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OPTIMIZATION RESEARCH ON BIODEGRADATION OF HYDROCARBON POLLUTIONS IN WEATHERING SOIL SAMPLES FROM MANUFACTURED GAS PLANT (MGP)

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Abstract: The aim of this work is purification of contaminated soil from manufactured gas plants MGP, which are not used. Prepared chromatographic methodologies, which allow qualitative identification and quantitative determination of individual aliphatic and aromatic (TAH, PAH) hydrocarbons in the soil from the selected MGP, are presented. The results of the research on remediation of the soil polluted with petroleum hydrocarbons (TAH, PAHs) in semi-field conditions are discussed. Application of basic bioremediation and bioaugmentation with indigenous microorganisms, enriched with PAH biodegradable fungi, resulted in reduction in amount of 5- and 6-polycyclic aromatic hydrocarbons. The research enables control of remediation progress, selection of optimal doses of biogenic compounds and determines the time of the process. The entire cycle of soil remediation was monitored with the use of gas chromatography (GC). Estimation of biodegradation degree of individual aliphatic hydrocarbons (alkanes) was based on changes in their concentration in the soil and determined by biodegradation indicators: C_{17}/pristane and C_{18}/phytane ratios. Attention was paid particularly to biodegradation of polycyclic aromatic hydrocarbons (PAH), which were observed in substantial concentrations in the polluted soil. The elaborated chromatographic methodology of PAH determination in the soil enabled estimation of a biodegradation rate referring to individual compounds. Moreover, attempts to create a TAH biodegradation model with the use of C_{30}–17α(H),21β(H)-hopane were undertaken.

INTRODUCTION

Huge pollution of Manufacturing Gas Plant (MGP) areas was caused by gas production from coal, which usually lasted for over thirty years. The failure during production phase and the improbable usage and storage of wastes resulted in soil pollution with total aliphatic hydrocarbons (TAH) and polycyclic aromatic hydrocarbons (PAHs). The pollutants that do not biodegrade are a source of a serious threat, especially when the usage of the area is changed [32].

Hydrocarbon pollutants (TAH, PAH) are prone to ageing due to numerous processes such as biodegradation, evaporation and dissolution [8, 9, 35].

When selecting the treatment procedure for hydrocarbon pollutants, analysis of laboratory results and groundwater environment should be taken into account. Such interdisciplinary analysis should indicate the optimum treatment of hydrocarbon derivative contaminants [28, 38, 50, 77].

Recently, increasing number of projects has been devoted to degradation of hydrocarbons using bacteria extracted from strongly contaminated natural environment. Such biological methods are quite popular due to their low costs and high effectiveness [14, 42, 43, 49, 62].

The biodegradation of hydrocarbon pollutants in contaminated environment depends on the intensity of biotic and abiotic agents including the following: chemical properties of soil, concentration and chemical structures of hydrocarbon contaminants, concentration of biogenic substances (nitrogen, phosphorus), temperature, oxygen content, humidity, pH of water, concentration of organic compounds, presence of toxic chemicals, quantitative and qualitative composition of microorganisms in soil [5, 17, 18, 20, 29, 48, 66, 67, 73].

The chemical structure of hydrocarbon pollutants as well as physicochemical conditions of the contaminated soil influence their amount and bioavailability. PAH bioavailability strictly depends on soil ageing, that is how long contaminants have been in contact with soil, diffusing through organic matter or soil micropores [11, 19, 22, 42, 55].

Basic biodegradation, which means activation of natural bacterial microflora through adding nutrition substances to the soil environment, brought satisfactory results not only in TAH biodegradation [20, 50, 52, 66] but also reduction in PAHs amount by 45.6–64.5% [65, 69–71].

In order to accelerate the bioremediation process of oil contaminants (TAH, PAHs), biotechnological processes are led with the use of cultures of microorganisms (which were isolated from the polluted area) [1, 26, 42, 53, 62] or bacterial cultures created in a laboratory (commercial biopreparations) [36].

Microorganisms from the group of *Pseudomanas sp., Sphingomonas sp., Nocardiodes sp., Mycobacterium sp., Rhododococcus sp.* belong to most active bacterial cultures able to PAH biodegradation [2, 12, 21, 24, 25, 30, 31, 37, 40, 46, 60, 72, 74, 78]. Also, satisfactory results of PAH biodegradation in soil were obtained due to the use of biopreparations of indigenous microorganisms [23, 42, 62]. Research was led in order to enrich bacterial cultures (able to TAH and PAH biodegradation) with several types of fungi. Their influence on microorganisms was reduced as much as possible. Inoculation by indigenous microorganisms enriched with *Pleurotus ostreatus* and *Dichomitus sgualens* caused more advanced biodegradation of 4- and 5-rings PAH [62]. Similar tests on the soil from the old (not working) manufacturing gas plant (MGP) were made with the use of biopreparations, which were combination of bacterial cultures (*Pseudomonas putida*) and fungi: *Irpex lacteus* or *Pleurotus ostreatus*. They considerably reduced PAH amount [43]. Satisfactory effects of PAH biodegradation in MGP areas were obtained due to detergents combined with fungus-based (*Phananerochete chrysosporium*) biopreparations [7, 75].

This work concerns laboratory ex-situ method research of biodegradation of hydrocarbon contaminants (TAH, PAH) taken from areas of an old MGP. The tests were done with the use of basic bioremediation and inoculation of biopreparations based on indigenous microorganisms and fungi isolated from the soil. The obtained results enable elaboration of parameters of biodegradation process led in industrial condition with an in-site method [50, 53].

The entire cycle of soil purification ageing was monitored due to chromatographic methods of TAH and PAH determination, which enable not only determination of total amount of TAH and PAH but also quantitative determination of various hydrocarbons found in pollutants.

What is more, chromatographic analytical method is useful to determine changes in quantity of hydrocarbon pollutants (TAH and PAH) during consecutive phases of biodegradation. They also enable identification and quantitative determination of isoprene group hydrocarbons: pristane (Pr) and phytane (Ph). Changes in n-C_{17}/Pr and n-C_{18}/Ph ratio were taken as an indicator of a degree of n-alkanes biodegradation [6, 41, 50, 54].

FIRST-ORDER BIODEGRADATION MODEL

The biomarker C_{30}–17α(H),21β(H)-hopane, which is an isolated fraction from Californian deposit petroleum, was used in laboratory tests in order to find a simple mathematical model of TAH and PAH biodegradation to determine the degree of their biodegradation.

The use of C_{30}–17α(H),21β(H)-hopane biomarker in laboratory research of TAH and PAH biodegradation process in old soils taken from a manufactured gas plant enabled elimination of analytical errors in quantitative determination of TAH and PAH. Results of TAH and PAH chromatographic determinations, normalized by hopane, done during biodegradation, were used to create a first-order mathematical model describing TAH and PAH biodegradation process [34, 56, 57, 70, 71]. The model was described as follows:

$$C/C_H = (C/C_H)_0 \exp(-kt) \quad (1)$$

where: C – concentration of analyte,
C_H – concentration of hopane,
k – the first-order biodegradation rate constant for the analyte,
C/C_H – the time-varying hopane normalized concentration of the analyte,
$(C/C_H)_0$ – the theoretical value of that onset of biodegradation,
t – time of process lasting.

The analysis of non-linear regression with the use of the above mentioned equation (1) enabled determination of a constant of first-order biodegradation (k) and a coefficient of determination (r^2) which determines matching the measuring points to the theoretical curves of TAH and PAH biodegradation process.

The non-linear variation (ANOVA) with the Tukay Method on the confidence level $p < 0.05$ enables elimination of analytical (chemical and biological) data, which statistically are insignificant. The program "Statistica", version 7.1 was used.

ANALYTICAL METHODOLOGY

Analysis of oil contaminants is a difficult analytical task regarding a huge number and variety of products, which can be the source of pollution, and the quality and heterogeneity of an environmental matrix (soil).

A qualitative and quantitative method of determining the content of hydrocarbon derivatives in soil was developed. This method allows for control of the process of removal of hydrocarbon pollutants and preparation and application of an effective biopreparation consisting of indigenous microorganisms (TAH).

Collected samples were averaged and homogenized. Analytes were enriched after isolation. Isolation of aliphatic hydrocarbons analytes was done with the use of the method of solvent extraction modified ultrasonically. Dichloromethane was selected as the

optimum solvent [23, 29]. During simple solvent extraction of aliphatic hydrocarbons (TAH) the recovery ratio reached 85.5%. The use of ultrasonication in solvent extraction process resulted in increase of extraction rate of the petroleum substances to 95.9% [51]. The optimum time of reacting with ultrasonication (30 kHz) at 40°C varied from 20 to 35 minutes [9, 10, 39, 59]. In order to achieve desired extraction of analytes from the soil matrix, it is advisable to repeat extraction (2 or 3 times) with fresh doses of solvent. In the case 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.

Degree of analyte recovery was determined due to a substitute standard, which was o-terfenyl [44, 59]. The purification of analytes from polar substances was done with the use of sorption columns filled with sorbent called Florisil [15, 58].

Identification and quantitative analysis of n-alkanes (n-C_6 – n-C_{38}), hydrocarbons from isoprenoids group (Pr, Ph), and total aliphatic hydrocarbons (TAH) content was done on Clarus 500 GC/FID chromatograph, produced by Perkin Elmer, equipped with Quadrex 007-1 (30 m x 0.53 mm) capillary column by Panalytica, with the helium (as a carrier gas) flow of 20 cm^3/min. PPS injector and detector temperature was 290°C and 300°C.

The temperature program for aliphatic hydrocarbons was as follows: isothermal run: 28°C in 2 minutes, temperature increase: 28–150°C (rate of 10°C/minute), temperature increase: 105–258°C (rate of 5°C/minute), isothermal run: 10 minutes in temperature 285°C.

The temperature program for C_{30}–17α(H),21β(H)-hopane was as follows: isothermal run: 28°C in 2 minutes, temperature increase: 28–105°C (10°C/minute), temperature increase: 105–320°C (5°C/minute), isothermal run: 20 minutes in temperature 320°C.

In order to determine total quantity of aliphatic hydrocarbons (TAH), the set of calibration standards by Tusnovic Instruments was used. The certified standards by Supelco and Restek were used for quantitative determination of n-alkenes in hydrocarbon pollutants. The standards were: No D2887 – certified mixture of n-paraffin hydrocarbons n-C_6 – n-C_{44} and standard No A029668 certified mixture of Fuel Degradation Oil Mix n-C_{17}, pristane, n-C_{18}, phytane. The certified standard of C_{30}–17α(H),21β(H)-hopane by Supelco was used as a biomarker.

In TAH determination the degree of analyte recovery and the waste mass decrement rate while drying were taken into account.

In order to determine PAH in the soil from an area of a MGP, an aromatic fraction was isolated and a chromatographic method of determination was created. 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 (1:1) [61, 64, 70, 71], hexane:acetone (2:1) [63], hexane:acetone (9:1) [2], methylene chloride [45], hexane:dichlromethane (1:1) [16, 23], 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:

- analyte (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 cm^3;
- 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 cm^3 of acetone and toluene (3:1);
- analyte purification with the use of SPE column with Florisil (by Supelco) phase.

The extraction efficiency of the sample preparation step before PAHs analysis was between 89.7–95.7%, as determinated with the reference soil ERM-CCO13.

Chromatographic analysis, which enabled determination of 16 PAH, total amount of PAHs and C_{30}–17α(H),21β(H)-hopane, was done on Clarus 500 GC chromatograph produced by Perkin Elmer, equipped with RTX-440 capillary column, with temperature program: isothermal run: 40°C in 2 minutes; temperature increase: 40–240°C in 2 minutes; temperature increase: 40–240°C (rate 30°C/minute); temperature increase: 240–320°C (rate 8°C/minute); isothermal run: 320°C in 10 minutes; injector and FID detector temperature: 320°C.

Chromatographic analysis with the use of an internal-standard calibration method and qualitative and quantitative standards by Restek and Supelco (certified standard of C_{30}–17α(H),21β(H)-hopane) enabled identification and quantification of individual compounds of PAH, PAHs and C_{30}–17α(H),21β(H)-hopane.

CHARACTERISTICS OF INVESTIGATED SAMPLES

The soil from an area of a manufactured gas plant, which was out of exploitation, was used for research. Samples were taken near pits with aftergas tar, from the depth of 0.3–1.5 m under the surface, which were averaged and analyzed (W-1 sample).

Due to the methodology of chromatographic determination of total aliphatic hydrocarbons with the chain length from n-C_{10} to n-C_{38} and hydrocarbons from isoprenoid group Pr and Ph were found in the soil. TAH total amount was 5619.2 mg/kg dry mass (Tab. 1). The amount of identified hydrocarbons occurrence in pollutants (TAH) is presented in Figure 1.

Table. 1. Chromatographic analyses results of identified aliphatic hydrocarbons and isoprenoids occurrence in pollutants (TAH) in W-1 sample

Component	Content [mg/kg dry mass]	Component	Content [mg/kg dry mass]	Component	Content [mg/kg dry mass]
n-C_{10}	197.2	n-C_{18}	152.4	n-C_{26}	102.6
n-C_{11}	217.2	Phytane (Ph)	35.8	n-C_{28}	58.9
n-C_{12}	412.6	n-C_{19}	297.2	n-C_{30}	65.9
n-C_{13}	275.3	n-C_{20}	210.3	n-C_{32}	48.5
n-C_{14}	289.2	n-C_{21}	105.2	n-C_{34}	35.6
n-C_{15}	115.2	n-C_{22}	186.3	n-C_{36}	29.5
n-C_{16}	192.5	n-C_{23}	102.3	n-C_{38}	18.4
n-C_{17}	287.3	n-C_{24}	95.8	Unidentified	1940.2
Pristane (Pr)	78.9	n-C_{25}	68.9	TAH	5619.2

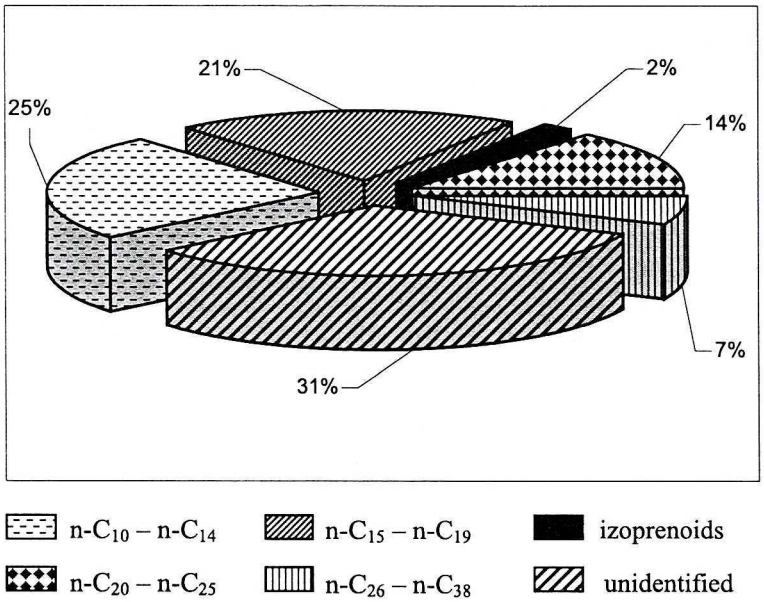


Fig. 1. Percentage of identified hydrocarbons composition in TAH pollutants

Chromatographic analysis enabled identification of 16 PAH, whose total amount was on a high level of 1136.98 mg/kg dry mass (Tab. 2.). The percentage concentration of polycyclic aromatic hydrocarbons (PAH) in relation with the number of aromatic rings is illustrated in the Figure 2. PAHs with: 4-ring (42%), 5-ring (33%), 3-ring (17%) dominated.

Table 2. Chromatographic analyses results of identified polycyclic aromatic hydrocarbons occurrence in pollutants (PAH) in a W-1 sample

Component	Content [mg/kg dry mass]	Component	Content [mg/kg dry mass]
Naphthalene (N)	9.81	4-ring PAHs	487.20
Acenaphthylene (Acl)	12.47	Benzo(b)fluoranthene (BbF)	119.52
Acenaphthene (Ac)	18.57	Benzo(k)fluoranthene (BkF)	43.21
Fluorene (Fluo)	10.87	Benzo(a)pyrene (BaP)	98.41
Phenanthrene (Fen)	62.75	Dibenzo(a,h)anthracene (DaA)	74.61
Anthracene (A)	72.45	5-ring PAHs	373.15
3-ring PAHs	192.31	Indeno(1,2,3,cd)pyrene (IndP)	13.32
Fluoranthene (F)	132.45	Benzo(g,h,i)perylene (BghiP)	52.12
Pyrene (Pir)	121.78	6-ring PAHs	74.51
Benzo(a)anthracene (BaA)	102.39	Total PAHs	1136.98
Chrysene (CH)	98.21		

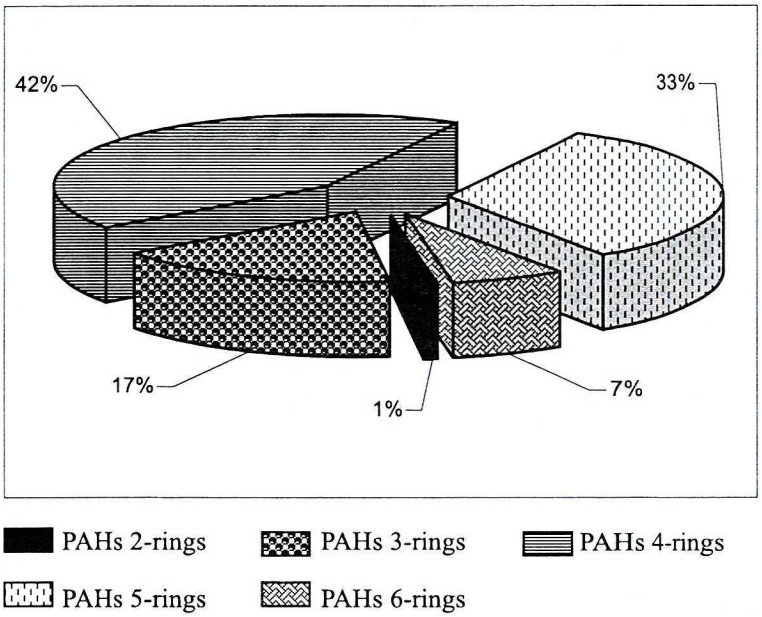


Fig. 2. Percentage of identified hydrocarbons composition in PAH pollutants

BTEX was also determined chromatographically: xylene – 32.0 mg/kg dry mass and phenol – 13.48 mg/kg dry mass, which are not above acceptable amounts required by soil standards for an industrial area.

It was found that in a W-1 soil sample nitrogen to phosphorous proportion was 1:8 while an optimum ratio is 10:1 [11, 14, 66, 67]. This means that there were no favonian conditions for indigenous microflora growth in the tested soil.

Moreover, the soil pH is 5.67 while an optimum level should be 7.5–7.8 [3]. Humidity is between 11.2 and 9.7% of weight and it is much above an acceptable range which is 8.5–22.4% for aerobic biodegradation process [47].

It was demonstrated in chemical analyses that the tested soil from the old MGP is highly degraded and the relation of carbon to nitrogen is C:N = 495:1.

Microbiological analyses done with the use of MPN method [68] (the most probable number of repetitions) proved the presence of microorganisms which oxidise hydrocarbon ($8.5 \cdot 10^5$ cfu/g dry mass) and fungi ($3.2 \cdot 10^4$ cfu/g dry mass) were the basis of biopreparation.

RESULTS OF TAH POLLUTANTS BIODEGRADATION

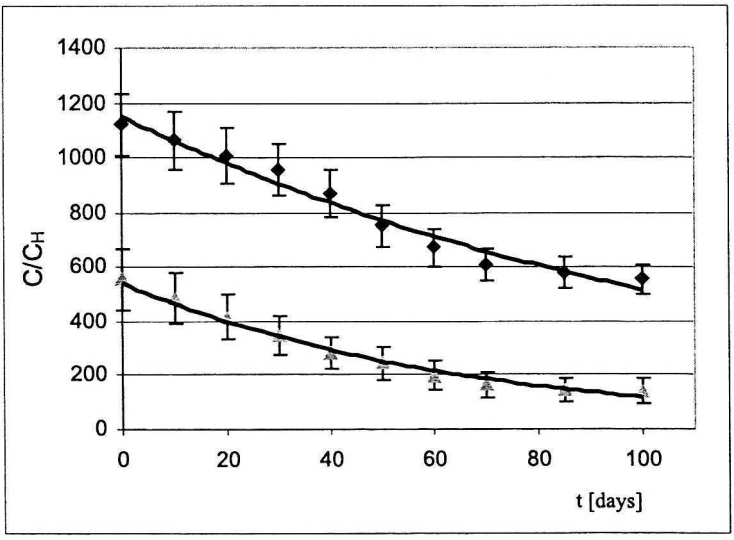
The W-1 soil sample from the MGP was the material in off-site laboratory tests, which enable control of hydrocarbons biodegradation, the choice of optimum dose of soil lime and mineral fertilizers. They are also useful in pre-determination of time of consecutive purification phases and effectiveness of indigenous microorganism-based biopreparations [50, 53].

The soil in the laboratory was treated with basic bioremediation stimulated by aeration and optimum nutrition and water conditions, the adequate basic and thermal condi-

tions. The soil contaminated by petroleum hydrocarbons needs much more oxygen than the pollution-free soil. Deficiency of oxygen is an essential factor which reduces intensiveness of bioremediation [13]. The soil lime and mineral fertilizer "Azofoska" (consisting of: NO_3-N – 7.5%, NH_3-N – 10.5%, P_2O_5 – 11%, K_2O – 19.1%, K_2SO_4 – 4.4%), determined on the basis of laboratory research, were added to the soil sample in order to obtain an optimum reaction of pH 7.5 with 1:10 nitrogen to phosphorous proportion.

Calcium from calcium carbonate conducts grained structure, which causes coagulation of soil colloids. Owing to this process, water-air conditions improve in the soil. Also, it changes reaction (pH) of the soil and increases life span of microorganisms through protection of their cell membranes from destructive influence of aliphatic hydrocarbons [27].

Temperature inside the prism was kept on the level of 17–20°C. This temperature was assumed as possible to obtain in local conditions during summer months in a moderate climate. Temperature influences the activity of microorganisms and effectiveness of hydrocarbon degradation. During bioremediation processes the optimum temperature range for majority of bacteria is 20–38°C [4, 13, 76]. Humidity was kept on the level close to optimum (20–25%) by water spraying. The methodology of chromatographic TAH determination in soil enables total control of oil pollutants biodegradation. As a result of ex-situ basic bioremediation process stimulated by optimum choice of its parameters, it can be stated that after 70 days huge reduction in TAH contaminants took place. There was 50.5% decrease (from 5619.2 to 2782.4 mg/kg dry mass). This level of contamination was kept during the further days of bioremediation process (Fig. 3). Owing to chromatographic analyses, degree of biodegradation of individual hydrocarbons in TAH contaminants was estimated.

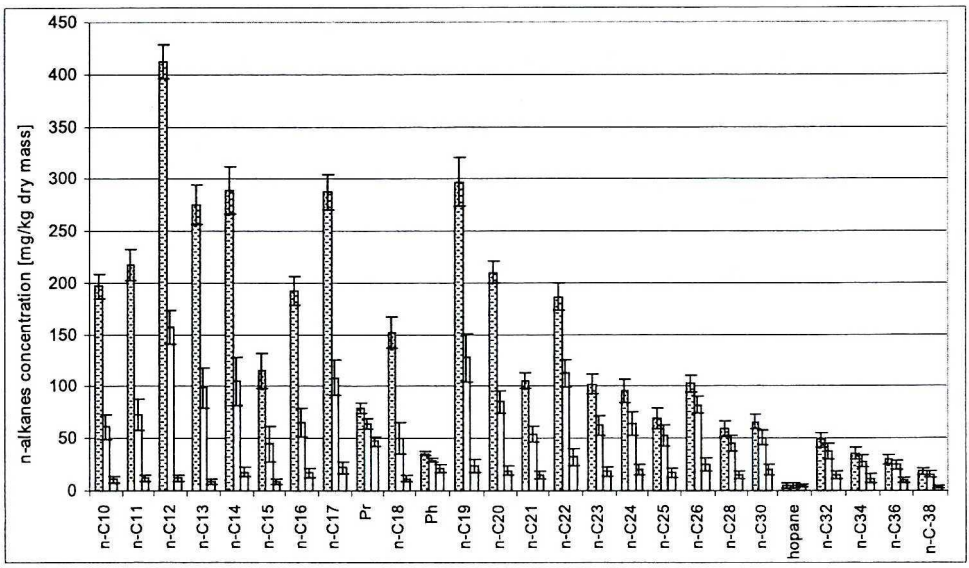


◆ W-2 sample – after basic bioremediation

△ W-3 sample – after bioaugmentation with WA-1 biopreparation

Fig. 3. Decline in TAH (C/C_H) after hopane normalization in consecutive stages of MGP soil biodegradation (number of repetitions n = 8–10, confidence level $p < 0.05$)

Owing to results of chromatographic analyses, it can be stated that during basic bioremediation the highest degree of reduction was observed for n-C_{10} – n-C_{20} aliphatic hydrocarbons and it was on the level of 57.1–69.0%. Reduction in n-C_{12} – n-C_{24} hydrocarbons is slightly lower: 32.5–48.7%. Heavier hydrocarbons n-C_{25} – n-C_{38} are biodegraded in a much lower degree: 15.2–23.1% (Fig. 4).



W-1 sample – initial soil sample

W-2 sample – soil after basic bioremediation

W-3 sample – soil after bioaugmentation with WA-1 biopreparation

Fig. 4. Comparison of n-alkane content changes in consecutive phases of MGP soil biodegradation in ex-situ conditions (number of repetitions n = 9–10, confidence level $p < 0.05$)

Isoprenoid group hydrocarbons are difficult to biodegrade and they can be used as an index of biodegradation degree – relations of n-C_{17}/Pr and n-C_{18}/Ph are reduced in basic bioremediation process respectively from 3.581 to 1.698 and from 4.240 to 1.690.

Basic bioremediation means only activation of natural microflora of the polluted area, which was used to reduce initial TAH concentration. Amount of microorganisms increased from $8.5 \cdot 10^5$ to $7.6 \cdot 10^6$ cfu/g dry mass of soil.

Microbiological research done on the soil samples from MGP together with results of chromatographic analyses enabled the creation of WA-1 biopreparation from isolated, selected and multiplied indigenous microorganisms prone to biodegradation of previously identified hydrocarbon contaminants. In order to create the biopreparations, during microbiological research the following parameters were taken into account: mobility of microorganisms, ability to grow in oxygen and oxygen-free conditions, ability to grow in a wide range of temperatures (4–10°C), ability to use hydrocarbons as the only source of carbon in biotic processes [50].

WA-1 bioprepartation was an active suspension with concentration of $1 \cdot 10^9$ cfu/cm^3. Results of investigation in order to find possible pathogenic microorganisms were negative. The biopreparation included 28 bacterial cultures extracted from a W-1 soil sample and they were among others: *Pseudomonas sp. Acinetobacter sp., Alcaligenes sp., Sphingomonas sp., Noacardiodes sp., Mycobacterium sp., Rhodococcus sp., Flavobacterium sp.*

Inoculation phase with WA-1 biopreparation in laboratory conditions was led during 100 days (Fig. 3.). It enabled TAH amount reduction to the level of 704.1 mg/kg dry mass, which is 74.7% reduction in contaminants. The results of chromatographic analysis indicate that bacteria prefer the n-C_{13} – n-C_{22} aliphatic hydrocarbons and that the rate of biodegradation of these compounds is higher than for others. Their concentration was decreased by 71.3–92.3%. Long-chain hydrocarbons (n-C_{23} – n-C_{30}) are slightly less biodegradable (concentration decrease was within 61.4–70.2%) whereas n-alkanes – which contain over 30 atoms of carbon per molecule – are rather resistant to biodegradation [18, 33], however, their concentration also decreased to 54.2–58.2% (Fig. 4). The above presented results prove high effectiveness of WA-1 biopreparation and its wide range of usage due to reduction in almost all n-alkanes amount in hydrocarbon contaminants in the soil.

As a result of bioaugmentation with WA-1 biopreparation based on indigenous microorganisms n-C_{17}/Pr decreased from 1.695 to 0.476 while n-C_{18}/Ph was reduced from 1.690 to 0.549. It means that biodegradation takes place on a satisfactory level.

C_{30}–17α(H),21β(H)-hopan was used in laboratory tests in order to create a mathematical model of TAH bioremediation process. According to the biomarker, normalization of TAH concentration was led during biodegradation process.

TAH biodegradation process can be defined as follows:

$$C/C_H = (C/C_H)_0 \exp(-kt)$$

Table 3 presents factors of the above mentioned formula (k, $(C/C_H)_0$) and the correlation ratio (r^2) for consecutive purification phases.

Table 3. Comparison of coefficients of equation describing a TAH biodegradation model evaluated for consecutive cleaning stages (a W-1 soil sample)

Purification process	k [d^{-1}]	$(C/C_H)_0$	Determination coefficient (r^2)
Basic bioremediation	0.0080 ± 0.003	1151 ± 67.5	0.9664
Bioaugmentation with WA-1 biopreparations	0.0152 ± 0.008	540 ± 9.8	0.9738

First-order biodegradation constant (k) is 0.0080 [d^{-1}] during basic bioremediation while it is 0.0152 [d^{-1}] during bioaugmentation with the use of indigenous microorganism-based WA-1 biopreparation. First-order biodegradation constant (k) is higher in bioaugmentation with WA-1 biopreparation, which means that the entire process is faster.

The correlation ratio (r^2) is close to one, so the matching of measuring points and curves, defined in the above mentioned equation, is satisfactory (Fig. 3).

Suitability of procedures during the attempt of elaborating a mathematical model of the TAH biodegradation is confirmed by reports in the literature [57, 69, 70].

RESULTS OF PAH POLLUTANTS BIODEGRADATION

The W-1 MGP soil is highly polluted by polycyclic aromatic hydrocarbons, which are above acceptable norms.

The ex-situ laboratory research on basic bioremediation process, stimulated by choice of optimum parameters, proved huge reduction in PAHs: from 1136.78 to 748.91 mg/kg dry mass during 100 days (Fig. 5). Chromatographic analysis makes determination of various PAH reduction degree possible. The lowest degree of the amount reduction was observed in hydrocarbons with the lowest number of rings (3) (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene) and it was on the level from 52.0 to 68.9%. Four-ring PAHs (fluoranthene, pyrene, benzo(a)anthracene, chrysene) were characterized by the much lower degree of reduction, which was 32.4–44.5%. It was observed especially for fluoranthene from 132.45 to 75.23 mg/kg dry mass and for pyrene – from 121.78 to 67.59 mg/kg dry mass (Fig. 6).

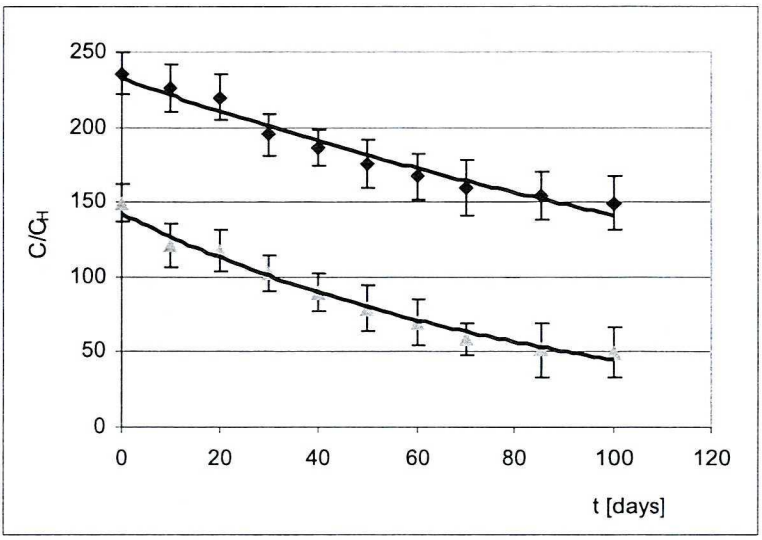
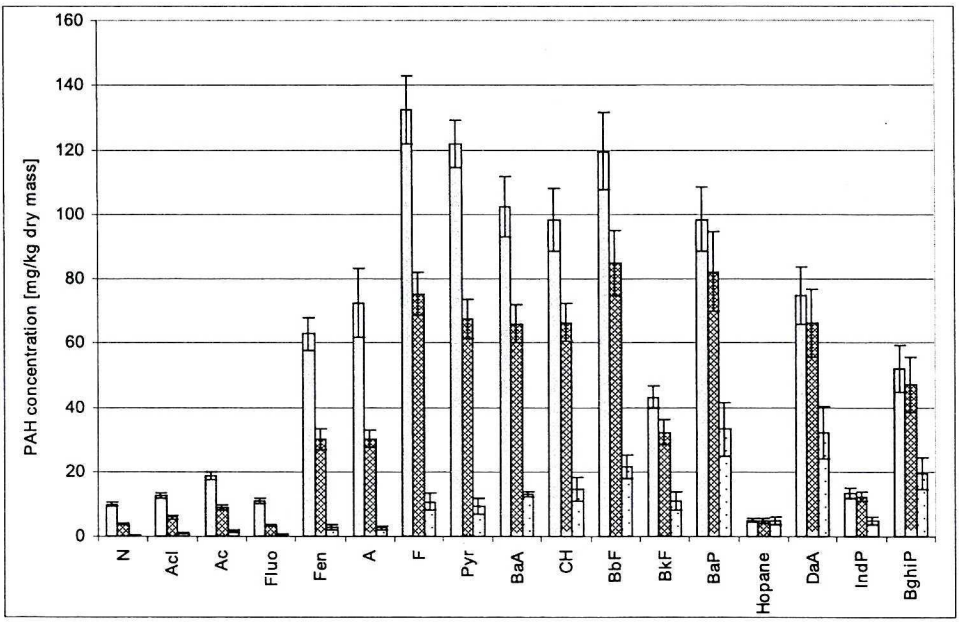


Fig. 5. Decline in PAHs after hopane normalization (C/C_H) in consecutive phases of MGP soil biodegradation (number of repetitions n = 8–10, confidence level $p < 0.05$)

Five-ring hydrocarbons (benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene) are more difficult to biodegrade. The effectiveness of this process is from 28.1 to 11.2%, which is clearly observed for benzo(b)fluoranthene and benzo(a)pyrene. Biodegradation of six-ring PAH (indeno(1,2,3,cd)pyrene and benzo(g,h,i)perylene) is on a much lower level of 7.2–9.8%.

In order to obtain higher reduction in the amount of polycyclic aromatic hydrocarbons, a WA-2 biopreparation based on indigenous microorganisms, enriched with PAH biodegradable fungus isolated from the cleaned area, was created. Fungi of *Pleurotus*

ostreatus and *Irpex lacteus* type, which are able to biodegrade PAH having higher number of rings in a molecule, were observed [43, 62]. A biopreparation, prepared as an active suspension $1 \cdot 10^9$ cfu/cm^3, was investigated in order to find possible pathogenic microorganisms.



W-1 sample – initial soil sample

W-2 sample – soil after basic bioremediation

W-3 sample – soil after bioaugmentation with WA-2 biopreparation

Fig. 6. Comparison of PAH content changes in consecutive phases of MGP soil biodegradation in ex-situ conditions (number of repetitions n = 8–10, confidence level $p < 0.05$)

Inoculation phase with WA-2 biopreparation reduced the amount of PAHs. According to the obtained results, it can be stated that there is gradual decrease in PAHs content during 100 days: from 704.16 to 204.6 mg/kg dry mass (Fig. 5). Chromatographic analyses prove that the highest degree of reduction was observed for 3-ring PAHs (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene): 81.2–91.2% while the lower degree of reduction was obtained for 4-ring PAHs (fluoranthene, pyrene, benzo(a)anthracene, chrysene) and it was 77.5–79.5%. It is observed especially for pyrene – from 44.58 to 9.31 and for fluoranthene – from 43.2 to 10.6 mg/kg dry mass. Also, huge reduction took place in 5-ring PAHs (benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene): 59.7–74.3%. Reduction degree for 6-ring PAHs (indeno(1,2,3,cd)pyrene, benzo(g,h,i)perylene) was on the level of 51.6–59.6% (Fig. 6). It is a huge achievement because these PAHs belong to carcinogenic aromatic compounds which are difficult to biodegrade.

It can be said that the inoculation process with a WA-2 biopreparation based on indigenous microorganisms, enriched with PAH biodegradable fungi isolated from the area

under purification, resulted in higher reduction in PAH amount in comparison to WA-1 biopreparation. This process was observed particularly for PAH which had a higher number (5 and 6) of rings in a molecule. The increase in WA-2 biopreparation effectiveness is especially high in the case of 5- and 6-ring PAH molecules. Effectiveness of indeno-(1,2,3,cd)pyrene biodegradation increased by 30%, of benzo(g,h,i)perylene by 27.5%, of benzo(b)fluoranthene by 27.3%, of benzo(a)pyrene by 26.6% and that of dibenzo(a,h)anthracene by 26.5% (Fig. 7).

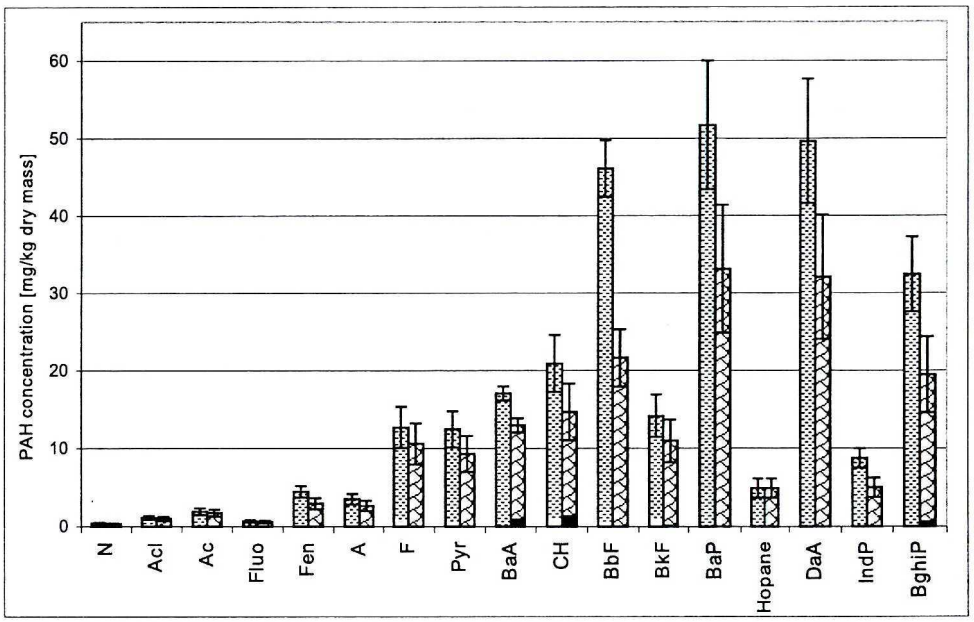


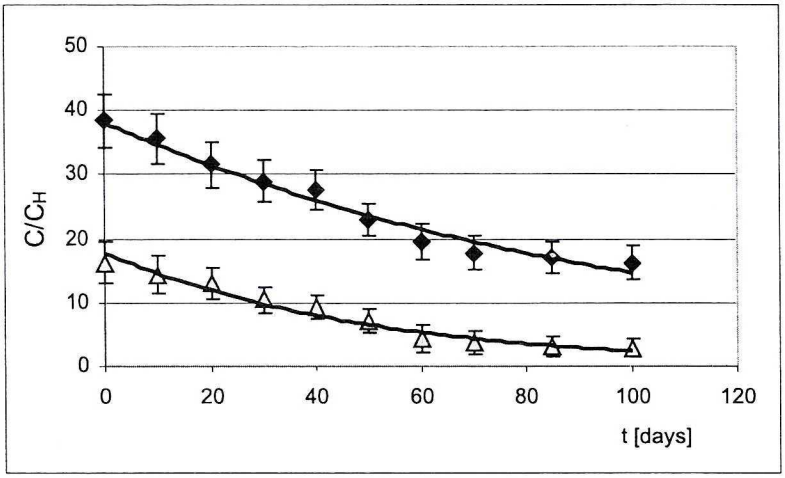
Fig. 7. Comparison of effectiveness of WA-1 and WA-2 biopreparations in ex-situ purification process of MGP soil (number of repetitions n = 8–10, confidence level $p < 0.05$)

In order to determine the rate of PAH biodegradation, a C_{30}–17α(H),21β(H)-hopane biomarker was used in laboratory tests. Normalization of 3-ring (Fig. 8), 4-ring (Fig. 9), 5-ring (Fig. 10) and 6-ring (Fig. 11) PAH concentration was led. Concentration during various phases of the purification process was changeable.

PAHs biodegradation process can be described as follows:

$$C/C_H = (C/C_H)_0 \exp(-kt)$$

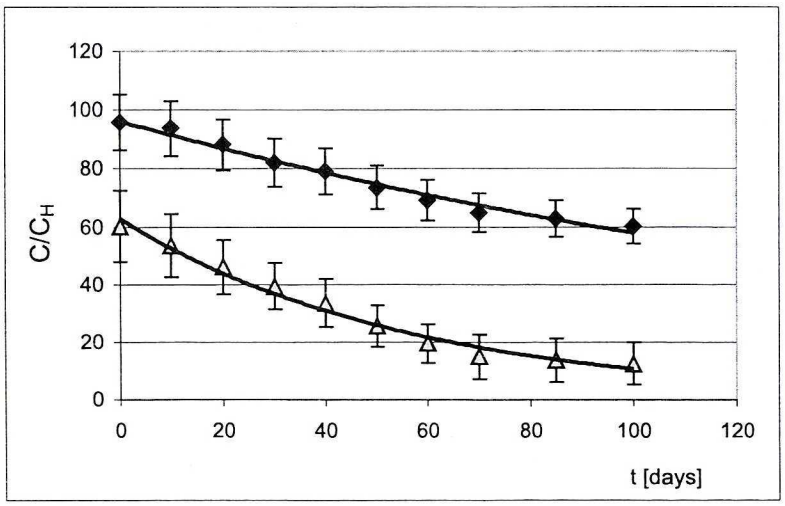
Their ratios in purification phases are presented in Table 4.



◆ sample W-2 – after basic bioremediation

△ sample W-3 – after bioaugmentation with WA-2 biopreparation

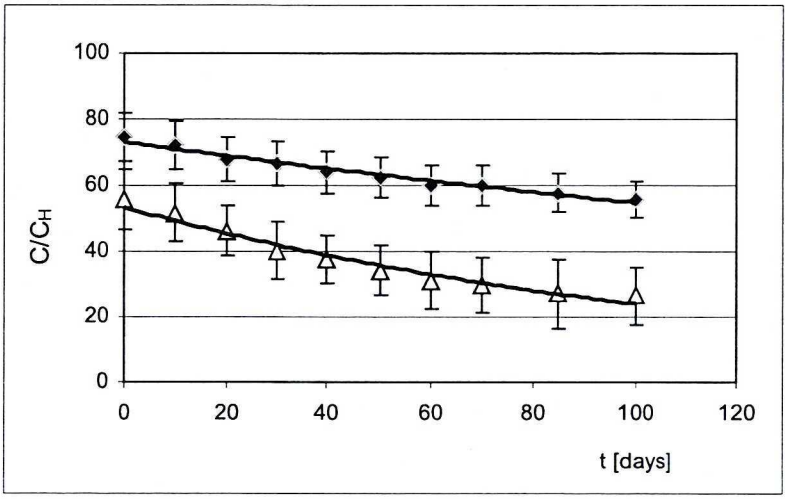
Fig. 8. Decline in 3-ring PAHs (C/C_H) after hopane normalization in consecutive phases of MGP soil biodegradation (number of repetitions n = 9–10, confidence level $p < 0.05$)



◆ sample W-2 – after basic bioremediation

△ sample W-3 – after bioaugmentation with WA-2 biopreparation

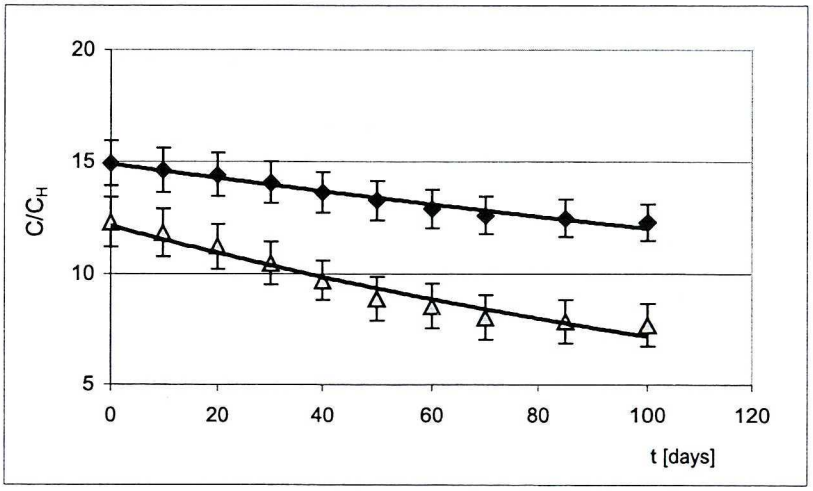
Fig. 9. Decline in 4-ring PAHs after hopane normalization (C/C_H) in consecutive phases of MGP soil biodegradation (number of repetitions n = 7–9, confidence level $p < 0.05$)



◆ sample W-2 – after basic bioremediation

△ Sample W-3 – after bioaugmentation with WA-2 biopreparation

Fig. 10. Decline in 5-ring PAHs after hopane normalization (C/C_H) in consecutive phases of MGP soil biodegradation (number of repetitions n = 8–10, confidence level $p < 0.05$



◆ sample W-2 – after basic bioremediation

△ Sample W-3 – after bioaugmentation with WA-2 biopreparation

Fig. 11. Decline in 6-ring PAHs after hopane normalization (C/C_H) in consecutive phases of MGP soil biodegradation (number of repetitions n = 7–10, confidence level $p < 0.05$)

Table 4. Comparison of coefficients of equation describing a PAHs (3-ring to 6-ring) biodegradation model evaluated for consecutive cleaning stages (a W-1 soil sample)

Purification stages	k [d^{-1}]	$(C/C_H)_0$	Determination coefficient (r^2)
Total PAHs			
Basic bioremediation	0.0050 ± 0.002	232.13 ± 33	0.9599
Bioaugmentation with WA-2 biopreparations	0.0147 ± 0.007	143.26 ± 18	0.9719
3-ring PAHs			
Basic bioremediation	0.0095 ± 0.004	38.11 ± 3.5	0.9644
Bioaugmentation with WA-2 biopreparations	0.0225 ± 0.011	17.18 ± 1.5	0.9813
4-ring PAHs			
Basic bioremediation	0.0051 ± 0.003	96.06 ± 8.9	0.9803
Bioaugmentation with WA-2 biopreparations	0.0205 ± 0.013	58.21 ± 10.2	0.9641
5-ring PAHs			
Basic bioremediation	0.0029 ± 0.002	73.03 ± 6.7	0.9718
Bioaugmentation with WA-2 biopreparations	0.0100 ± 0.011	54.96 ± 3.2	0.9664
6-ring PAHs			
Basic bioremediation	0.0021 ± 0.004	14.89 ± 2.6	0.9716
Bioaugmentation with WA-2 biopreparations	0.0069 ± 0.006	12.37 ± 1.8	0.9651

The first-order constant (k) of PAHs biodegradation for the phase of basic bioremediation is much lower than the constant for bioaugmentation with WA-2 biopreparation based on indigenous microorganisms and fungi. During the phase of a basic biodegradation, the biodegradation constant (k) for 3-ring PAHs is 0.0095 [d^{-1}], for 4-ring PAHs – 0.0051 [d^{-1}], for 5-ring PAHs it is 0.0029 [d^{-1}] and for 6-ring PAHs it is 0.0021 [d^{-1}]. In a bioaugmentation phase with WA-2 biopreparation the biodegradation constant (k) is higher (0.0225–0.0069 [d^{-1}]), which means a wide range of WA-2 effectiveness (Tab. 4).

While comparing first-order biodegradation constants (k) for PAH of various numbers of rings, it can be said that they are inversely proportional to the number of rings in a molecule, so a biodegradation constant (k) decreases when the number of rings grows.

Correlation ratios (r^2) are on a satisfactory level (0.9559–0.9803), which means that the curves of first-order biodegradation precisely match the measuring points (Figs 8–11.)

What is more, the comparison of the constants of first-order biodegradation for TAH and PAHs shows that TAH biodegradation is much higher particularly for a basic bioremediation stage. However, the biodegradation rate constants (k) for bioaugmentation process with a WA-2 biopreparation are similar for both TAH and PAH (Tabs 3 and 4).

CONCLUSIONS

The above presented laboratory results prove the correctness of MGP gradual purification which includes basic bioremediation based on activation of indigenous microbacterial flora by the choice of optimum parameters (N:P = 10:1, pH = 7.5, humidity 20–25%, temperature 17–20°C) and inoculation with the indigenous-based biopreparations enriched with isolated fungus able to PAH biodegradation.

Owing to chromatographic methodology of quantitative and qualitative determination of hydrocarbon contaminants, there is a possibility to control the biodegradation

process of aliphatic and aromatic hydrocarbons during their biodegradation in each phase of purification.

Combination of chromatographic analyses and microbiological research enables the preparation of biologicals based on indigenous miscroorgnisms and fungus, whose ingredients precisely match the chemical character of hydrocarbon pollutants (TAH, PAH) found in MGP soil.

Enrichment of biopreparations (based on indigenous microorganisms) in isolated fungi able to biodegrade PAH (*Pleurotus ostreatus, Irpex lacteus*) increased effectiveness of biopreparations, which was observed particularly for 5- and 6-ring PAHs.

The use of C_{30}–17α(H),21β(H)-hopane biomarker to normalize TAH and PAH concentrations in consecutive phases of biodegradation enabled the creation of a mathematical model defining kinetics of TAH and PAH biodegradation.

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BADANIA OPTYMALIZACYJNE BIODEGRADACJI ZANIECZYSZCZEŃ WĘGLOWODOROWYCH W ZESTARZAŁYCH GLEBACH Z GAZOWNI KLASYCZNEJ

Artykuł podejmuje zagadnienia związane z problemem oczyszczania gruntu skażonego w wyniku działalności klasycznych gazowni, które obecnie zostały wyłączone z eksploatacji. Przedstawiono opracowane metodyki chromatograficzne umożliwiające jakościową identyfikację i ilościowe oznaczenie poszczególnych węglowodorów alifatycznych i aromatycznych oraz oznaczenie ich sumarycznej zawartości (TAH, WWAs) w gruncie z terenu wytypowanej do badań gazowni. Omówiono wyniki badań w skali półtechnicznej oczyszczania gruntu skażonego zanieczyszczeniami węglowodorowymi (TAH, WWA) z wykorzystaniem bioremediacji podstawowej i bioaugmentacji poprzez inokulację mikroorganizmami autochtonicznymi wzbogaconymi o grzyby zdolne do biodegradacji WWA, co uwidacznia się w obniżeniu zawartości 5- i 6-pierścieniowych WWA. Prowadzone badania pozwalają prześledzić przebieg procesu oczyszczania gruntu, dobrać optymalne dawki substancji biogennych i określić ramy czasowe prowadzonego procesu. Cały cykl oczyszczania gruntu monitorowano za pomocą chromatografii gazowej (GC). Oceny stopnia biodegradacji poszczególnych węglowodorów alifatycznych (n-alkanów) dokonano na podstawie zmian zawartości oznaczonych chromatograficznie oraz za pomocą przyjętych wskaźników stopnia biodegradacji: C_{17}/Pr i C_{18}/F. Zastosowanie biomarkera C_{30}–17α(H),21β(H)-hopanu do normalizacji stężeń TAH i WWA w trakcie przebiegu poszczególnych etapów biodegradacji pozwoliło na opracowanie modelu matematycznego opisującego kinetykę biodegradacji TAH i WWA.