# Role of Fungi in Biodegradation of Petroleum Hydrocarbons in Drill Waste

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## Abstract

Petroleum substances are the main source of pollutants stored in old waste pits. They cause degradation of biological life in the area of storage. The aim of this article is to present laboratory research on petroleum pollutant biodegradation. The hydrocarbon contaminants in old drilling wastes came from the Graby-19 waste pit. The tests included basic bioremediation and inoculation with a biopreparation based on non-pathogenic species of bacteria and fungi, which were identified in molecular research. Multicriteria effectiveness estimation of petroleum pollutant biodegradation in the tested wastes enabled determination of a role of fungi in this process. Biopreparation enrichment was done in the final phase with non-pathogenic fungi isolated from the waste during purification. As a result, an increase in long-chain n-alkanes and aromatic hydrocarbon (BTEX and PAH) biodegradation was observed. Biodegradation rate constants for petroleum hydrocarbons, calculated on the basis of a mathematical model, can serve as proof of fungi usage in the bioremediation process.

### Introduction

Oil waste results in significant and increasing biological degradation of an active surface of the earth. In the area of Grabownica Plant there are numerous waste pits, dated from 1925 to 1950, in which drill wastes are stored. The wastes, contaminated with petroleum hydrocarbons, come from drillings done using a percussive method.

Nowadays, acceleration of petroleum hydrocarbon biodegradation through biotechnological processes with the use of effective bacteria cultures (isolated from severely contaminated areas) has been among the most common research. Due to relatively low costs and high effectiveness, biological methods have been applied in a technical scale [1-8]. However, a huge amount of petroleum pollutants (up to 200,000 mg TPH/kg dry mass) in weathered drill wastes stored in pits causes difficulties in bioremediation works.

Therefore, it has been claimed that the technological concept of waste purification, based on stage application of consecutive purification processes, will enable a gradual decrease in petroleum contaminant levels. This will lead to the use of successive methods for deeper purification of a polluted area [9].

Laboratory research on biodegradation of petroleum contaminants from weathered drill waste (*ex-situ* method) includes the following steps:
- basic/initial bioremediation stimulated by bioventilation (providing oxygen through aeration) and waste environment enrichment with biogenic ingredients in doses determined on the basis of laboratory tests, which aided growth of autochthonous microflora in temperature and humidity similar to field conditions,
- bioaugmentation with inoculation of initially purified waste done by biopreparation based on isolated, selected, and multiplied autochthonous microorganisms.

The above-mentioned tests enabled not only optimization of the consecutive stages of complex technology of

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waste purification, but also the effectiveness of the applied biopreparations and identification of a role of fungi used for modification of biopreparation based on autochthonous bacteria.

Kerosene is a complicated multi-constituent structure. Due to this fact, a mixture of microorganism cultures with a complex enzymatic system has to be applied in its degradation. It is recommended to use bacterial consortia in forms of a biopreparation based on autochthonous microorganisms isolated from the soil – in order to avoid antagonistic influence of autochthonous-soil microflora on extraneous microorganism cultures unadapted to a certain environment [5, 10-16].

In world literature there are numerous works devoted to research on biodegradation with the use of fungi [17-21]. The majority of filamentous fungi is unable to totally mineralize aromatic hydrocarbons; they only transform them into indirect products of decreased toxicity and increased susceptibility to decomposition with the use of bacteria. Among the filamentous fungi participating in aliphatic hydrocarbon biodegradation are *Cladosporium* and *Aspergillus*, whereas fungi belonging to *Cunninghamella*, *Penicillinum*, *Fusarium*, and *Aspergillus* can take part in aromatic hydrocarbon decomposition.

Prenafeta-Boldu and his co-workers [22, 23] researched the use of *Cladophialophoria* sp. fungi in monoaromatic hydrocarbons (BTEX) mineralization. The tests indicated that the decomposition is more dynamic for toluene, ethylobenzene, and m-xylene than in the case of benzene. Introduction of *Rhodococcus* sp. bacteria resulted in a significant increase in mineralization of benzene. Metabolic profiles and inhibitors of mutual interaction depict that toluene, ethylobenzene, and m-xylene were degraded in a lateral chain by the same monoxygenase enzyme.

Rot fungi (eg. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*) produce extracellular enzymes (eg. lignic peroxydase) that participate in decomposition of a lignic cell-wall in plants and in oxygenation of various xenobiotics. The microorganisms transform PAH into chinone derivates, and – in consecutive stages – splitting of an aromatic ring of chinones with their complete mineralization takes place.

PAH biodegradation is much more effective after enrichment of *Pseudomonas putida* bacteria with *Pleurotus ostreatus* and *Iprex lacetus* fungi. An increase in biodegradation can be noticed particularly in the case of 5-6-chain aromatic hydrocarbons, where reduction increase in their content ranged from 16% to 19% [8, 19, 24].

Common fungi with the ability of aromatic compound (BTEX, PAH) biodegradation include *Phanerochaete chrysosphorium* [21, 25]. Interaction among fungi and bacteria is profitable for the process of petroleum hydrocarbon mineralization.

Also, research on the biodegradation of PAH with biopreparations created on the basis of autochthonous microorganisms, has been enriched with following fungi species: *Dichomitus squalens* and *Pleurotus ostreatus*. Satisfactory effects of mineralization of PAH have been observed, particularly in the case of 5-6-chain PAH [20].

In the research presented in this article, it has been assumed that deeper purification of contaminated drill waste can be obtained through the application of fungi (isolated from an area under purification) in a final stage of the process. The fungi were used in order to enrich the biopreparation created on the basis of autochthonous bacteria. The aim of modification of the biopreparation was to biodegrade groups of petroleum wastes that were particularly resistant to biological decomposition (BTEX, PAH, and aliphatic long-chain hydrocarbons).

## Research Material and Methodology

Weathered 50-year-old waste, obtained from the Graby-19 pit after initial remediation (including drainage), was used as material in laboratory research. It caused a decrease in petroleum pollutant content from 78,145 to 31,724 mg TPH/kg dry mass, which is a level enabling application of biological purification methods.

Chromatographic analysis indicated that 76.8% of petroleum contaminants are $nC_6$-$nC_{36}$ alkanes. There is diversification in contents of consecutive n-alkane groups ($nC_6$-$nC_{12}$=20.4%; $nC_{13}$-$nC_{19}$=23.7%; $nC_{20}$-$nC_{25}$=11.8%; $nC_{26}$-$nC_{36}$=3.7%; izoprenoids Pr and F =1.4%; unidentified hydrocarbons = 39.9%).

There were observed monoaromatic hydrocarbons (BTEX = 36.4 mg/kg dry mass), including benzene in the amount of 23.8 mg/kg dry mass, ethylbenzene = 7.5 mg/kg, toluene = 7.6 mg/kg, and xylenes = 5.0 mg/kg. Polycyclic aromatic hydrocarbons were in trace amounts (PAH=1.378 mg/kg dry mass with 88.5% of naphthalene).

Moreover, the proportions of bioorganic substances (based on chemical analyses) were on a level: C:N:P = 100:1:5 – different from optimal values (C:N:P = 100:10:1). This means that microbiological processes are inhibited and autochthonous bacteria flora will not be activated without correction of these element contents.

Laboratory research on biodegradation of oil pollutants in Graby-19 weathered waste was led in semi-industrial conditions in specifically constructed research sites. Foil was laid directly on a floor in order to protect against the spread of petroleum pollutants and to avoid contamination of the place. On the foil, a platform was situated (and protected with foil). The construction enabled draining of water and streaming liquid contaminants from a prism. A layer of gravel was placed on the platform. Inside the layer, a system of perforated pipes was installed. The air from a compressor was pumped through the pipes in order to provide proper aeration of the ground. Next, a prism (150 kg) was formed from soil taken from the Graby-19 waste pit after initial remediation. Research on basic bioremediation consisted of nutrient dosages (nitrogen and phosphorus) in a form of Azofoska, mineral fertilizer which contained: nitrogen total (13.6%), $NO_3$ nitrogen 5.5 %, $NH_3$ nitrogen 8.1%, $P_2O_5$ 6.4%, $K_2O$ 19.1% (in $K_2SO_4$ form), MgO 4.4% (as $MgSO_4$), and microelements (Fe 0.17%, Mn 0.27%, Cu 0.18%, Zn 0.045%, and Mo 0.09%). The dosage was done in order to obtain optimum proportions of nutrients that had

been determined through monitoring alternations in parameters such as dehydrogenase activity and a decrease in petroleum contaminant content in the waste.

During purification, waste moisture was kept on a level from 10 to 20% through water spraying, whereas reaction was stabilized on a level of 7.5 to 7.8 through dosage of fertilizer lime. The prisms were covered with a foil tunnel, which enabled maintaining constant temperature (from 17 to 20ºC), possible to obtain in terrain conditions. Effluent from the prisms was collected in a waste container installed below the level of the platform.

The next stage of purification included inoculation done with biopreparation based on autochthonous microorganisms (G-19-1) – the first series. Then the cleaned waste, collected in the prism, was divided into 2 prisms and the second series of inoculation was applied:

Prism A – inoculation with autochthonous bacteria-based biopreparation (G-19-1)

Prism B – inoculation with autochthonous bacteria-based biopreparation enriched with isolated non-pathogenic fungi species (G-19-2)

Petroleum hydrocarbon biodegradation was controlled through monitoring of determined physical-chemical parameters, particularly taking into account chromatographic analyses of oil and microbiological contaminants.

## Biopreparation Characteristics

Creation of the professional biopreparation, based on autochthonous bacteria and fungi taken from the area of the waste pit severely contaminated with petroleum pollutants, was the aim of microbiological research. It was stated that the classical tests had to be extended by research based on molecular techniques [26-28]. Assuming huge biodiversity of the created biopreparations, leading these kinds of research is essential to precise identification of microorganisms through coding gene sequences: 16S rDNA for bacteria and 18S rDNA for fungi.

The microbiological research included initial microbiological analyses of samples, which meant determination total bacteria number, the number of bacteria-degrading petroleum hydrocarbons, and fungi. The analyses aimed at isolation of the microorganisms able to use aliphatic and aromatic hydrocarbons as the only source of carbon. The next stage included obtaining pure microorganism strains degrading oil compounds. In order to lead generic and specific identification of the isolated microorganisms, standard techniques of microorganism identification with classical methods were applied. They included analyses of morphological, physiological, and biochemical features. Then, PRC reaction (polymerase chain reaction) and coding sequences analysis (16S rDNA for bacteria and 18S rDNA for fungi) were done as confirmation. The obtained data was compared with GenBank data, with the use of BLAST software.

15 bacteria strains were isolated from Graby-19 waste. They were able to use kerosene and hydrocarbons as the only source of carbon. Among all the isolated microorganisms, the vast majority were representatives of *Actinomycetales* (*Actinobacteria* type) – common in soil environment, able to decompose petroleum hydrocarbons. Genus of *Rhodococcus* had a particularly significant number of representatives. The isolated strains were able to use not only aliphatic but also aromatic hydrocarbons, though mutual tracks of the usage of these hydrocarbons are not common. Apart from the above-mentioned strains, four active fungi strains were isolated: *Aspergillus*, *Fusarium*, *Cladosphorium*, and *Phanerochaete*.

In creation of a professional biopreparation, special attention ought to be paid to the security of its application. The molecular research enabled species determination of the isolated and selected microorganisms, which led to elimination of pathogenic species from biopreparation. The microorganisms were classified according to American type culture collection (ATCC) as biosafety level-1; there is no evidence of causing illnesses in healthy adults.

The classified species include a strain of *Agrobacterium tumefaciens* that has specific features. First, its ability to use hydrocarbons as the only source of carbon has not been thoroughly verified by documentary evidence. Second, the bacteria can grow (similar to *Pseudomonas rhodesiae*) in oxygen-free conditions, which is useful in bioremediation done using an *in-situ* method. However, probably the most significant feature of the bacteria are the ability to genes horizontal transfer. This means that it can spread genes able to degrade hydrocarbons (which were acquired during adaptation to oil contaminant-dominated conditions) to eucariotic organisms. The bacteria belong to pathogenic plants; therefore, it was not included in G-19-1 biopreparation.

Among the identified fungi were pathogenic species of *Fusarium oxysporum*, which was not included in G-19-2 biopreparation.

In the Department of Microbiology of Oil and Gas Institute, two biopreparations were created for the Graby-19 waste pit: G-19-1 autochthonous bacteria-based biopreparation, which was modified with selected non-pathogenic fungi (G-19-2) isolated from the waste under purification. The biopreparation included bacteria and fungi species presented in Table 1.

The created biopreparations were tested in laboratory research (inoculation) on a soil and ground prism after initial bioremediation.

## Research Results

Initial bioremediation, consisting of natural microflora activation in Graby-19 waste, enabled a decrease in petroleum hydrocarbons (TPH) from 31,724 to 17,016 mg/kg dry mass. The TPH decrease degree in initial bioremediation, done in laboratory conditions, was as follows: 17.5% after two weeks, 28.8% after 4 weeks and 38.2% after eight weeks. After this time biodegradation rates decreased and were on an approximate level. In addition, there was a decrease in BTEX contents in 20.1%, PAH contents in 16.2%, and phenols content in 28.2% (Table 2).

Table 1. Microorganism identification in G-19-1 and G-19-2 biopreparations.

| Strain identification – classical method | Strain identification – sequence methods | Identity percentage/GenBank most probable sequences | ATCC safety category |
|---|---|---|---|
| G-19-1 biopreparation | | | |
| *Bacillus* sp. | *Paenibacillus borealis* | 98% /HM563046 | 1 |
| *Klebsiella* sp. | *Raoultella planticola* | 98%/AF181574 | 1 |
| *Microccoccus* sp. | *Micrococcus luteus* | 99%/AM992194 | 1 |
| *Mycobacterium* sp. | *Mycobacterium frederiksbergense* | 99%/AF544628 | 1 |
| *Nocardia* sp. | *Rhodococcus corynebacterioides* | 99%/EU438932 | 1 |
| *Rhizobium* sp. | *Rhizobium daejeonense* | 97%/DQ089696 | 1 |
| *Rhodococcus* sp. | *Rhodococcus erythropolis* | 100%/AJ237967 | 1 |
| *Rhodococcus* sp. | *Rhodococcus erythropolis* | 99%/AB546303 | 1 |
| *Pseudomonas* sp. | *Pseudomonas rhodesiae* | 98%/AB495138 | 1 |
| G-19-1 biopreparation enriched with selected fungi species (G-19-2 biopreparation) | | | |
| *Aspergillus* sp. | *Aspergillus sydowii* | 99%/AY373869 | 1 |
| *Cladosporium* sp. | *Cladosporium cladosporioides* | 99%/EF577236 | 1 |
| *Phanerochaete* sp. | *Phanerochaete chrysosporium* | 98%/AF475147 | 1 |

Table 2. Comparison of results of chemical, microbiological, and enzymatic analyses done during Graby-19 waste purification – laboratory research (*ex-situ* method).

| Time of process [weeks.] | pH | TPH [mg/kg dry mass] | BTEX [mg/kg dry mass] | WWA [mg/kg dry mass] | Phenols [mg/kg dry mass] | Dehydrogenase activity [µg TF/g dry mass/24 hrs] | Cellulose activity [µg Gcl/g dry mass/24 hrs] | Number of bacteria degrading hydrocarbons [cfu/g dry mass] | Number of fungi [cfu/g dry mass.] |
|---|---|---|---|---|---|---|---|---|---|
| Sample from G-19 waste pit after initial remediation | | | | | | | | | |
| | 6.53 | 31,724 | 36.4 | 1.378 | 6.98 | 13.4 | 5.8 | $1.1 \cdot 10^5$ | $1.3 \cdot 10^2$ |
| Sample from waste prism after basic bioremediation | | | | | | | | | |
| 2 | 6.75 | 26,147 | 35.8 | 1.289 | 6.47 | 19.5 | 6.5 | $2.3 \cdot 10^5$ | $1.9 \cdot 10^2$ |
| 4 | 7.20 | 22,584 | 31.4 | 1.258 | 6.24 | 24.8 | 8.7 | $2.9 \cdot 10^5$ | $2.4 \cdot 10^2$ |
| 8 | 7.54 | 19,587 | 30.7 | 1.187 | 5.91 | 31.5 | 10.8 | $3.1 \cdot 10^5$ | $2.7 \cdot 10^2$ |
| 12 | 7.84 | 17,016 | 29.1 | 1.154 | 5.01 | 38.5 | 13.5 | $3.9 \cdot 10^5$ | $3.2 \cdot 10^2$ |
| Sample from waste prism after 1st series of inoculation with G-19-1 biopreparation | | | | | | | | | |
| 14 | 7.72 | 13,784 | 25.4 | 1.099 | 4.71 | 46.2 | 16.5 | $6.8 \cdot 10^5$ | $5.2 \cdot 10^2$ |
| 18 | 7.59 | 7,147 | 21.7 | 0.845 | 3.55 | 51.8 | 21.4 | $9.9 \cdot 10^5$ | $7.4 \cdot 10^2$ |
| 20 | 7.38 | 4,987 | 17.8 | 0.752 | 2.94 | 63.3 | 26.4 | $2.3 \cdot 10^6$ | $9.5 \cdot 10^2$ |
| Sample from waste prism A after 2nd series of inoculation with G-19-1 biopreparation | | | | | | | | | |
| 22 | 7.24 | 3,842 | 16.5 | 0.644 | 2.56 | 75.1 | 29.5 | $5.5 \cdot 10^6$ | $1.9 \cdot 10^3$ |
| 26 | 7.16 | 2,814 | 13.1 | 0.558 | 2.04 | 89.1 | 31.5 | $7,8 \cdot 10^6$ | $2.8 \cdot 10^3$ |
| 28 | 7.08 | 2,014 | 9.8 | 0.488 | 1.65 | 97.4 | 33.8 | $9.9 \cdot 10^6$ | $3.5 \cdot 10^3$ |
| Sample from waste prism B after 2nd series of inoculation with G-19-2 biopreparation | | | | | | | | | |
| 22 | 7.21 | 3,157 | 14.2 | 0.524 | 2.37 | 79.1 | 35.8 | $5.6 \cdot 10^6$ | $6.9 \cdot 10^3$ |
| 26 | 7.12 | 1,675 | 9.14 | 0.411 | 1.69 | 91.5 | 48.9 | $7.7 \cdot 10^6$ | $9.5 \cdot 10^3$ |
| 28 | 6.92 | 998 | 6.54 | 0.298 | 0.75 | 105.5 | 68.4 | $9.7 \cdot 10^6$ | $2.8 \cdot 10^4$ |

According to microbiological monitoring, it can be said that there was an increase in biological activity of the waste, which meant dehydrogenase activity increased from a level of 13.4 to 38.5 µg TF/g dry mass/24 hrs in correlation with bacteria total number growth. During twelve weeks of bioremediation, done in laboratory conditions, there was a increase in the number of hydrocarbon-degrading bacteria from $1.1 \cdot 10^5$ to $3.9 \cdot 10^5$ cfu/g dry mass and an increase in the number of fungi (Table 2). Basic bioremediation enabled a decrease in petroleum hydrocarbon content to a level where inoculation with autochthonous microorganism-based bio-preparations would be more effective [9].

Inoculation with G-19-1 biopreparation resulted in TPH content decrease from 17,016 to 4,987 mg/kg dry mass in eight weeks. The TPH decrease degree, obtained through inoculation with G-19-1 biopreparation (1st series), was as follows: 18.8% after two weeks, 57.2% after six weeks, and 87.2% after eight weeks. There was a significant decrease in BTEX (in 38.8%), PAH (in 33.1%), and phenol (in 41.3%) contents (Table 2).

As a result of the process, a number of hydrocarbon-degrading bacteria increased to a level of $2.3 \cdot 10^6$ cfu/g dry mass, and their number was in correlation with the decrease rate of petroleum hydrocarbon contents (Table 2). Moreover, the notified dehydrogenase activity growth to a level of 63.3 µg TF/g dry mass/24 hrs was in correlation with a decrease in oil contaminants included in Graby-19. In the experiment, a number of fungi increased to $9.5 \cdot 10^2$ cfu/g dry mass (Table 2).

Inoculation with G-19-1 biopreparation (2nd series) did not give satisfactory results. The obtained TPH decrease was from 4,987 to 2,014 mg/kg dry mass, which does not meet requirements determined in soil and ground quality standards.

However, application of the autochthonous microorganisms-based biopreparation (G-19-2), enriched with chosen fungi species (2nd series), caused further TPH decrease from 4,987 to 998 mg/kg dry mass. During the initial stage of the second series of inoculations, TPH biodegradation rate was high: there was a decrease in TPH contents by 36.6% after 2 weeks, and by 79.9% after 8 weeks. Inoculation with G-19-2 biopreparation enabled not only TPH decrease to a low level, but it also resulted in a higher degree reduction in comparison to the first series (inoculation with the autochthonous bacteria-based biopreparation), which was as follows (Table 2): BTEX by 66.9%, PAH by 59.2%, and phenols by 74.4%. The number of petroleum hydrocarbon-degrading bacteria increased to $9.7 \cdot 10^6$ cfu/g dry mass. In addition, according to microbiological monitoring, it can be said that adaptation of microorganisms (bacteria and fungi) introduced in a form of G-19-2 biopreparation was satisfactory. Increase in dehydrogenase activeness (which was 105.5 µg TF/g dry mass/24 hrs in the final phase of the process) means a high level of biological activeness of Graby-19 waste (Table 2).

According to comparison in analyses of cellulose activity in soil from Graby-19, it can be observed that there was significant increase in activity after the second series of inoculations with G-19-2 biopreparation (Table 2). The analysis of this parameter enables estimation of whether there is proper growth of fungi in the waste/soil under purification because the fungi are responsible for cellulose decomposition in soil conditions. As it can be observed, the presence of the fungi in G-19-2 biopreparation is related to growth in cellulose activity, which means a proper adaptation process of fungi to the soil environment and increasing the process of waste/soil purification.

Due to the results of chromatographic analyses, it can be said that during initial bioremediation of Graby-19 waste/soil and earth, the highest biodegradation degree was observed in the case of $nC_9$-$nC_{17}$ aliphatic hydrocarbons (from 59.9 to 67.4%), whereas for $nC_{18}$-$nC_{23}$ hydrocarbons it was slightly lower (from 47.6 to 55.7%). Heavier hydrocarbons with $nC_{24}$-$nC_{36}$ chains underwent decomposition in a much lower degree, because a decrease in their contents was from 10.8 to 19.8% (Fig. 1). Reductions in unidentified hydrocarbon contents (alkenes, izoparaffins, cycloparaffins, etc.) was at 29.8%.
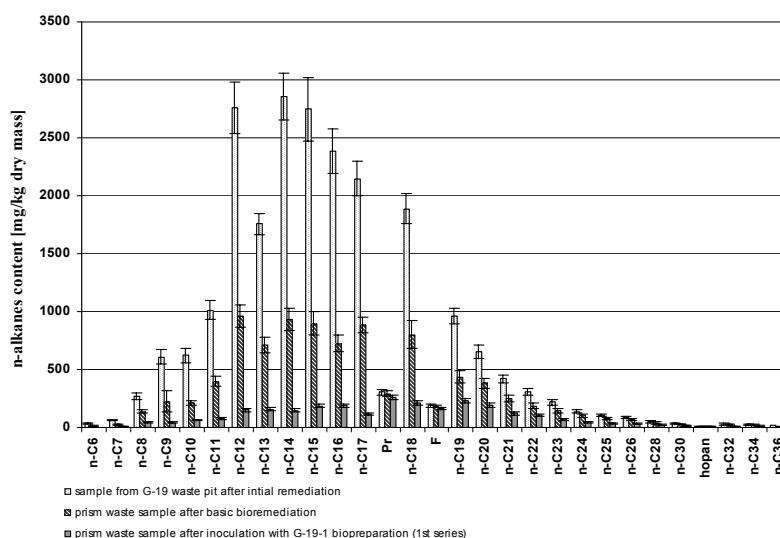


Fig. 1. Comparison of identified n-alkane contents in Graby-19 waste after consecutive purification stages in laboratory conditions – *ex-situ* method (repetition number = 8-10, p<0.05).

Table 3. Comparison of n-alkane biodegradation degree indicators $nC_{17}/Pr$ and $nC_{18}/F$ in Graby-19 waste after consecutive purification stages – laboratory research (*ex-situ* method).

| Sample from G-19 waste pit after initial remediation | | | |
|---|---|---|---|
| n-C$_{17}$/Pr =11.985 (±0.857) | | n-C$_{18}$/F =5.874 (±0.468) | |
| Sample from waste prism after basic bioremediation | | | |
| n-C$_{17}$/Pr =4.125 (±0.351) | | n-C$_{18}$/F =3.621 (±0.247) | |
| Sample from waste prism after 1$^{st}$ series of inoculations with G-19-1 biopreparation | | | |
| n-C$_{17}$/Pr =0.501 (±0.061) | | n-C$_{18}$/F =1.335 (±0.105) | |
| Sample from waste prism A after 2$^{nd}$ series of inoculations with G-19-1 biopreparation | | Sample from waste prism B after 2$^{nd}$ series of inoculations with G-19-2 biopreparation | |
| n-C$_{17}$/Pr =0.246 (±0.034) | n-C$_{18}$/F =0.301 (±0.042) | n-C$_{17}$/Pr =0.041 (±0.003) | n-C$_{18}$/F =0.069 (±0.005) |

Satisfactory effectiveness of petroleum pollutant biodegradation during basic bioremediation has been proved by $nC_{17}/Pr$ and $nC_{18}/F$ biodegradation degree indicators, which were significantly decreased (Table 3): $nC_{17}/Pr$ – from a level of 11.985 to 4.125 and $nC_{18}/F$ – from 5.874 to 3.621.

According to the results of chromatographic analyses, in the case of inoculation with G-19-1 biopreparation, led during 8 weeks (one series), the highest biodegradation degree was observed for $nC_8$-$nC_{19}$ aliphatic hydrocarbons (on a level from 74.2 to 86.4%). Satisfactory biodegradation degree was noted in the case of $nC_{20}$-$nC_{30}$ hydrocarbons (from 56.4 to 61.3%). Hydrocarbons containing more than 30 atoms of carbon in a molecule, and belonging to hardly-degraded compounds, underwent decomposition on a lower level, from 31.2 to 43.52% (Fig. 1). Unidentified hydrocarbons content was decreased by 51.6%.

During the second series of inoculation with G-19-2 biopreparation, a further decrease in oil pollutants took place due to reduction in $nC_8$-$nC_{23}$ n-alkanes content on a level from 72.5 to 83.7%, whereas $nC_{24}$-$nC_{36}$ hydrocarbons were degraded to a level from 51.3 to 63.2% (Fig. 2), despite a significant decrease in properties of indicators of n-alkane biodegradation degrees to the following levels (Table 3):

- after inoculation with G-19-1 biopreparation (1$^{st}$ series): $nC_{17}/Pr$=0.501, $nC_{18}/F$=1.335
- after inoculation with G-19-2 biopreparation (2$^{nd}$ series): $nC17/Pr$=0.246, $nC18/F$=0.301

No decrease in oil contaminants to a level of acceptable properties determined by soil/earth standards was obtained.

Modification of G-19-1 biopreparation with selected fungi species (G-19-2) and application of the biopreparation in the second series of inoculations resulted in petroleum contaminant reduction to acceptable properties determined by soil/earth standards and comparison in chromatographic analyses (Fig. 2). The use of the biopreparation enriched with fungi (G-19-2) increased biodegradation dynamics for all analyzed
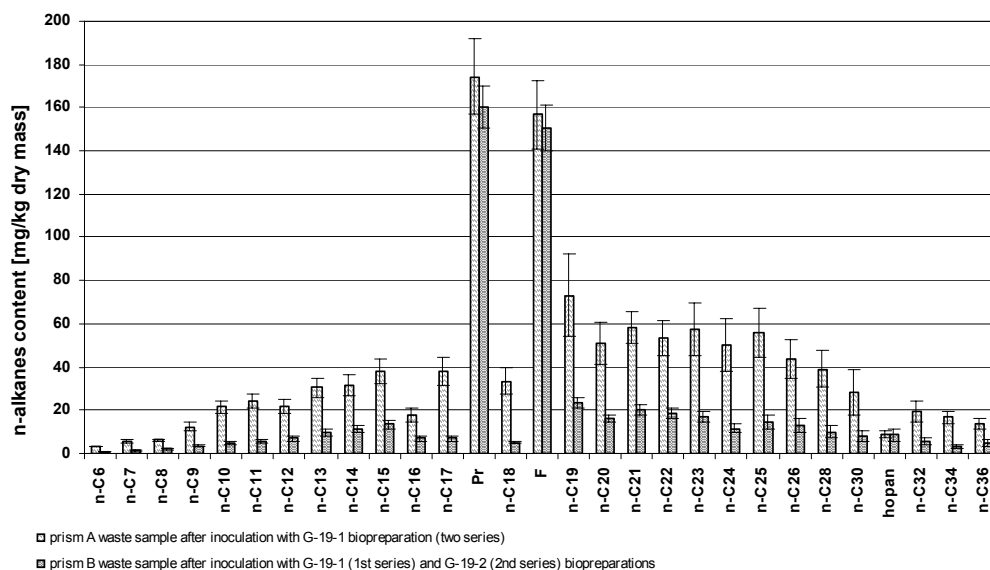


Fig. 2. Comparison of G-19-1 and G-19-2 biopreparation effectiveness during Graby-19 waste inoculation in laboratory conditions – *ex-situ* method (repetition number = 8-10, p<0.05).

Table 4. Biodegradation rate constant for petroleum hydrocarbons in Graby-19 waste after consecutive purification stages – laboratory research (*ex-situ* method).

| Purification phase | TPH | | S n-$C_8$-n-$C_{22}$ | | S n-$C_{23}$-n-$C_{36}$ | | BTEX | |
|---|---|---|---|---|---|---|---|---|
| | k [$d^{-1}$] | $r^2$ | k [$d^{-1}$] | $r^2$ | k [$d^{-1}$] | $r^2$ | k [$d^{-1}$] | $r^2$ |
| Basic bioremediation | 0.0,058 ± 0.003 | 0.9968 | 0.0079 ± 0.004 | 0.9947 | 0.0018 ± 0.002 | 0.9784 | 0.0038 ± 0.002 | 0.9641 |
| Inoculation with G-19-1 bioremediation (two series) – prism A | 0.0152 ± 0.007 | 0.9845 | 0.0204 ± 0.010 | 0.9847 | 0.0101 ±0.006 | 0.9648 | 0.0095 ± 0.005 | 0.9741 |
| Inoculation with G-19-1(1ˢᵗ series) and G-19-2 (2ⁿᵈ series) – prism B | 0.0208 ± 0.009 | 0.9747 | 0.0267 ± 0.013 | 0.9745 | 0.0134 ± 0.006 | 0.9618 | 0.0157 ± 0.008 | 0.9578 |

hydrocarbon groups (n-alkanes and BTEX). The fastest biodegradation rate was noted in the case of n$C_9$-n$C_{19}$ aliphatic hydrocarbons: 88.3-96.1%, whereas for n$C_{20}$-n$C_{36}$ hydrocarbons the biodegradation rate was insignificantly slower: 79.1-85.8% (Fig. 2). There was also a decrease in unidentified hydrocarbon content by 74.3%.

Apart from this, biodegradation degree indicators after inoculation with G-19-1 biopreparation (1ˢᵗ series) and G-19-2 biopreparation (2ⁿᵈ series) were much more significantly decreased than in the case of inoculation with G-19-1 biopreparation done in two series, and they were as follows: n$C_{17}$/Pr=0.041, n$C_{18}$/F=0.069 (Table 3).

Basic bioremediation resulted in reduction of aromatic compounds, which was on a level from 19.2 to 21.3%. The highest biodegradation degree was observed in cases of toluene and benzene.

Inoculation with G-19-1 biopreparation (one series in eight weeks) caused a decrease in BTEX contents in a range from 39.1 to 50.2% (benzene: 49.1%, toluene: 49.1%), whereas the use of G-19-2 biopreparation (second series) increased biodegradation effectiveness in the case of monoaromatic compounds to a level from 61.3 to 81.3%. The highest biodegradation degree was noted for toluene (81.3%) and benzene (78.3%), whereas decomposition rates for ethylobenzene and xylene were much slower (Fig. 3).
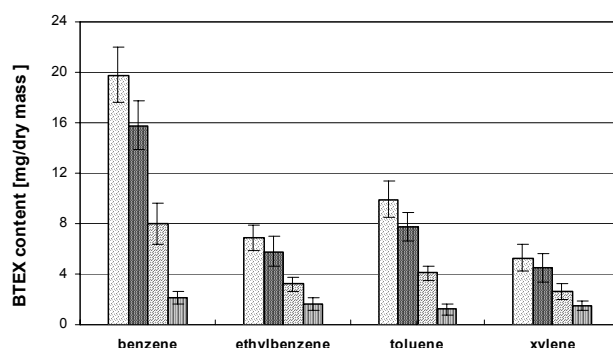


☐ G-19 waste sample after initial remediation
▪ prism waste sample after basic bioremediation
☐ prism A waste sample after two series of inoculations with G-19-1 biopreparation
☐ prism B waste sample after inoculation with G-19-1 (1st series) and G-19-2 (2nd series) biopreparations

Fig. 3. Alternation in BTEX contents after consecutive stages of Graby-19 waste purification in laboratory conditions – ex-situ method (repetition number = 9-10, p<0.05).

## Mathematical Model of Petroleum Contaminants Biodegradation

During laboratory research (*ex-situ* method), a $C_{30}$-17α(H)21β(H)-hopane biomarker was used in order to normalize analyte concentrations (TPH, Σ n$C_8$-n$C_{22}$, Σ n$C_{23}$-n$C_{36}$, BTEX) and to prepare a mathematical model of biodegradation of petroleum contaminants in Graby-19. The biomarker enabled total estimation of biodegradation degrees of oil hydrocarbons. Results of chromatographic determination of hydrocarbons, normalized according to hopane, served as a basis of the first-order mathematical model presented below:

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

...where:

$C$ – analyte concentration [mg/kg dry mass]
$C_H$ – hopane concentration [mg/kg dry mass]
$(C/C_H)_0$ – normalized analyte concentration in starting point of biodegradation
$k$ – first-order biodegradation constant [$d^{-1}$]
$t$ – time of the process (days) [d]

Calculated constants of first-order biodegradation ($k$) for the process (including basic bioremediation and inoculation with biopreparations based on autochthonous bacteria and fungi) enable monitoring and comparison in kinetics of biodegradation in the case of contaminant groups in consecutive stages of Graby-19 waste purification. In addition, according to comparison in first-order biodegradation constants ($k$), effectiveness of the autochthonous bacteria-based biopreparation (modified with fungi isolated from the waste) can be determined [29, 30].

During basic bioremediation, the first-order TPH biodegradation constant ($k$) was at a low level of 0.0058 [$d^{-1}$], whereas correlation ratio was 0.9968 (Table 4).

The next stage of Graby-19 waste purification, including inoculation (done in two series) with the autochthonous bacteria-based biopreparation (G-19-1), resulted in an increased TPH biodegradation rate. There was increase in a biodegradation rate constant to a level of 0.0152 [$d^{-1}$]. Modification in the autochthonous microorganisms-based biopreparation, enriched with non-pathogenic fungi species (G-19-2) isolated from waste pit soil, and application of the

biopreparation in the second series of inoculation increased TPH biodegradation rate. The biodegradation rate constant reached a level of 0.0208 [d$^{-1}$] (Table 4).

The biodegradation constant of the first-order (k) for $\Sigma$ nC$_8$-nC$_{22}$ hydrocarbons group is the highest, particularly after purification, including inoculation with G-19-2 biopreparation based on autochthonous bacteria and fungi (second series) and reaches a level of 0.0267 [d$^{-1}$]. However, during basic bioremediation the biodegradation constant is much lower: 0.0079 [d$^{-1}$] (Table 4). The presented results serve as proof that hydrocarbons of that chain length are easily biodegradable.

The biodegradation constants of the first-order for heavier hydrocarbons of nC$_{23}$-nC$_{36}$ chain length reached the lowest level (0.0018 [d$^{-1}$]) during basic bioremediation, which means low effectiveness of biological decomposition of these compounds.

There was significant increase in the biodegradation rate constant for heavier hydrocarbons nC$_{23}$-nC$_{36}$ after inoculation with G-19-2 biopreparation, and it was on a level of 0.0134 [d$^{-1}$]. This means that the autochthonous bacteria- and fungi-based biopreparation has a wide range of activity because even long-chain nC$_{23}$-nC$_{36}$ hydrocarbons undergo biodegradation (Table 4).

Monoaromatic hydrocarbon (BTEX) biodegradation during basic bioremediation takes place at a low level when the biodegradations rate constant is 0.0038 [d$^{-1}$]. Inoculation with the biopreparation based on autochthonous bacteria and fungi has essential influence to biodegradation rate of aromatic compounds (BTEX), because the biodegradation rate constants increase to a level of 0.0157 [d$^{-1}$] (Table 4).

The above-presented values of the first-order biodegradation constants of petroleum hydrocarbons in Graby-19 waste are similar to results given by many foreign researchers, who applied a similar model in order to describe oil contaminant biodegradation during bioremediation stimulated by adding biogenic substances [15, 31]. This article presents research extending the use of the mathematical model of biodegradation for consecutive stages of pollutant biodegradation – inoculation with biopreparations based on autochthonous bacteria and biopreparations enriched with fungi. This innovative attitude enables complete observation of the purification process and determination of biodegradation effectiveness for consecutive hydrocarbon groups, with the application of a variety of biopreparations. As in previous research results, the mathematical model proves the usefulness of introduction of the biopreparation enriched with fungi during a final stage of purification.

Similar research was done on wastes taken from other waste pits situated in the area of the oil and gas plant in Grabownica. In addition, a biopreparation based on autochthonous bacteria and enriched with non-pathogenic fungi species (*Penicillium chrysogenum*, *Hypocrea virens*, *Trichoderma asperellum*, *Phanerochaete chrysosporium*) was applied. The obtained results were similar, which means that the fungi should not be ignored in developing effective strategies of bioremediation.

## Conclusions

1. The innovative attitude toward effective estimation of petroleum contaminant biodegradation during waste purification was done by applying chromatographic methodology. It enabled identification and quantity determination of individual hydrocarbons included in contaminants, and resulted in determination of a biodegradation level of hydrocarbon groups. What is more, it was possible to discover the crucial role of fungi in oil hydrocarbon biodegradation.

2. Microbiological research was extended with tests based on molecular techniques in order to obtain precise identification of microorganism species. The sequence of genes coding (16SrRNA for bacteria and 18SrRNA for fungi) enabled creation of biopreparations. The professional biopreparations included a select range of non-pathogenic bacteria and autochthonous fungi, which were able to quickly adapt to the polluted environment, had high biochemical activity in oil hydrocarbons biodegradation, and a wide spectrum of activity.

3. Modification in the autochthonous bacteria-based biopreparation consists of the introduction of selected non-pathogenic fungi species taken from the area under purification. It resulted in an increase in both the effectiveness and activity spectrum of the biopreparation through an increase in biodegradation degree of long-chain aliphatic and aromatic hydrocarbons (BTEX and PAH).

4. The created mathematical model of petroleum hydrocarbon biodegradation enables complete monitoring of the purification process and determination of biodegradation effectiveness for particular hydrocarbon groups with the application of various biopreparations. The model serves as proof that the use of the biopreparation enriched with fungi in the final stage of the purification process brings satisfactory results.

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