various taxonomic groups (bacteria, crustaceans and land plants), representing all trophic levels: producers, consumers and decomposers (Tab. 1) enabled complex estimation of condition of a waste pit area under purification.

Table 1. Toxicological tests estimation of soil from waste pit

Trophic level	Life form	Test	Test reaction	Time
Decomposers	*Vibro fischeri*	Microtox® SPT	luminescence inhibition	15 min.
Consumers	*Heterocypris incongruens*	Ostracodtoxkit™	growth inhibition, death	6 days
Producers	*Sorghum Saccharatum*	Phytotoxkit™	sprouting, early growth	3 days
	Lepidium sativum			
	Sinapis alba			

DESCRIPTION OF THE BIOREMEDIATION PROCESS

High content of petroleum pollutants in waste indicated that purification would be a long-lasting and difficult process. Introduction of the phase technology of purification was necessary in order to decrease the contamination level gradually, which enabled application of the consecutive phases aiming at deeper purification of the waste [34].

The laboratory research (*ex-situ* method) enabled optimisation of the following stages of a complex purification technology and served as a basis for *in-situ* purification. Due to seasonal and changeable atmospheric conditions, the conception of wastes purification in industrial conditions (*in-situ* method) ought to be verified through lengthening the period of the consecutive stages of purification.

Purification of soil taken from Graby-12 waste pit, led in industrial conditions with the *in-situ* method, included the following phases:

– 1st year of purification – initial remediation (drainage), which resulted in satisfactory effects of decrease in petroleum contaminants in surface and deeper soil layers;

– 2nd year of purification – basic bioremediation stimulated by biogenic elements dosage (mineral fertilizer "Azofoska"); doses were indicated on the basis of laboratory tests (C:N:P = 100:10:1), pH correction = 7.4–7.8, humidity in the range of 20–25%, 2-series inoculation with autochthonous bacteria-based G-12-1 biopreparation;
– 3rd year of purification – 2-series inoculation with autochthonous bacteria-based G-12-2 biopreparation enriched with selected fungi.

The typical microbiological research, broadened by sequence analysis of DNA coding 16S rRNA for bacteria and 18S rRNA for fungi, resulted in creation of the G-12-1 biopreparation based on isolated and reproduced autochthonous bacteria species (*Micrococcus luteus, Rhodococcus erythropolis, Rhizobium daejeonense, Pseudomonas veronii, Gardonia Terrae)*. The biopreparation was enriched with selected species of fungi *(Penicillium chrysogenum, Phanerochaete chrysosporium)* – G-12-2 biopreparation.

During creation of the professional biopreparation, attention should be paid particularly to safety of its usage. Molecular research enabled determination of species affinity of the isolated and selected microorganisms. On the basis of a classification list used by American Type Culture Collection (ATCC) (Biosafety Level-1: cases of illnesses in healthy adults were not found), pathogenic species of microorganisms were eliminated from the biopreparation.

The applied biopreparations were able to fast adaptation in contaminated environment, had high biochemical activeness in petroleum hydrocarbons biodegradation, a wide spectrum of activeness and high effectiveness in decrease in oil pollutants content.

Each step of petroleum-contaminated soil purification process was controlled by the chromatographic method, which enables to determine the quality and quantity of hydrocarbon compounds (TPH, BTEX, PAH) in soil. This analytical method is used for observation of decrease in every single hydrocarbon pollutant quantity in purification process. During the process of biological purification, indirect metabolites of diversified biological activeness are created. Some of the metabolites might be more toxic than an initial product. Furthermore, they can be potentially mutagenic and carcinogenic. Taking these properties into consideration, in the purification done in Graby-12 waste pit, typical monitoring (chemical, chromatographic and microbiological) was aided by toxicological tests.

ANALYTICAL METHODOLOGY

Chemical analysis

Chromatographic analytical method allows for identification and quantification of hydrocarbon pollutants (TPH, n-C_6 – n-C_{44}, pristane, phytane) in soil. Due to this method, there is a possibility to control the process of hydrocarbon pollutants degradation. In addition, it is useful for the preparation of effective microbial consortia consisting of indigenous microorganisms and their application [33, 34].

Collected samples were averaged and homogenized. Isolation of petroleum analytes was done with application of the method of solvent extraction modified ultrasonically. Dichloromethane was selected as the optimum solvent. During simple solvent extraction of petroleum hydrocarbons (TPH), the recovery ratio reached 75.7%. The use of ultrasonication in solvent extraction process resulted in increase in the petroleum sub-

stances recovery to 95.9%. The optimum time of reaction with ultrasonication (30 kHz) at 40°C varied from 20 to 35 minutes; in the course of sonication no artefacts were found. Concentrations of analytes in solvent extracts were below detection levels and had to be increased above the traceable limit (enrichment). The recovery ratio of analytes was determined with the use of o-terphenyle. Columns with florisil sorbent were used to purify analytes from polar substances. The Clarus 500 (GC/FID Perkin Elmer) chromatograph with a QUADREX capillary column 007-1 (30m x 0.53 mm, Panalytica) and a helium flow rate of 20 mL min^{-1} was used for identification and quantitative determination of n-alkanes and hydrocarbons from an isoprenoid group. Evaluation of total concentration of hydrocarbons was done on the basis of the data of chromatographic analyses. Temperatures of a PPS injector and detector were 290°C and 300°C. The schedule of temperature changes for aliphatic hydrocarbons was as follows: 28°C – constant temperature for 2 minutes, 28°C–105°C – temperature increase rate 10°C min^{-1}, 105°C–285°C – temperature increase rate 5°C min^{-1}, 285°C – constant temperature for 20 minutes. A set of calibration standards (Tusnovic Instruments) was used for quantitative evaluation of total petroleum hydrocarbon (TPH) content, whereas certified standards (Supleco, Restek) were used for quantitative determination of other alkanes (certified n-paraffin mixture No. D2887 consisting of: nC_6 – nC_{44} and certified standard mixture No. A029668: Fuel Oil Degradation Mix – nC_{17}, pristane, nC_{18}, phytane).

In order to determine BTEX content in soil, an analytical method with an application of Clarus 500 gas chromatograph with HeadSpace TurboMatrix 16 autosampler was used.

A soil sample of 3 g was placed in an ampoule which was closed and put in an autosampler. Before the analysis, the sample had been heated for 10 minutes in temperature of 90°C in the presence of a carrier gas – helium. The chromatographic analysis was done with the use of Clarus 500 chromatograph, produced by Perkin Elmer, with the following parameters; injector temperature = 200°C, detector temperature = 280°C, capillary column RT-TCEP 60 m x 0.25 μm, oven temperature run: 60°C – isothermal run in 5 min, temperature growth 5°C/min in a range from 60°C to 100°C, isothermal run in 10 min.

A chromatographic method of determination was created in order to determine PAH isolated from the soil taken from the waste pit. This method enables identification of separated PAH and their quantitative determination. Research on isolation of an aromatic fraction was done with the use of numerous solvents chosen due to literature: hexane : acetone (9:1) [34], hexane : isopropanol (2:5) [3], hexane : dichloromethane (1:1) [16], dichloromethane: acetone (1:1) [20], petroleum benzine – fraction 40–60°C.

As an optimum solvent petroleum benzine (40–60°C), made by Merck, was used.

PAH determination method was as follows:

- analytes (PAH) isolation with the use of a continuous extraction (according to Soxhlet), petroleum benzine 40–60°C fraction and evaporation of a sample to 5 ml;
- PAH fraction separation with the use of 2-phase columns Bakerbond SPE PAH Soil, containing phases: 500 mg Cyjano/100 mg Silica Gel;
- PAH elution with the use of mixture of solvents 3 x 3 ml of acetone and toluene (3:1);
- analytes purification with the use of SPE column with Florisil (by Supelco) phase.

The extraction efficiency of the sample preparation stage (before PAH analysis) was in the range from 89.7 to 95.7%, as determined with the use of the reference soil ERM-CCO13.

Chromatographic analysis, which enabled determination of 16 PAH and total amount of PAHs, was done on Clarus 500 GC chromatograph produced by Perkin Elmer, equipped with RTX–440 capillary column, with oven run temperature program: 40°C – isothermal run in 2 minutes; temperature increase 40–240°C (rate 25°C/minute); temperature increase 240–320°C (rate 10°C/minute); 320°C – isothermal run in 10 minutes; injector and detector (FID) temperature: 320°C. A set of calibration standards, produced by Restek, was applied in order to identify and determine quantity of 16 PAH.

Ecotoxicological analysis

Microtox Solid Phase Test, produced by SDI Company (USA), enables a direct contact of luminescence bacteria *Vibro fischeri* with a soil sample, which leads not only to determination of substances dissolved in water, but also to the recognition of lipophilicity systems and poorly dissolved systems in water. Two grams of soil were mixed with 100 ml deionised water, mixing was carried out until a dark colour was observed in the solution. Subsequently, the soil extract was filtered with Whatman no. 40 and the filtrate was analysed in a Delta Tox analyser.

A screening test was done according to a standard procedure, with the use of lyophilised bacteria *Vibro fischeri*, which can be stored for a year in a temperature of minus 20°C and then used for testing immediately after suspending in deionised water. A basic toxicity test is applied to samples, which appeared to be toxic in the screening test.

Professor Persoone's team from Belgium created tests called Toxkits. Organisms are delivered as criobiotical forms: rotifers as cysts, crustaceans as spore-forming eggs, algae as cells blocked on a carrier and protected against growth by a special solution [8, 25].

Ostracodtoxkit(F)™ test belongs to direct contact tests of chronic toxicity estimation with the use of crustaceans *Heterocypris incongruens.* The direct contact test is done with the application of young bottom crustaceans *(Heterocypris incongruens)*, which hatched from cysts during 52 hours (according to the producer's procedure). The test is carried out on 6-hole polystyrene microplates. A standardised nutrient is an algae suspension, which is applied to the suspension in portions of 2 ml. Portions of 10 ostracods are added to each cell of the microplate. 0.5 g of a control soil is applied to the row A, whereas 0.5 g of the soil sample is added to the consecutive rows. Incubation lasts for 6 days in a temperature of 25°C. Results reading consists of estimation of life microforms amount in each hole of the microplate and measurement of their length. Growth inhibition of *H. incongruens* was calculated as:

$$GI = 100 - (\frac{A}{B} * 100) \quad (1)$$

where: A – ostracods length increment in the tested soil,
B – ostracods length increment in the reference soil.

The dried solid samples were testes for their phytotoxic properties by application of plant organisms *Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba* (Phytotoxkit™ by Microbiotests) as test species. Ten seeds from each species were placed in flat shallow transparent test plates consisting of two compartments, where the lower one was able to maintain the test soil saturated to the water holding capacity. Also, a control

sample was prepared with the use of reference OECD soil. All samples were prepared in triplicates. Test plates with the seeds were incubated for 3 days at 25°C. The inhibition in seed germination and root growth was calculated according to the following equation:

$$I = \frac{(A - B)}{B} * 100 \quad (2)$$

where: I – inhibition (%),
A – seed germination or root length in the control soil,
B – seed germination or root length in the test soil.

Numerous petroleum contaminants and metabolites created in biodegradation of pollutants include carcinogenic compounds.

In order to estimate potentially mutagenic properties of the soil from the waste pit, Ames Test was applied. It enables determination of reverse mutations from histydyne auxotrophy to prototrophy in mutants, which are test strains of *Salmonella typhimurium* [19, 22, 36].

The test strains of the bacteria can be characterised by various types of mutation in genes, which are responsible for histidine synthesis. Therefore, the presence of histidine in a substratum is required in order to provide the growth of the strains. Generally, TA98 and TA100 strains are used. They include proper mutations consisting of so-called reading frame displacement, pair base substitution and two mutagenes (2-nitro-fluoren and sodium azide). The test strains are mutants, which are unable to histidine synthesis. A mutagenous substance causes reversion in mutation and histidine synthesis can take place, which results in appearance of revertants colonies in the histidine-free basis.

In order to lead Ames Test, a high-tech microplate AMES MPF test was applied. It can be used for detection of genotoxic activity of soil contaminated with petroleum substances and metabolites, which are the effects of bioremediation. Delivered microforms of T-98 and T-100 *Salmonella typhimurium* undergo strict quality control (genotype and phenotype). The test results in colour reaction, where yellow means return mutation, whereas scarlet – the lack of mutation.

Petroleum contaminants from the samples were extracted with the use of dichloromethane, which was then vaporised and the obtained deposit was dissolved in dimethylosulfooxygen (DMSO).

As an activator, S9 microsomal fraction from a rat liver was used. The role of the activator is to transform the tested substance, which leads to disclosure of its potentially mutagenous properties.

RESEARCH MATERIAL CHARACTERISTICS

In the area of an oil plant in Grabownica, there are 150 weathered waste pits, which were partially neutralised through mixing the wastes with soil. However, due to their high content of petroleum contaminants, the pits are still a serious threat to the natural environment.

Drill wastes, stored in Graby-12 waste pits of a capacity of 1 733 m^3, are a result of a shallow hole drilling (more than 1000 m under the surface) with the use of a percussive and mud-rotary methods in deepening. The wastes include rock spoil, drilling mud components and oil from drilled-out petroliferous layers (after drilling). In drillings, only silt

muds (created from water and silt minerals such as bentonite), unmodified with chemicals, were applied. On the basis of physico-chemical and mineralogical research, archival documents on drilling and chromatographic analyses of petroleum pollutants included in wastes, the wastes have been classified as the soil and ground contaminated with petroleum substances (code: **ex 17 05 03**).

According to chromatographic analyses of petroleum contaminants (TPH) included in waste samples taken from several points (1–40) of depth in a range from 0 to 0.50 m under the surface from Graby-12 pit, three areas of a varied contamination degree have been determined (Fig. 1.):

area I: 59 270 – 99 960 mg TPH/kg dry mass;
area II: 111 822 – 192 480 mg TPH/kg dry mass;
area III: 71 125 – 112 657 mg TPH/kg dry mass.

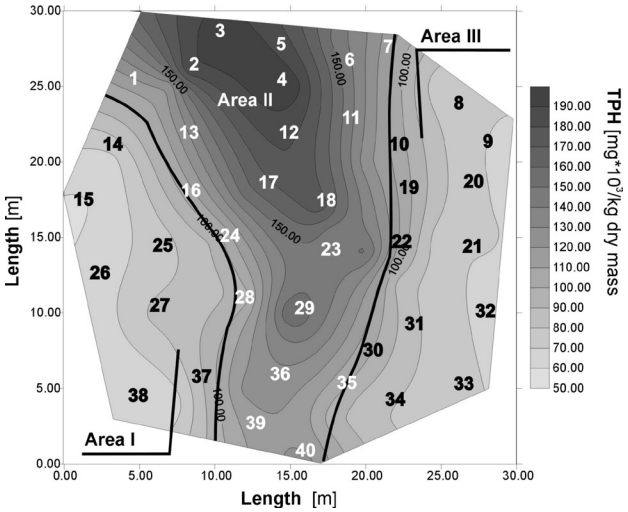

Fig. 1. Petroleum hydrocarbons (TPH) concentration in surface level (interval 0–50 cm) in Graby-12 waste pit with marked sampling points

Analyses of samples taken from deeper layers proved that soil and earth at the depth of 0.80 m under the surface were contaminated in a range from 31 248 to 89 794 mg TPH/kg dry mass. Only at the depth of 1.8 m under the surface, TPH contents were close to a soil standard level.

The chromatographic analysis indicated that nC_6 – nC_{36} chain alkanes in averaged samples taken from various depth intervals ranged from 72.8 to 89.4% petroleum pollutants.

In Graby-12 waste, monoaromatic hydrocarbons (BTEX) were in a range from 34.712 to 95.445 mg/kg dry mass. The hydrocarbons content decreases with the depth of the pit. Benzene and toluene were present in prevalent amounts, much more above soil standards. There were trace amounts of multi-ring aromatic hydrocarbons (PAH): from 4.711 to 1.741 mg/kg dry mass (Tab. 2). Naphthalene was among identified hydrocarbons of the highest content (3.45–1.258 mg/kg dry mass), whereas other hydrocarbons were identified in trace amounts below soil standards.

CHROMATOGRAPHIC RESEARCH RESULTS

Purification of the waste from the waste pit, done with an *in-situ* method, was monitored through chromatographic analyses of petroleum hydrocarbons and through toxicological estimation of the wastes under purification in consecutive stages of bioremediation.

Initial bioremediation, consisting of drainage led in the first year of purification in the area of Graby-12 waste pit, brought satisfactory results of decrease in petroleum contaminants contents in surface and deeper layers of the soil as well.

The *in-situ* method, led in industrial conditions in Graby-12 waste pit in the second year of purification, included initial bioremediation and inoculation with G-12-1 biopreparation. It resulted in a significant decrease in TPH content in consecutive areas of the waste pit in a range from 5.812 to 20.943 mg/kg dry mass (Tab. 2).

Table 2. Petroleum pollutants content in research material samples (soil from Graby-12 waste pit)

Sample	Content [mg/kg dry mass]								
	TPH			**BTEX**			**Σ PAH**		
	Area I	Area II	Area III	Area I	Area II	Area III	Area I	Area II	Area III
A*	55 725	185 508	99 860	34.712	95.445	67.178	1.741	4.711	3.125
B*	4 258	12 785	9 734	5.812	20.943	14.575	0.258	2.702	2.058
C*	998	1 035	1 023	1.412	5.848	3.459	0.106	0.279	0.218

A* – crude soil sample from Graby-12 waste pit – before bioremediation
B* – soil sample from Graby-12 waste pit – during *in-situ* bioremediation (2nd year of purification)
C* – soil sample from Graby-12 waste pit – after bioremediation (3rd year of purification)

Despite diversity in petroleum hydrocarbons contamination, the course of initial bioremediation was similar in the consecutive areas of Graby-12 waste pit.

During initial bioremediation, stimulated with biogenic substances dosage in Area II of Graby-12 waste pit, biodegradation of $nC_6 – nC_{19}$ chain aliphatic hydrocarbons was the fastest (42.3–59.7%), whereas in the case of heavier hydrocarbons ($nC_{20} – nC_{36}$) biodegradation was in a range from 6.1 to 29.2%. However, inoculation with G-12-1 biopreparation (aided by surface spraying and deep injection) resulted in biodegradation of $nC_{10} – nC_{21}$ aliphatic hydrocarbons as the fastest process (57.2–69.8%), whereas for heavier hydrocarbons ($nC_{22} – nC_{36}$) decrease in TPH content was from 38.4 to 58.9% (Fig. 2).

The third year of Graby-12 waste pit purification included initial bioremediation and inoculation with G-12-2 biopreparation done in two series, which resulted in satisfactory decrease in TPH content in consecutive areas of the waste pit: from 998 to 1 035 mg/kg dry mass (Tab. 2).

Inoculation with G-12-2 biopreparation led in the third year of purification in Area II, which was the most contaminated part of Graby-12 waste pit, resulted in TPH contents reduction as follows: 77.1–85.8% in the case of $nC_{10} – nC_{21}$ hydrocarbons and 54.7–61.9% for $n\text{-}C_{22} – nC_{36}$ hydrocarbons (Fig. 3).

Consecutive stages of purification done in Graby-12 areas resulted in significant decrease in monoaromatic hydrocarbons (BTEX) content. After the process, their content was on the level from 1.412 to 5.848 mg/kg dry mass (Tab. 2). The highest biodegradation degree was observed in the case of benzene (75.8–89.1%) and toluene (69.7–75.7%) (Fig. 4).

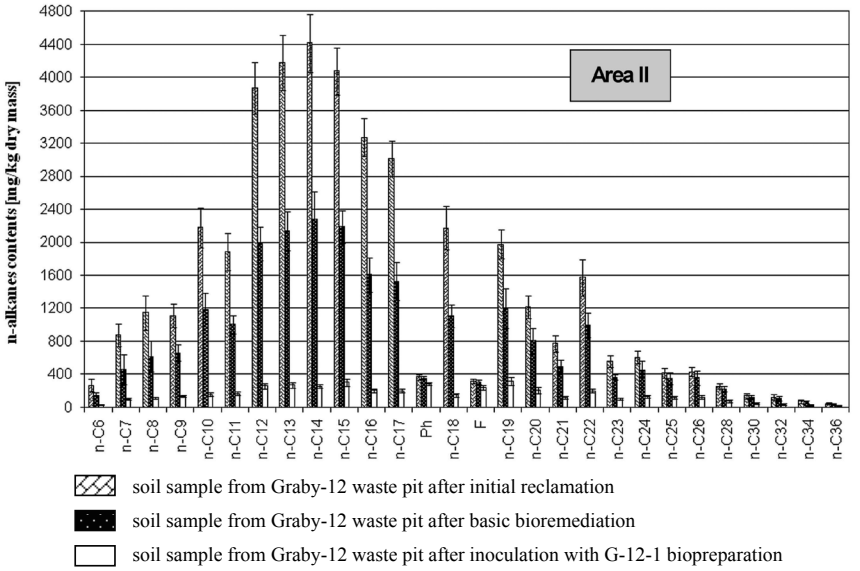

Fig. 2. Comparison in identified n-alkane content in averaged samples from Graby-12 waste pit after consecutive purifying stages – industrial conditions, *in-situ* method, 2nd year of purifying (repetition number n = 9–10, $p < 0.05$)

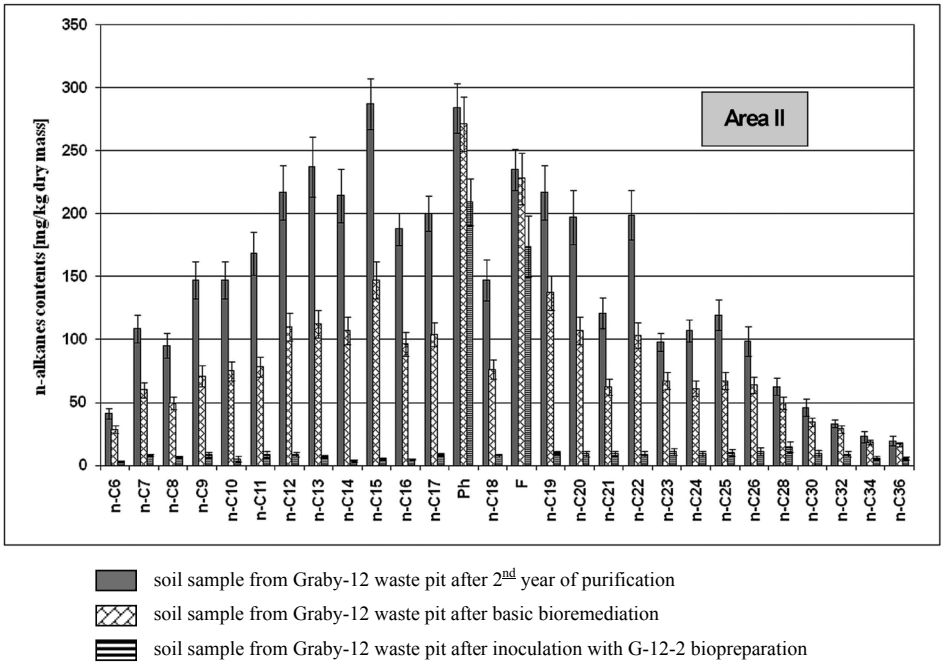

Fig. 3. Alternation in number of hydrocarbon-degrading bacteria in averaged samples from Graby-12 waste pit after consecutive purification stages – industrial conditions, *in-situ* method, 3rd year of purifying

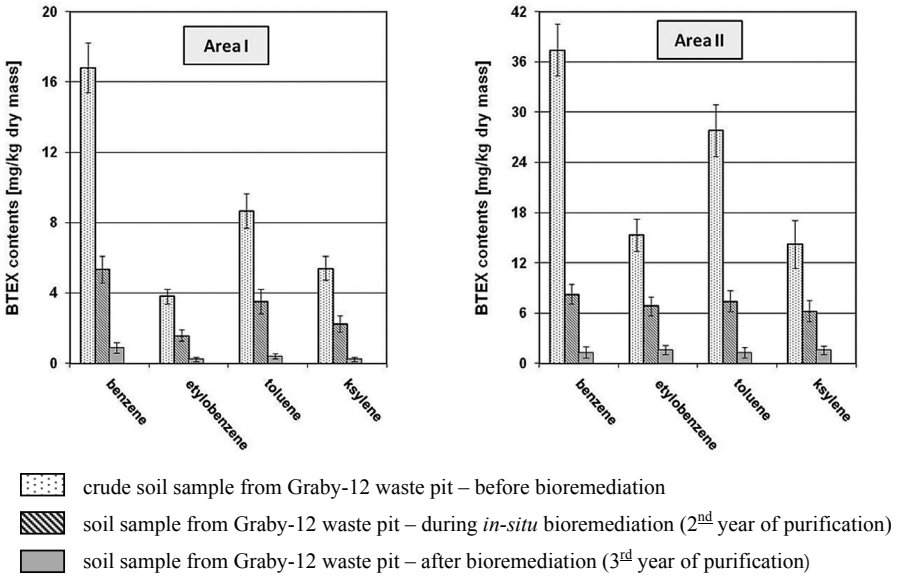

Fig. 4. Comparison in BTEX content after consecutive purifying stages (soil from waste pits) – industrial conditions, *in-situ* method (repetition number n = 8–10, $p < 0.05$)

During microbiological degradation of PAH in the second year of purification, done with the use of G-12-1 biopreparation, there was a decrease in PAH content in a range from 28.5 to 37.5%. Inocculation in the third year of cleaning, done with G-12-2 biopreparation enriched with non-patogenous fungi, caused reduction in PAH content by 58.9–86.7%.

The observed decrease in aromatic hydrocarbons contents (BTEX and PAH) indicates that the inoculation biopreparations included bacteria and fungi species, which were capable of biodegradation of aliphatic and aromatic hydrocarbons. The observation was followed by research.

TOXICOLOGY RESEARCH RESULTS

Initial screening research with application of Microtox® SPT enabled selection of samples of essential toxicity effect (luminescence decrease level > 50%). Next, the toxic samples were the object of basic tests with dilution, which enabled EC_{50} determination (Fig. 5, Tab. 3).

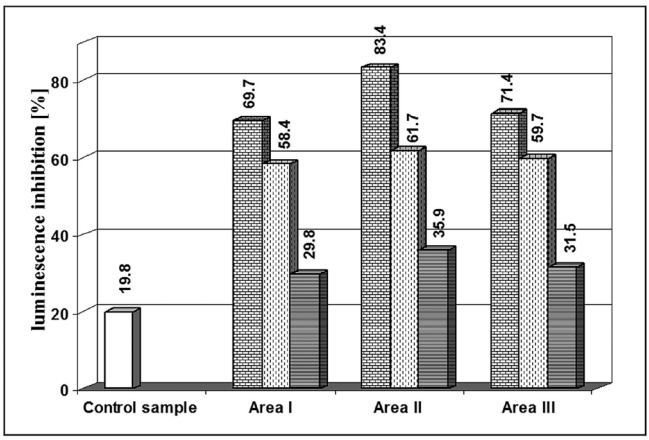

Fig. 5. Influence of water extracts from Graby-12 soil samples on luminescence inhibition during and after bioremediation in industrial conditions (*in-situ* method)

Table 3. Properties of effective concentration causing effect of 50% luminescence inhibition, obtained for water extracts from Graby-12 soil samples during and after bioremediation in industrial conditions (*in-situ* method)

Parameter	Homogenous soil sample from Area I (n = 3)			Homogenous soil sample from Area II (n = 3)			Homogenous soil sample from Area III (n = 3)		
	A*	B*	C*	A*	B*	C*	A*	B*	C*
EC_{50} [mg waste/dm^3]	6 480	24 300	N.T.	3 510	18 090	N.T.	4 950	21 150	N.T.
TU	13.2	3.67	–	25.6	4.97	–	18.1	4.25	–

A* – crude soil sample from Graby-12 waste pit – before bioremediation
B* – soil sample from Graby-12 waste pit – during *in-situ* bioremediation (2nd year of purification)
C* – soil sample from Graby-12 waste pit – after bioremediation (3rd year of purification)
N.T. – no toxicity effect

The crude soil samples, taken from consecutive areas of Graby-12 waste pit, appeared to be toxic for areas II and III of higher contamination with petroleum hydrocarbons. Contaminants included in the samples resulted in over 50% inhibition in luminescence in a range from 71.4 to 82.4%. According to dilution tests results, effective concentration (EC_{50}) is in a range from 3 510 to 6 480 mg waste/dm^3 (TU = 13.2–25.6) (Tab. 3). The properties cause 50% luminescence inhibition in the samples obtained for water extracts of wastes samples from Graby-12 pit (particularly from areas II and III).

A toxicity analysis of water extracts from the soil and ground, taken in a bioremediation process, done with an *in-situ* method (after the second year of purification), revealed increase in toxicity degree of TU soil to a level from 3.62 to 4.97. The results indicate that the toxicity could come from metabolites created during bioremediation. The soil sam-

ples, after a period of three-year purification, did not inhibit test bacteria luminescence to a degree, which could indicate the presence of toxic compounds. It means that determination of EC_{50} properties was not possible.

In order to measure toxicity of the soil and ground contaminated with petroleum substances taken from Graby-12 waste pit, a crustacean *Heterocypris incongruens* was applied. The representative of a trophic level of consumers was used as a biomarker. Soil and ground toxicity estimation was done in various stages of purification, and the obtained results have been presented in Table 4.

Table 4. Results of Ostracodtoxkit F™ test done on Graby-12 soil samples during and after bioremediation in industrial conditions (*in-situ* method)

Parameter	Control sample	Homogenous soil sample from Area I (6 repetitions)			Homogenous soil sample from Area II (6 repetitions)			Homogenous soil sample from Area III (6 repetitions)		
		A*	B*	C*	A*	B*	C*	A*	B*	C*
Death rate										
Average death [%]	10.0	48.3	33.3	23.3	66.6	38.1	28.8	58.3	36.6	25.0
Variation rate of average death [%]	–	2.98	3.95	5.98	2.16	3.75	4.15	2.32	2.82	4.96
Growth inhibition										
Average initial length value [μm]	221	199	202	219	168	198	204	157	205	210
Average organisms length growth ΔL [μm]	424	234	298	401	**X**	282	374	**X**	295	399
Average growth inhibition [%]	–	44.9	29.7	5.4	**X**	33.4	11.7	**X**	30.4	5.8

A* – crude soil sample from Graby-12 waste pit – before bioremediation
B* – soil sample from Graby-12 waste pit – during *in-situ* bioremediation (2nd year of purification)
C* – soil sample from Graby-12 waste pit – after bioremediation (3rd year of purification)

In the case of the crude soil and ground, taken from areas II and III in Graby-12 waste pit, a death rate of bioindicators was in a range from 53.3 to 66.6%, which means high toxicity of the soil contaminated with petroleum hydrocarbons (99 860–153 428 mg/kg dry mass). An insignificant death rate has been notified in the case of the soil sample taken from Area I (48.3%), whereas inhibition in crustaceans growth was at level of 44.9%.

Purification (done in the second year), which included inoculation with autochthonous bacteria-based biopreparation (G-12-1), resulted in death rate decrease to a range from 33.3 to 38.1%, whereas growth inhibition was from 29.7 to 33.4%. The results presented above, have revealed crucial toxicity effect, despite decrease in contamination to a level of 9 734 mg/kg dry mass, which can suggest presence of indirect metabolites, created during petroleum hydrocarbons biodegradation.

Research done on unified samples from Graby-12 pit (areas I, II and III) proved decrease in death rate of life forms to a level from 23.3 to 28.8% after purification. The second parameter (growth inhibition) has been decreased to a significantly low level from 5.4 to 11.7%, which indicates the lack of soil toxicity.

The phytotoxicity research with the use of Phytotoxkit Test revealed significant differences between the individual samples and test plants. The obtained results have been presented in Table 5.

Table 5. Results of Phytotoxkit™ Test done on Graby-12 soil samples during and after bioremediation in industrial conditions (*in-situ* method)

Parameter	Control sample	Homogenous soil sample from Area I (3 repetitions)			Homogenous soil sample from Area II (3 repetitions)			Homogenous soil sample from Area III (3 repetitions)		
		A*	B*	C*	A*	B*	C*	A*	B	C*
Lepidium sativum										
Sprouting [%]	97.6	13.3	66.6	96.9	6.6	56.6	93.3	9.0	61.7	96.9
Average root length [mm]	41.23	0.98	25.31	35.95	0.07	20.45	31.95	1.19	22.45	34.45
Growth inhibition [%]	–	97.6	38.61	12.84	99.8	50.37	29.74	97.1	45.54	16.44
Variation rate [%]	32.4	53.4	49.8	33.8	62.4	47.2	39.8	54.6	48.3	37.8
Sinapis alba										
Sprouting [%]	100.0	86.6	93.6	96.6	80.0	90.0	93.9	90.0	93.3	93.3
Average root length [mm]	38.42	16.25	27.27	44.28	13.45	23.24	40.25	15.23	26.95	45.28
Growth inhibition [%]	–	57.70	29.12	–15.2	65.12	39.52	–4.7	60.35	29.82	–17.8
Variation rate [%]	25.85	39.5	35.5	29.5	37.2	41.2	28.3	38.7	31.2	35.2
Sorghum saccharatum										
Sprouting [%]	100	93.3	96.9	96.9	86.6	93.3	93.3	93.3	96.9	96.9
Average root length [mm]	52.73	18.52	35.25	46.81	12.55	30.91	42.29	20.12	3638	44.95
Growth inhibition [%]	–	65.33	31.96	11.28	76.23	41.33	18.69	61.83	31.0	14.71
Variation rate [%]	49.5	52.3	42.3	41.2	51.8	43.2	39.2	51.7	39.7	36.3

A* – crude soil sample from Graby-12 waste pit – before bioremediation
B* – soil sample from Graby-12 waste pit – during *in-situ* bioremediation (2nd year of purification)
C* – soil sample from Graby-12 waste pit – after bioremediation (3rd year of purification)

The analysed soil samples from Graby-12 waste pit before the beginning of purification (crude samples) were toxic towards *Lepidium sativum*. They caused inhibition in root growth in a range from 97.1 to 99.8%, which corresponds with a huge content of petroleum hydrocarbons in the samples.

Despite a significant decrease in petroleum pollutants in all waste pit areas, growth inhibition of *Lepidium sativum* was at level from 38.61 to 50.37%. This result can indicate that cress is more sensitive to metabolites in soil and petroleum hydrocarbons, which were not biodegraded.

The amount of sprouted seeds for a crude sample was in a range from 6.6 to 13.3%, whereas during bioremediation it increased to a level from 56.6 to 66.6%. Research done after bioremediation works (which lasted for three years) has revealed satisfactory results: percentage of sprouted cress seeds was at a high level from 93.3 to 96.0%, whereas inhibition in root growth was in a range from 12.84 to 29.74%. *Sorghum saccharatum* and *Sinapis alba* have appeared to be much less sensitive to soil pollution. In the case of *Sinapis alba*, which had the highest immunity to toxic influence of contaminants presented in soil samples, sprouted seeds percentage was high for all analysed samples: from 86.6 to 90.0%. Root growth inhibition in crude samples taken from consecutive areas was

at a level from 57.70 to 65.12%, whereas in samples obtained during bioremediation it ranged from 29.1 to 39.52%. In samples taken after bioremediation, the growth of root length was from 4.7 to 17.8% higher than in a control sample. High immunity to petroleum pollutants has been observed in the case of *Sorghum saccharatum* as well. Sprouted seeds percentage for pure soil was in a range from 86.6 to 93.3%, whereas the second tested parameter, root growth inhibition, was at a level from 61.83 to 76.23%. A quite high degree of root growth inhibition during bioremediation, done after initial stages of biological purification, can indicate presence of toxic metabolites, which resulted from petroleum contaminants biodegradation. After bioremediation, root growth inhibition for *Sorghum saccharatum* was at a low level from 11.28 to 18.69%, which means lack of toxicity of soil and ground taken from the waste pit under purification.

Ecotoxicological tests present alternation in petroleum-contaminated soil toxicity during purification done with an in-situ method in Graby-12 waste pit area.

In the area of remediated Graby-12 waste pit, which can be used as a forested or agricultural terrain in the future, biological monitoring is necessary. This process ought to include research on presence of mutagens and carcinogens. Ames Plate Test was applied in order to estimate mutagenous properties of soil and ground contaminated with petroleum hydrocarbons in crude samples taken from Graby-12 waste pit (areas II and III) and samples after bioremediation.

With the use of a calculation programme offered by the producer of Ames Test, research results have been presented in a graphic form: exemplary results for Area II as numbers of revertants induced on a histidine-less basis as a result of a contact with petroleum pollutants in a sample extract. Mutagenic samples have been marked with an asterisk (Fig. 6).

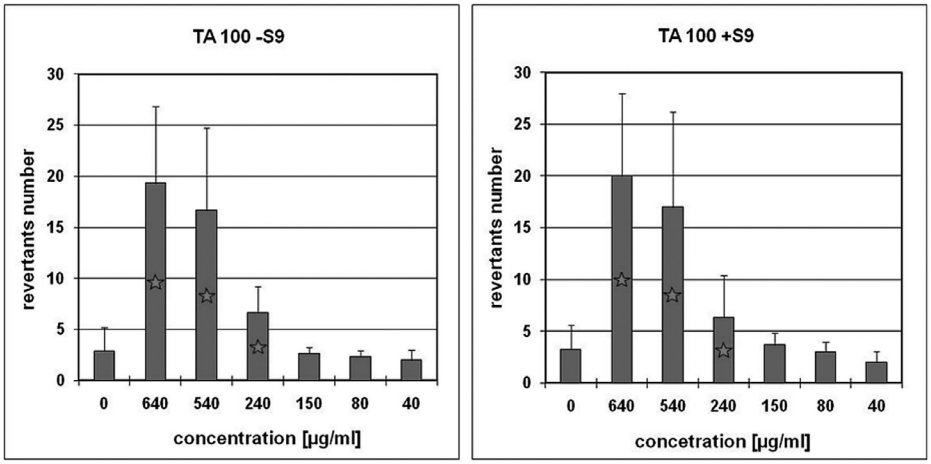

Fig. 6. Influence of petroleum pollutants to induced revertants number – crude soil from Graby-12 waste pit (area II, $n = 6$, $p < 0.05$)

Pure soil and ground samples from Graby-12 waste pit, with higher concentration of petroleum hydrocarbons, appeared to be mutagenous due to the fact that a number of return mutations was increased ($p < 0.05$) for TA-100 strain. TA-98 strain did not give a

positive answer, which means the lack of revertants growth. Furthermore, the use of an activator (S9 microsomal fraction) had an insignificant influence on increase in return mutations number.

A mutagenity ratio for the samples from Area II (considered as mutagenous) was in a range from 3.34 to 6.19, whereas in the case of Area II samples (with a higher degree of contamination) it was at a level from 2.29 to 6.63.

Potential mutagens and carcinogens have not been observed in the soil and ground samples from Graby-12 waste pit (Fig. 7.).

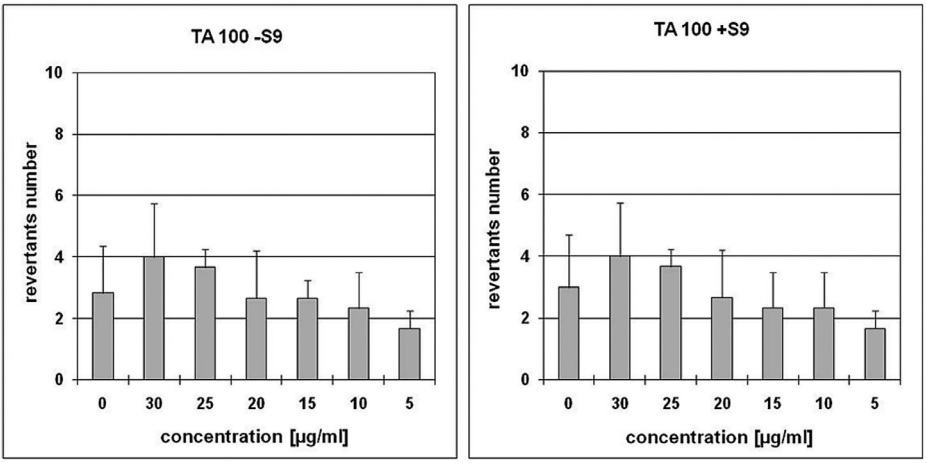

Fig. 7. Influence of petroleum pollutants to induced revertants number soil from Graby-12 waste pit after purification process – industrial condition (*in-situ* method) (area II, $n = 6$, $p < 0.05$)

The number of revertants induced without histidine was insignificantly higher (less than twice) than the number of spontaneous mutants in a control basis; therefore, the samples cannot be classed as mutagenous. Due to Ames Test results, it can be said that bioremediation resulted in decrease in substances of potentially mutagenous properties.

CONCLUSIONS

1. The obtained research results have proved high effectiveness of consecutive stages of Graby-12 soil and ground purification done with the use of an *in-situ* method.
2. Estimation of purification effectiveness has been verified in toxicological tests. The use of bioindicators of diversified taxonomic groups (bacteria, crustaceans and species plant) representing all trophic levels (producers, consumers and decomposers) enabled complex estimation of condition of Graby-12 environment.
3. The results of toxicological tests, carried in Graby-12 waste pit after purification, have shown that the soil is not toxic.
4. Owing to research results done with the use of Ames Test, it can be claimed that soil bioremediation caused decrease in substances which could be potentially mutagenous.

5. The obtained results enable observation of indirect metabolites creation and alternation in toxic properties during the process of soil purification. Moreover, the results will give information whether the soil can become reforested.

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ZASTOSOWANIE BIOTESTÓW DO OCENY PRZEBIEGU PROCESU BIOREMEDIACYJNEGO ZASTARZAŁEGO ODPADU WIERTNICZEGO

Zanieczyszczenia ropopochodne stanowią złożoną mieszaninę związków o zróżnicowanych własnościach biologicznych, które mogą być przyczyną niekorzystnych dla człowieka i organizmów ż ywych zmian zachodzących w skażonych ekosystemach. W wyniku prowadzonych prac bioremediacyjnych (obejmujących bioremediację stymulowaną substancjami biogennymi oraz inokulację biopreparatami sporządzonymi na bazie mikroorganizmów autochtonicznych i grzybów) powstają metabolity o zróżnicowanej i słabo poznanej aktywności biologicznej. Niektóre z nich mogą być bardziej toksyczne niż substrat wyjściowy i posiadać własności mutagenne lub rakotwórcze. Ocenę skuteczności stosowanych zabiegów remediacyjnych na dołach urobkowych uzupełniono o monitoring toksykologiczny. Prowadzony przy użyciu organizmów żywych jako biowskaźników reprezentujących wszystkie poziomy troficzne (producentów, konsumentów i reducentów) danego ekosystemu, co pozwala na kompleksową ocenę stanu badanego środowiska glebowego. Celem badań było wykorzystanie biotestów (Microtox®, Ostracodtoxkit F™, Phytotoxkit™ i test mutagenności Ames'a) do oceny przebiegu procesu bioremediacji zastarzałych odpadów wiertniczych oraz określenie wpływu pośrednich metabolitów

powstających w procesach bioremediacyjnych na biocenozę glebową. Uzyskane wyniki badań pozwoliły na prześledzenie zmian własności toksycznych w trakcie prowadzonych procesów oczyszczania gleby z dołów urobkowych oraz na stwierdzenie przywrócenia rekultywowanych terenów do użytkowania leśnego.