

Ph.D. Thesis
Expanded abstract

**Effectiveness of Techniques Enhancing the
Biodegradation of Petroleum Hydrocarbon
Contaminants in Soil from a Waste Pit Area**

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This doctoral dissertation is based on seven thematically coherent scientific publications, the details of which are presented below.

- I. **Katarzyna Wojtowicz**, T. Steliga, P., Kapusta, J. Brzeszcz, T. Skalski, *Evaluation of the Effectiveness of the Biopreparation in Combination with the Polymer γ -PGA for the Biodegradation of Petroleum Contaminants in Soil*, *Materials*, Volume 15(2), 400, 2022.
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- II. **Katarzyna Wojtowicz**, T. Steliga, T. Skalski, P., Kapusta, *Influence of Biosurfactants on the Efficiency of Petroleum Hydrocarbons Biodegradation in Soil*, *Sustainability*, Volume 17(14), 6520, 2025.
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- III. **Katarzyna Wojtowicz**, T. Steliga, P., Kapusta, J. Brzeszcz, *Oil-Contaminated Soil Remediation with Biodegradation by Autochthonous Microorganisms and Phytoremediation by Maize (*Zea mays*)*, *Molecules*, Volume 28(16), 6104, 2023. DOI: 10.3390/molecules28166104
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- IV. **Katarzyna Wojtowicz**, T. Steliga, P., Kapusta, *Evaluation of the Effectiveness of Bioaugmentation-Assisted Phytoremediation of Soils Contaminated with Petroleum Hydrocarbons Using *Echinacea purpurea**, *Applied Sciences (Basel)*, Volume 13(24), 13077, 2023.
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- V. **Katarzyna Wojtowicz**, T. Steliga, J. Brzeszcz, J. Fyda, T. Skalski, P., Kapusta, *Phytoremediation of soil contaminated with petroleum hydrocarbons using the wild plants *Scirpus sylvaticus* and *Cirsium oleraceum*, supported by bioaugmentation*, International Biodeterioration & Biodegradation, Volume 206, 106217, 2026.
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- VI. **Katarzyna Wojtowicz**, T. Steliga, P., Kapusta, J. Brzeszcz, *The Role of Graphene Oxide and Zinc Oxide Nanoparticles in Enhancing the Effectiveness of Phytoremediation of Petroleum Hydrocarbon-Contaminated Soils Using *Lolium perenne**, Molecules, Volume 31(5), 890, 2026. DOI: 10.3390/molecules31050890
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- VII. T. Steliga, **Katarzyna Wojtowicz**, P., Kapusta, J. Brzeszcz, *Assessment of Biodegradation Efficiency of Polychlorinated Biphenyls (PCBs) and Petroleum Hydrocarbons (TPH) in Soil Using Three Individual Bacterial Strains and Their Mixed Culture*, Molecules, Volume 25(3), 709, 2020.
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1 Introduction

Increasing industrialisation, population growth, and high demand for petrochemical products have contributed to intensive economic development, which has simultaneously become one of the main causes of soil pollution and degradation. A particularly significant environmental issue is soil contamination with petroleum hydrocarbons, primarily associated with the extraction, transport, and processing of crude oil (Mekonnen et al., 2024; Ossai et al., 2020; Singha and Pandey, 2021). In addition, the lack of recycling of waste oils and the disposal of hazardous oily waste in landfills without proper management in the 1970s led to the formation of so called historical contamination sites (waste pits), which are particularly difficult to remediate. The presence of petroleum hydrocarbons in soils is associated not only with significant risks to the natural environment, but also to the health of humans and animals (Daâssi & Qabil Almaghribi, 2022). For this reason, recent years have seen intensive research aimed at improving existing methods and developing new approaches for the remediation of soils contaminated with petroleum substances.

A particularly representative example of a mining area contaminated with petroleum hydrocarbons is the Grabownica field, where long term drilling activity resulted in numerous waste pits used in the past for the deposition of drilling waste contaminated with petroleum hydrocarbons (Steliga, 2009). The prolonged storage of these wastes directly within the waste pits led to persistent soil contamination and created a real threat to the soil and groundwater environment, which justified remediation efforts aimed at reducing the level of contamination and restoring the functional properties of the degraded land. Despite the availability of many bioremediation methods used for cleaning soils contaminated with such chemical compounds, in the case of soils originating from waste pit areas of the Grabownica field, their effectiveness proved insufficient.

At the Oil and Gas Institute – National Research Institute, a staged in situ treatment technology for the remediation of drilling waste contaminated with petroleum compounds, deposited in historical waste pits, was developed. This approach enables a substantial reduction in petroleum hydrocarbon concentrations (Steliga et al., 2012, 2018a). However, in several waste pits, after a three year period of bioremediation activities, residual petroleum contamination levels remained in the range of 4,025 to 22,125 mg/kg dry matter, indicating that an adequate degree of remediation had not been achieved. Consequently, there was a need to develop methods enabling more advanced cleanup of waste pit soils, focused on enhancing the degradation efficiency of both aliphatic and aromatic hydrocarbons, as well as reducing soil toxicity.

In this context, it was *hypothesised that deeper remediation of waste pit soils could be achieved through the modification of existing bioremediation techniques and the development of integrated strategies that enhance the efficiency of petroleum hydrocarbon biodegradation.*

The scientific objective of this work was to identify and analyse key parameters enabling a comprehensive assessment of the effectiveness of the proposed soil bioremediation strategies for waste pit sites.

The applied objective was to develop effective and environmentally friendly methods for the remediation of soils contaminated with petroleum hydrocarbons, allowing the restoration of functional properties of degraded areas. Research conducted on real soil samples from waste pits contaminated with aged petroleum hydrocarbons provided reliable results, increasing their potential applicability under industrial conditions in the future.

Specific objectives:

- Evaluation of the effect of additives (γ -PGA and selected biosurfactants) modifying the inoculation process with a biopreparation based on autochthonous bacteria, aimed at increasing the efficiency of petroleum hydrocarbon biodegradation in soil from waste pit sites.
- Determination of the effect of enriching the bacterial biopreparation with selected strains of autochthonous fungi on the intensification of biodegradation processes of aliphatic hydrocarbons and polycyclic aromatic hydrocarbons.
- Introduction of phytoremediation using cultivated plants in combination with bioaugmentation as a strategy enabling deeper remediation of waste pit soils.
- Application of phytoremediation using wild plant species, supported by inoculation with a bacterial biopreparation based on autochthonous bacteria, to achieve deeper remediation of historically contaminated waste pit soils.
- Characterisation of bacterial and ciliate communities in soil following phytoremediation processes using wild plant species.
- Assessment of the effect of selected nanomaterials on the efficiency of bioremediation processes supported by bioaugmentation, and evaluation of their application potential in phytoremediation of waste pit soil.
- Assessment of the influence of polychlorinated biphenyls (PCBs) co-occurrence on the course and efficiency of petroleum hydrocarbon biodegradation.

2 Materials and methods

2.1 Research materials

The research material in articles I–VII consisted of:

- Real soil samples collected from waste pit sites (G6, G11, G44, G70) as well as from an area of natural crude oil seepage, all characterised by high concentrations of petroleum hydrocarbons. In article VII, commercially available PCB-contaminated soil was additionally used.
- Biopreparations based on autochthonous bacteria or a combination of autochthonous bacteria and fungi, as well as individual autochthonous strains including *Mycolicibacterium frederiksbergense* IN53, *Rhodococcus erythropolis* IN129, and *Rhodococcus* sp. IN306 originating from the Negev Desert.
- Additives enhancing contaminant bioavailability: γ -PGA, biosurfactants (rhamnolipids, surfactin, commercially available preparations JBR 425 and JBR 320, and a proprietary PSR mixture), as well as graphene oxide nanoparticles (GO-NP) and zinc oxide nanoparticles (ZnO-NP).
- Phytoremediation plants: *Zea mays*, *Echinacea purpurea*, *Lolium perenne*, *Scirpus sylvaticus*, *Cirsium oleraceum*.

2.2 Research methods

Assessment of the effectiveness of the discussed bioremediation strategies, depending on the article, was based on a diverse set of analytical methods including respirometric, chromatographic, toxicological, microbiological, and biological analyses, which enabled a multifaceted characterization of soil remediation processes in waste pit areas.

3 Effect of γ -PGA on the efficiency of petroleum hydrocarbon biodegradation in waste pit soil supported by bioaugmentation - Article I

Biodegradation using biopreparations based on autochthonous microorganisms is one of the fundamental and widely applied methods for the remediation of soils historically contaminated with petroleum hydrocarbons. Under appropriate biostimulation and control of environmental parameters, this approach enables a substantial reduction in contaminant concentrations (Greco Lucchina et al., 2026; Mekonnen et al., 2024).

In Article I (Wojtowicz et al., 2022), comprehensive studies on the biodegradation of petroleum contaminants were presented for soil from waste pit G11, characterised by high concentrations of TPH (19,774.23 mg/kg dry matter), BTEX (17.35 mg/kg dry matter), and PAHs (27.03 mg/kg dry matter). The soil was subjected to inoculation with biopreparation A1, developed on the basis of autochthonous bacteria, as well as to a modified inoculation procedure involving the additional application of γ -PGA to the soil, with the aim of increasing contaminant bioavailability and consequently improving remediation efficiency.

The study was conducted under both laboratory and semi technical conditions. Laboratory tests included respirometric analyses, on the basis of which the most optimal biopreparation to γ -PGA ratio was selected, equal to 1:1, providing the highest increase in biological activity in the system. This ratio was subsequently applied in soil inoculation in semi technical ex situ windrow experiments, conducted on two experimental piles in order to compare the effectiveness of standard biopreparation inoculation with the modified approach including γ -PGA addition.

The semi technical study included monitoring changes in petroleum hydrocarbon concentrations (n alkanes, BTEX, PAHs) during treatment using chromatographic analyses. It was demonstrated that modification of the inoculation process through the introduction of a γ -PGA solution together with the biopreparation had a measurable effect on the biodegradation efficiency of all petroleum hydrocarbons identified in the soil (Table 1). Furthermore, the identification of isoprenoid hydrocarbons (pristane and phytane), which are poorly biodegradable compounds, enabled an assessment of the effectiveness of the proposed bioremediation strategies based on changes in the n C₁₇/Pr and n C₁₈/Ph ratios.

Tabela 1 Comparison of the biodegradation efficiency of selected groups of petroleum hydrocarbons in soil inoculated with biopreparation A1 and with biopreparation A1 and γ -PGA

Petroleum hydrocarbons	Efficiency of biodegradation in soil inoculated with a biopreparation A1 [%]	Efficiency of biodegradation in soil inoculated with a biopreparation A1 and γ -PGA [%]
TPH	66,03	79,21
Unidentified hydrocarbons	59,49	71,84
\sum nC ₉ –nC ₂₁	86,52 - 94,59	96,57 - 99,55
\sum nC ₂₂ –nC ₃₀	44,62 - 77,77	80,35 - 88,35
\sum nC ₃₁ –nC ₄₀	25,13 - 40,60	46,43 - 65,73
BTEX	80,08	90,19
benzene	91,75	95,89
toluene	95,54	98,89
ethylbenzene	93,15	97,69
xylenes	71,06 – 78,69	85,14 – 89,88
\sum PAHs	38,86	51,18
2 ring PAHs	71,58	81,06
3 ring PAHs	63,73 – 64,44	68,63 – 72,22
4 ring PAHs	32,33 – 42,78	44,65 – 56,24
5 ring PAHs	30,06	40,30 – 43,17
6 ring PAHs	24,62	35,48 – 37,83

The semi technical scale studies presented in Article I were supplemented with toxicological monitoring using Phytotoxkit™, Ostracodtoxkit F™, Microtox® SPT, and Ames tests, enabling assessment of the proposed bioremediation strategies on the soil biocenosis and determination of their environmental safety.

Comprehensive investigations of soil bioremediation from the waste pit presented in Article I demonstrated that the proposed modification of the soil inoculation technique with biopreparation A1, involving the additional introduction of γ -PGA into the soil matrix, may

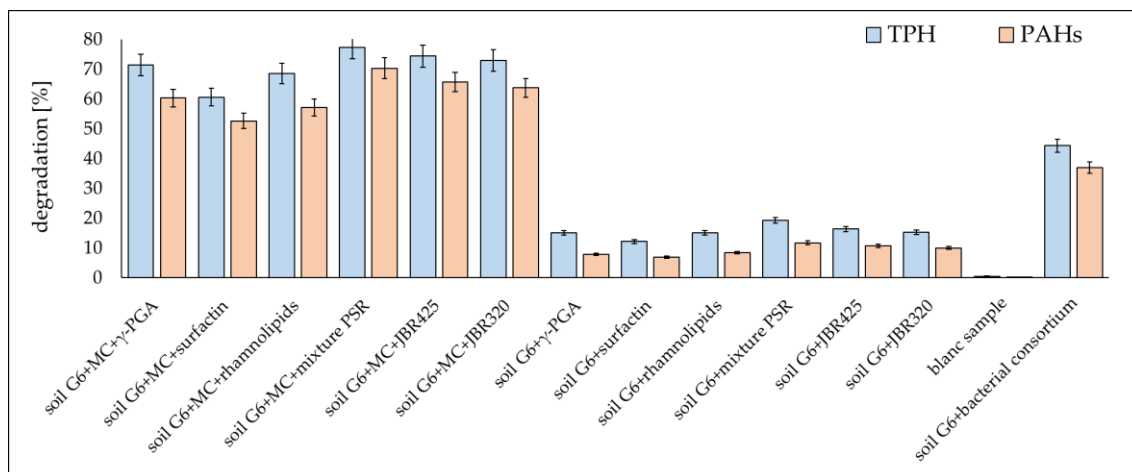
enhance the efficiency of petroleum hydrocarbon biodegradation and improve the overall quality of waste pit soil. The obtained results indicate the potential applicability of this approach under field conditions and provided the basis for further studies aimed at evaluating the effectiveness of inoculation modifications using other biodegradation enhancing agents, including biosurfactants.

4 The role of biosurfactants in improving the efficiency of petroleum hydrocarbon biodegradation in waste pit soil – Article II

Available literature data indicate that the introduction of biosurfactants into the system may improve the efficiency of petroleum hydrocarbon biodegradation in soils. Biosurfactants are biological surface active compounds that reduce interfacial tension, increase hydrocarbon solubility, and facilitate the desorption of contaminants from the soil matrix (Badmus et al., 2021; Zajic et al., 1983). Therefore, in Article II (Wojtowicz et al., 2025) studies were conducted to evaluate the possibility of modifying the inoculation process with a biopreparation through the application of biosurfactants such as JBR 425 and JBR 320 preparations, rhamnolipids, surfactin, γ -PGA, and a proprietary PSR mixture, in order to improve the efficiency of petroleum hydrocarbon biodegradation in waste pit soil.

The article presents comprehensive laboratory-scale bioremediation studies of soil from waste pit G6 subjected, depending on the experimental setup, to bioaugmentation with an autochthonous bacteria based biopreparation MC, modified bioaugmentation with biopreparation MC supplemented with a selected biosurfactant (PSR proprietary mixture, JBR 425, JBR 320, rhamnolipids, surfactin, or γ -PGA), and two biosurfactant concentrations in the inoculation mixture of 1 g/dm³ and 5 g/dm³, as well as treatments involving supplementation with selected biosurfactants at 1 g/dm³ and 5 g/dm³ as control samples.

The conducted studies, using respirometric and chromatographic analyses of soil for the determination of aliphatic hydrocarbons and PAHs, showed that modification of the inoculation technique with a biopreparation based on autochthonous bacteria, involving the introduction of the tested biosurfactants into the system, improved the efficiency of TPH biodegradation by 9.19–21.57% (at a biosurfactant concentration of 1 g/dm³) and 16.31–32.97% (at a biosurfactant concentration of 5 g/dm³), as well as PAHs by 6.7–24.74% (at a biosurfactant concentration of 1 g/dm³) and 15.67–33.35% (at a biosurfactant concentration of 5 g/dm³) compared with bioaugmentation alone (Fig.).



Rys. 1 Comparison of the degrees of TPH and PAH biodegradation in the investigated reaction systems at a biosurfactant concentration of 5 g/dm³ (number of replicates n = 7–10, p < 0.05) (Wojtowicz et al., 2025) .

Respirometric and chromatographic results also demonstrated that among the tested biosurfactants, the highest potential for application in enhancing petroleum hydrocarbon biodegradation in the studied soil was exhibited by the PSR mixture composed of rhamnolipids, surfactin, and γ -PGA.

In addition, Article II assessed the safety of the proposed bioremediation strategies and their impact on reducing soil toxicity. For this purpose, a suite of toxicological tests (Phytotoxkit™, Ostracodtoxkit F™, and Microtox SPT) was applied, enabling toxicity evaluation with respect to organisms representing all trophic levels.

Comprehensive results presented in Article II, including respirometric, chromatographic, and ecotoxicological analyses of soil after experiment completion, demonstrated that modification of the autochthonous bacteria based biopreparation through the addition of biosurfactants improved the efficiency of bioremediation of petroleum contaminated soil and may be applied in the future for further remediation of waste pit sites. Furthermore, based on observations from Articles I and II, integrated bioremediation strategies combining bioaugmentation and phytoremediation were developed with the aim of achieving deeper remediation of waste pit soils.

5 Effect of enrichment of a bacterial biopreparation with selected autochthonous fungal strains on the efficiency of petroleum hydrocarbon biodegradation in waste pit soil and the application of phytoremediation for its further cleanup – Articles III and IV

The composition of a biopreparation is a key factor determining the efficiency of petroleum hydrocarbon biodegradation in contaminated soils. Available literature indicates that enrichment of a bacterial autochthonous biopreparation with selected fungal strains may significantly enhance this process due to the ability of fungi to produce extracellular enzymes

and to degrade a wide spectrum of complex organic compounds, including petroleum hydrocarbon fractions (Steliga et al., 2018; Soto-Mancilla et al., 2026).

In Article III (Wojtowicz et al., 2023b), results are presented on the biodegradation of aliphatic hydrocarbons and PAHs in soil collected from a waste pit area, which was subjected to inoculation with two types of autochthonous microorganism based biopreparations, namely a bacterial biopreparation B1 and a bacterial–fungal biopreparation B2. The *ex situ* windrow experiments, supported by chromatographic and toxicological analyses, enabled a comparison of the effectiveness of the applied inoculation mixtures in the bioremediation process of waste pit soil.

It was demonstrated that the proposed modification consisting of enriching the autochthonous bacteria based biopreparation with selected autochthonous fungal strains improved the biodegradation efficiency of all petroleum hydrocarbons. The greatest effect was observed for hydrocarbons with carbon chain lengths of n-C₂₂–n-C₃₀, heavy hydrocarbons with carbon chain lengths of n-C₃₁–n-C₃₆, as well as PAHs (Table 2). Furthermore, ecotoxicological assessment of the soil using the standard toxicological test battery comprising Phytotoxkit™, Ostracodtoxkit F™, and Microtox SPT confirmed the effectiveness of the proposed biopreparations in improving soil functional properties and demonstrated their environmental safety.

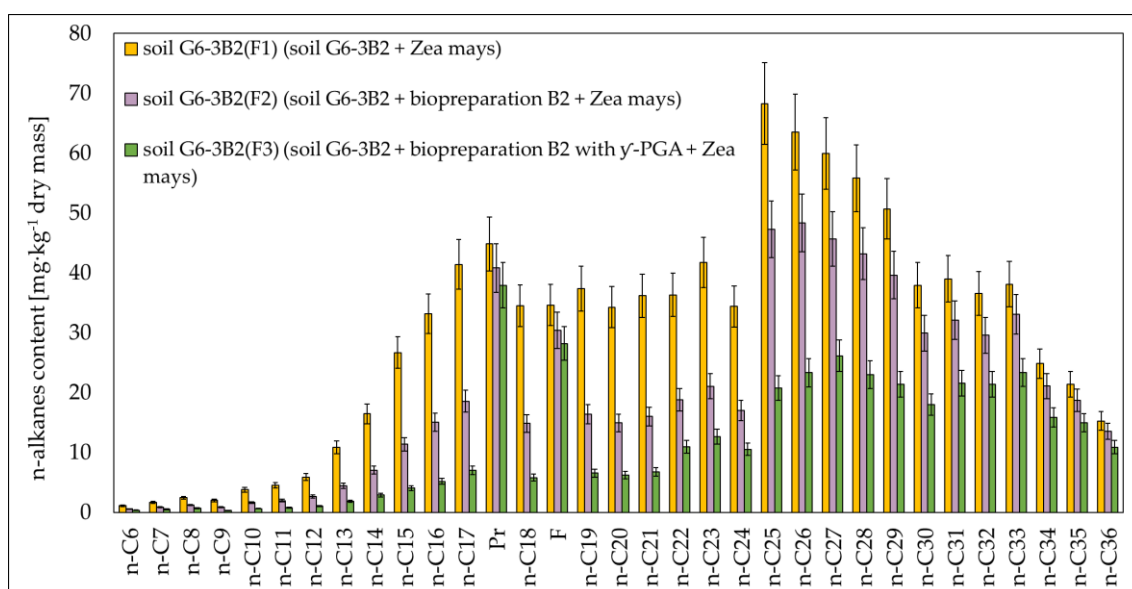
Tabela 2 Comparison of the biodegradation efficiency of selected groups of petroleum hydrocarbons in soil inoculated with biopreparations B1 and B2.

Petroleum hydrocarbons	Biodegradation efficiency in soil inoculated with biopreparation B1 [%]	Biodegradation efficiency in soil inoculated with biopreparation B2 [%]
TPH	31,85	41,67
Unidentified hydrocarbons	31,44	37,97
nC ₆ –nC ₉	53,96 – 63,99	78,50 – 65,17
nC ₁₀ –nC ₂₁	34,78 – 64,58	54,08 – 78,00
nC ₂₂ –nC ₃₀	10,25 – 32,44	22,05 – 47,63
nC ₃₁ –nC ₄₀	9,23 – 10,67	14,77 – 18,65
∑PAHs	27,41	34,73
2 ring PAHs	34,49	43,81
3 ring PAHs	24,40 – 30,82	30,12 – 38,05
4 ring PAHs	15,22 – 21,71	18,79 – 26,80
5 ring PAHs	11,42 – 11,46	16,56 – 16,61
6 ring PAHs	7,96 – 8,37	14,77 – 15,27

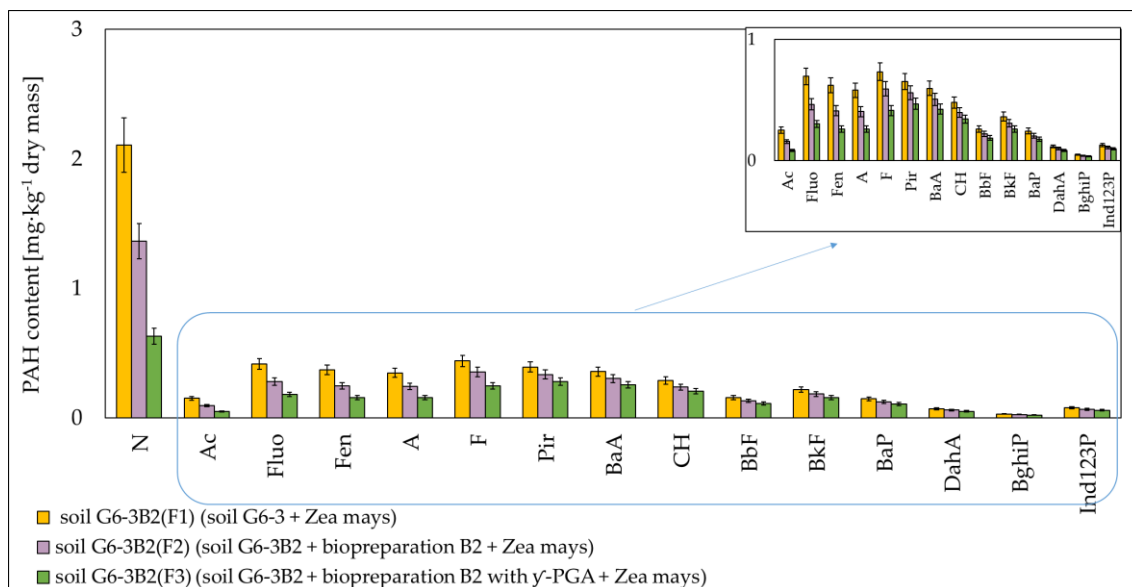
An important aspect of the studies presented in Articles III and IV was the introduction of another bioremediation technique, namely phytoremediation, which involves the use of plants together with microorganisms inhabiting the rhizosphere to uptake, accumulate, or biodegrade toxic substances (Barathi & Vasudevan, 2001; Tonelli et al., 2022). Phytoremediation experiments were conducted under semi technical conditions using a pot cultivation system and two plant species: *Zea mays* (Article III) and *Echinacea purpurea* (Article IV). As in the previous articles, the results were based on chromatographic analyses

and toxicological testing of soil samples. However, the scope of the study was expanded to include plant morphological analysis and, in Article IV, determination of petroleum contaminant concentrations in the roots and shoots of *Echinacea purpurea*.

The phytoremediation study presented in Article III demonstrated that combining bioaugmentation with the bacterial–fungal biopreparation B2 and phytoremediation using *Zea mays* resulted in high biodegradation rates of petroleum contaminants in waste pit soil, reaching 49.08% for TPH and 40.74% for PAHs after six months of treatment. Furthermore, it was shown that the inoculation modification strategy described in Article I, involving the additional introduction of a γ -PGA solution into the system, when combined with phytoremediation, enabled even higher remediation efficiency. This was confirmed by the biodegradation rates of TPH and PAHs achieved at the end of the experiment, amounting to 65.35% and 60.80%, respectively. The chromatographic analyses presented in the article further indicated that phytoremediation positively influenced the biodegradation efficiency of both aliphatic hydrocarbons (Fig. 2) and PAHs (Fig. 3), demonstrating its potential application for the further remediation of soils in waste pit areas.

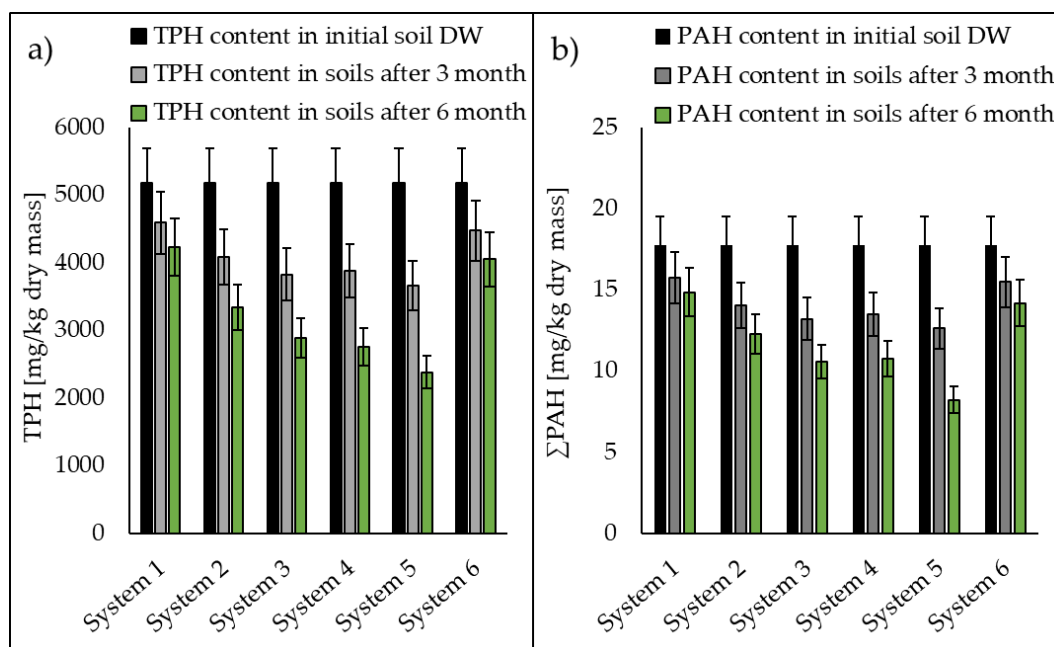


Rys. 2 Comparison of n-alkane concentrations determined in soil subjected to phytoremediation using *Zea mays* (Wojtowicz et al., 2023b).



Rys. 3 Comparison of PAH concentrations determined in soil subjected to phytoremediation using *Zea mays* (Wojtowicz et al., 2023b)

The phytoremediation studies discussed in Article IV (Wojtowicz et al., 2023a), conducted using *Echinacea purpurea*, confirmed that combining phytoremediation with an appropriately developed biopreparation based on autochthonous microorganisms enables high biodegradation efficiencies for both petroleum hydrocarbons and PAHs in waste pit soil. Furthermore, additional enrichment of the system with substances that improve contaminant bioavailability (γ -PGA) allowed for a further increase in remediation efficiency (Fig. 4).



Rys. 4 Determined concentrations of (a) TPH and (b) PAHs in waste pit soil subjected to phytoremediation using *Echinacea purpurea* under different soil amendment variants (System 1, no inoculation; System 2, inoculation with biopreparation B1; System 3, inoculation with biopreparation B1 and γ -PGA; System 4, inoculation with biopreparation B2; System 5, inoculation with biopreparation B2 and γ -PGA; System 6, inoculation with γ -PGA) (Wojtowicz et al., 2023a).

Expanding the scope of analyses to include the determination of TPH and PAH concentrations in the roots and shoots of *Echinacea purpurea* confirmed the active involvement of plants in the remediation of hydrocarbon contaminated soils. It was demonstrated that small amounts of petroleum contaminants can be taken up from the soil by phytoremediation plants and transported to plant tissues, where they may accumulate or undergo further biochemical transformations. The extent of contaminant accumulation in plant tissues was found to depend on the soil inoculation method applied.

The proposed phytoremediation approach supported by bioaugmentation was further verified using toxicological tests performed on soil after completion of the remediation process. The results confirmed a correlation between the reduction in contaminant concentrations and the decrease in soil toxicity.

The phytoremediation results presented in Articles III and IV demonstrated that the efficiency of bioremediation of soils originating from waste pit areas can be significantly enhanced through the combined application of multiple bioremediation techniques, including bioaugmentation, phytoremediation, and modification of the inoculation process using additives such as γ -PGA. The obtained results provided the basis for further studies focused on the application of phytoremediation supported by bioaugmentation as a strategy for achieving deeper remediation of soils from waste pit sites.

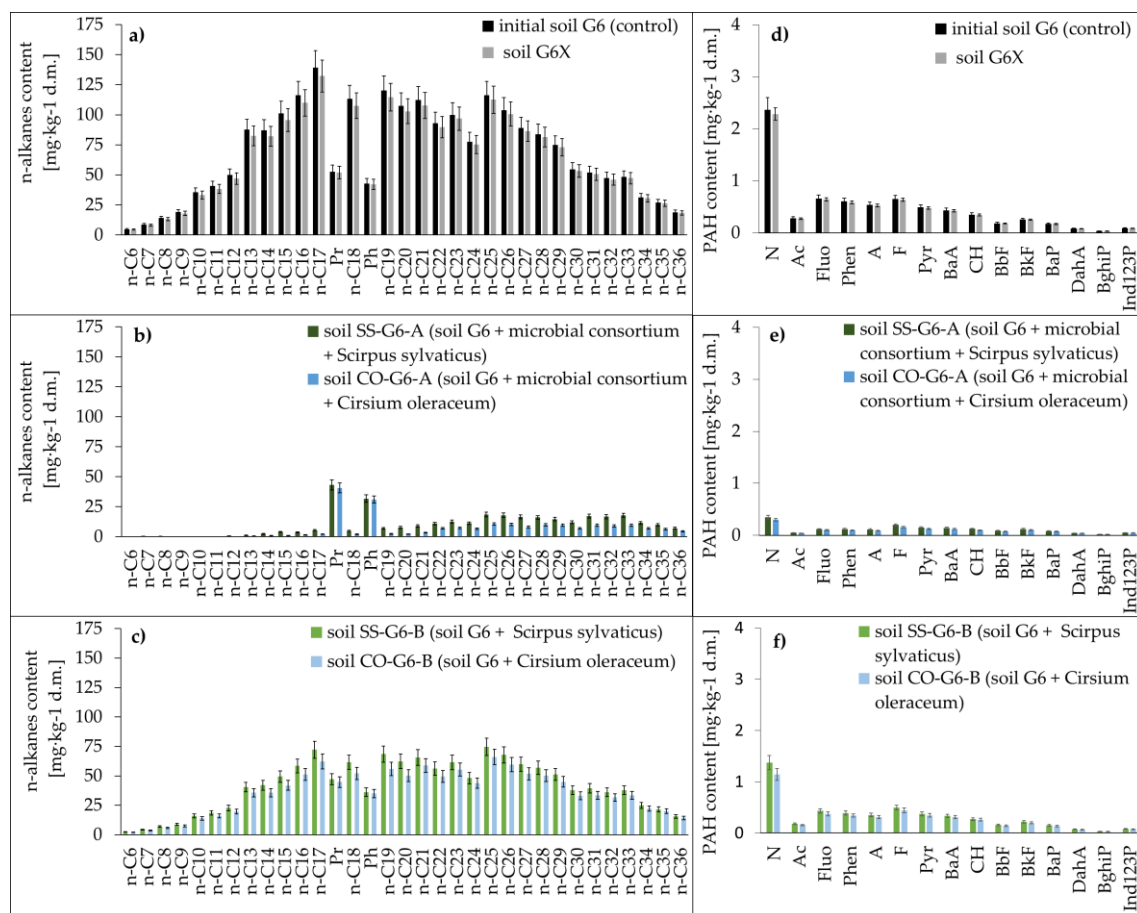
6 Application of Wild Plant Species to Enhance the Efficiency of Phytoremediation of Soils Contaminated with Petroleum Hydrocarbons – Article V

The results presented in Articles III and IV demonstrated that combining phytoremediation with soil bioaugmentation using a biopreparation based on autochthonous microorganisms enables high efficiency of bioremediation of waste pit soils. In Article V (Wojtowicz et al., 2026a), the applicability of this approach for further remediation of waste pit areas was continued; however, wild plant species, namely *Scirpus sylvaticus* and *Cirsium oleraceum*, collected from a natural crude oil seepage site, were used as phytoremediation plants.

The phytoremediation experiments described in Article V were conducted under semi technical conditions, and the assessment of the effectiveness of the proposed bioremediation strategies was based on chromatographic analyses of soil as well as plant roots and shoots with respect to petroleum hydrocarbon content, biomass and plant growth rate measurements, toxicological test results, and analysis of bacterial and ciliate biodiversity in the remediated soils.

Chromatographic analyses of soil after experiment completion demonstrated that the use of wild vegetation adapted to growth in oil contaminated environments in phytoremediation supported by bioaugmentation enables higher remediation efficiency of waste pit soils compared to the use of *Zea mays* and *Echinacea purpurea*. The obtained biodegradation rates after completion of the experiment reached 89.47% for TPH and 75.52% for PAHs in soil inoculated with biopreparation MC and planted with *Scirpus sylvaticus*, and 93.96% for TPH

and 79.55% for PAHs in soil inoculated and planted with *Cirsium oleraceum*. These results indicate high biodegradation efficiency for both aliphatic and aromatic hydrocarbons. Additionally, detailed analysis of individual petroleum hydrocarbon fractions confirmed satisfactory removal efficiency for all n alkanes and PAHs present in the soil (Fig. 5).

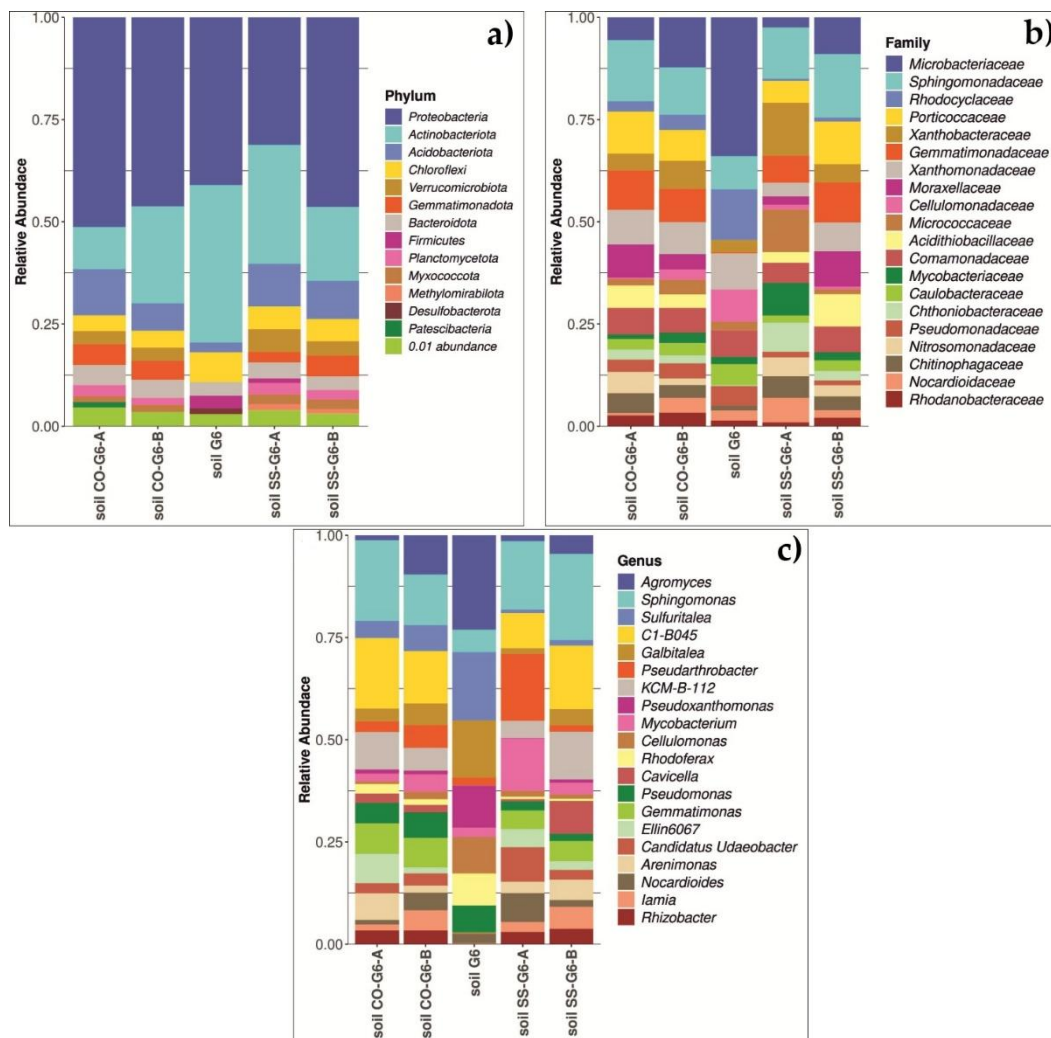


Rys. 5 Determined concentrations of (a, d) initial and control soil, (b, e) soil subjected to phytoremediation supported by bioaugmentation, and (c, f) soil subjected to phytoremediation only, for n-alkanes and PAHs (Wojtowicz et al., 2026a).

In addition, morphological analysis of the phytoremediation plants revealed correlations between plant growth and the efficiency of petroleum hydrocarbon biodegradation in soil. It was shown that the growth rate and biomass increase of *Scirpus sylvaticus* and *Cirsium oleraceum* were significantly higher in inoculated soils than in soils not subjected to biopreparation treatment. Furthermore, chromatographic analyses of roots and shoots of *Scirpus sylvaticus* and *Cirsium oleraceum* with respect to TPH and PAH content enabled assessment of the influence of morphological and anatomical plant traits on the uptake and transport of contaminants from soil into plant tissues. It was found that the concentrations of TPH and PAHs in the roots and shoots of *Cirsium oleraceum* grown under conditions comparable to *Scirpus sylvaticus* were markedly higher, indicating a greater capacity of this species for contaminant accumulation and translocation. These differences may be related to variations in root system architecture and species specific physiological characteristics. Moreover, the results suggest that soil inoculation with a biopreparation enhanced contaminant bioavailability, thereby facilitating their uptake by plants from the substrate.

An integral part of verifying the effectiveness of the proposed bioremediation strategy was the assessment of changes in the ecotoxicological properties of soil using Phytotoxkit™, Ostracodtoxkit F™, Microtox SPT, and MARA tests. The obtained results confirmed a significant reduction in the adverse effects of the remediated soil on test organisms, as indicated by a decrease in toxicity units (TU) observed across all analysed parameters.

Additionally, in Article V, to enable a comprehensive evaluation of both phytoremediation and bioaugmentation supported phytoremediation processes, the basic research scope presented in the other articles was extended to include characterization of soil microorganisms. Microbiological analyses revealed changes in soil microbial diversity after the remediation process, varying depending on the applied phytoremediation variant and observable at the genus, family, and gene levels (Fig. 6).



Rys. 6 Effect of phytoremediation and bioaugmentation on the relative abundance of bacterial taxa: (a) phylum, (b) family (20 most abundant), (c) genus (20 most abundant) (Wojtowicz et al., 2026a).

It was also demonstrated that bacteria absent both in the initial soil and in the applied biopreparation appeared in the remediated soils. This may indicate that, along with the transfer of phytoremediation plants from a crude oil contaminated area, microorganisms associated with their rhizosphere were introduced into the treated soil. The additional introduction of

microorganisms originating from an oil contaminated environment may have been one of the factors contributing to the enhanced efficiency of petroleum hydrocarbon biodegradation. Moreover, the assessment of ciliate diversity and abundance in soils after treatment enabled not only the evaluation of remediation effectiveness using microbiological indicators, but also the identification of potential bioindicators of ecosystem recovery and soil system stability.

The comprehensive results of studies on phytoremediation supported by bioaugmentation presented in Article V enabled verification of the effectiveness of the proposed bioremediation strategy in relation to soils contaminated with petroleum products. It was demonstrated that a key factor determining process efficiency was the use of plant species naturally occurring in oil contaminated environments, which were characterised by a high degree of adaptation to environmental conditions and potentially promoted the development of rhizospheric microorganisms. Phytoremediation performed without bioaugmentation showed limited effectiveness, whereas its combination with bioaugmentation led to a marked intensification of biodegradation processes, improved hydrocarbon removal efficiency, and reduced toxicity of the treated soils. The obtained results confirm the validity of the synergistic use of plants and microorganisms in remediation processes and indicate the high application potential of the developed strategy under field conditions

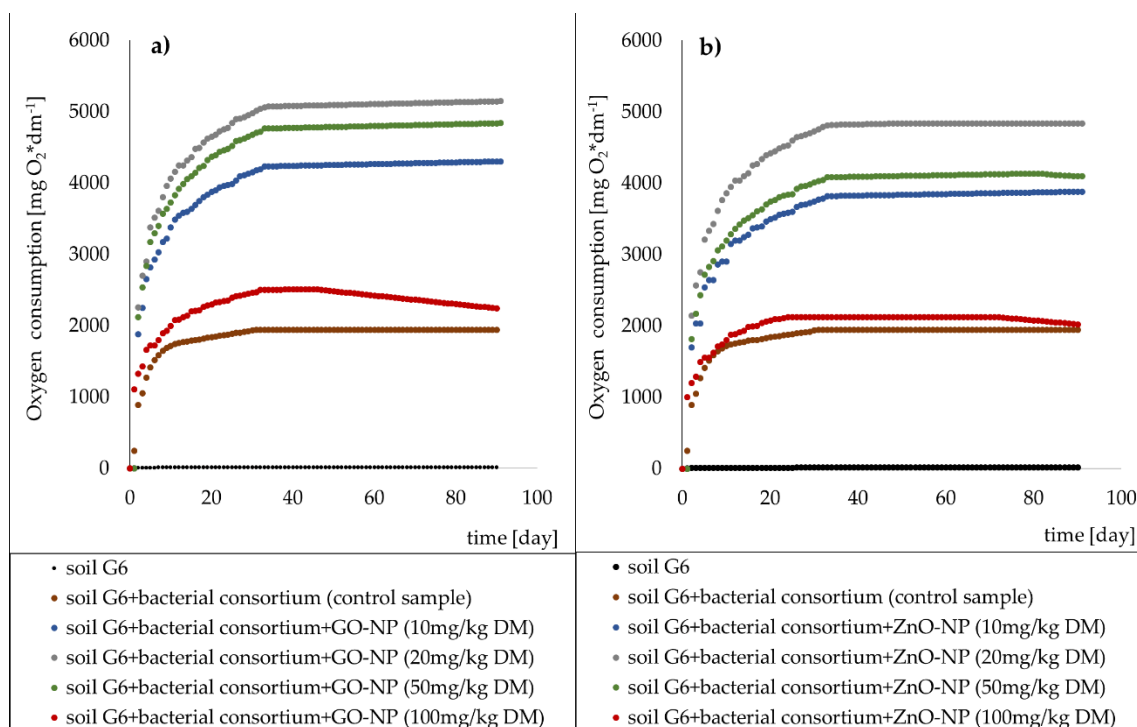
7 Effect of nanomaterials on the efficiency of bioremediation of waste pit soil – Article VI

Currently, an increasing number of studies in the field of soil bioremediation indicate that nanomaterials, due to their physicochemical properties, can significantly improve the efficiency of remediation of soils contaminated with petroleum hydrocarbons. Their influence on the mobility of organic contaminants in the soil environment, enhancement of bioavailability, mitigation of environmental stress, and modification of nutrient availability has been demonstrated.

Article VI (Wojtowicz et al., 2026b) presents the results of studies on the biodegradation of petroleum contaminants in waste pit soil subjected to inoculation with biopreparation MC and enrichment with nanomaterials, namely graphene oxide (GO-NP) or zinc oxide (ZnO-NP). The experiments were conducted at laboratory scale using respirometric and chromatographic analyses. A key aspect of the laboratory studies was the application of different nanomaterial dosages for soil amendment, which enabled assessment of their impact on biodegradation processes and determination of the dosage ensuring the highest efficiency of petroleum hydrocarbon removal.

The results of respirometric (Fig. 7) and chromatographic analyses confirmed that the combination of inoculation with soil enrichment using the tested nanomaterials enabled a significant improvement in the efficiency of TPH and PAH biodegradation, which, depending on the nanomaterial dose, ranged from 50.41–70.47% and 40.48–56.19% for GO-NP, respectively, and from 49.69–67.68% and 40.45–54.94% for ZnO-NP, respectively. Furthermore, the laboratory scale studies enabled the selection of an optimal nanomaterial dose

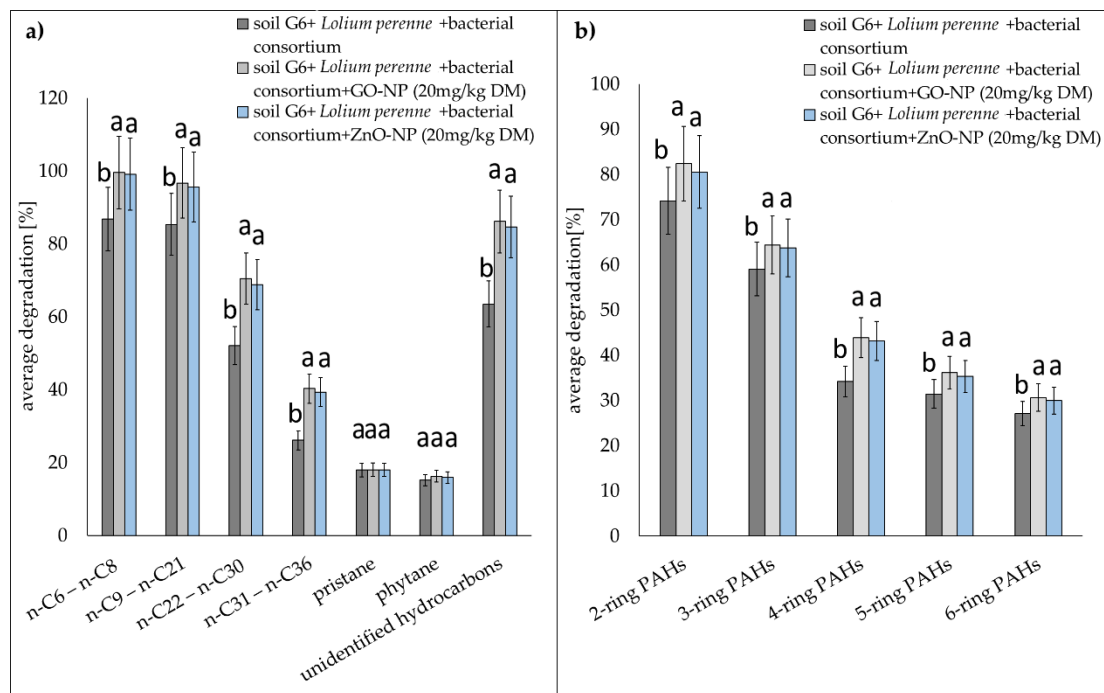
of 20 mg/kg dry matter, which was subsequently used in further experiments combining phytoremediation, bioaugmentation, and soil enrichment with nanomaterials



Rys. 7 Dependence of oxygen consumption [mg/dm^3] on time in G6 soil inoculated with biopreparation and enriched with (a) GO-NP and (b) ZnO-NP (Wojtowicz et al., 2026b).

The phytoremediation studies supported by bioaugmentation and nanomaterials discussed in Article VI were conducted using *Lolium perenne* as the phytoremediation plant. The evaluation of the proposed bioremediation strategy was based on chromatographic analyses of soil for aliphatic hydrocarbons and PAHs, as well as determination of these compounds in the roots and shoots of *Lolium perenne*, which additionally enabled assessment of their distribution within the soil–plant system. Furthermore, plant biomass analysis after phytoremediation and ecotoxicological assessment of the soil were performed, allowing for a comprehensive evaluation of the effectiveness of the applied remediation strategy.

The results demonstrated that the combination of phytoremediation with bioaugmentation and soil enrichment with nanomaterials resulted in a further increase in the efficiency of waste pit soil remediation. The biodegradation rates of TPH and PAHs in soil planted with *Lolium perenne* and inoculated with a biopreparation were 63.22% and 61.86%, respectively, whereas enrichment with GO-NP increased TPH degradation to 81.85% and PAH degradation to 73.19%. The application of ZnO-NP resulted in corresponding values of 80.39% and 70.66%, indicating a significant enhancement of the phytoremediation process under nanomaterial amended conditions.



Rys. 8 Mean biodegradation degree of selected groups of (a) aliphatic hydrocarbons and (b) PAHs in G6 soil subjected to phytoremediation using *Lolium perenne* under different soil amendment variants. Different lowercase letters indicate statistically significant differences between variants according to one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test ($p < 0,05$) (Wojtowicz et al., 2026a).

The results of petroleum hydrocarbon biodegradation efficiency correlated with the results of morphological analysis and biomass assessment of *Lolium perenne*, which were consistent with those presented in Articles IV and V, confirming that higher biodegradation efficiency was accompanied by better plant development and increased biomass production.

Analysis of roots and shoots of *Lolium perenne* with respect to TPH and PAH content showed higher concentrations of petroleum contaminants in plants grown in soils subjected to inoculation and nanomaterial enrichment. This confirmed the hypothesis that nanomaterials may enhance the bioavailability of contaminants for phytoremediation plants.

The effectiveness of the proposed bioremediation strategy for waste pit soils involving soil enrichment with nanomaterials was verified using toxicological analyses, which were particularly important in the context of phytoremediation supported by nanomaterials (GO-NP, ZnO-NP) due to their still insufficiently understood impact on soil environments and other living organisms.

The results presented in Article VI indicate that the application of nanomaterials at appropriately selected doses, as a supplement to bioaugmentation and phytoremediation supported by bioaugmentation, may represent an interesting alternative to traditional biological methods for the remediation of waste pit soils. The obtained results demonstrate the high potential of this strategy, reflected in increased efficiency of petroleum hydrocarbon biodegradation and improved quality parameters of remediated soils. At the same time, due to the relatively novel application of nanomaterials in such systems and the lack of complete knowledge regarding their long term effects in soil environments, further research is warranted

on their field scale application, including their stability, interactions with soil biota, and potential long term environmental impacts.

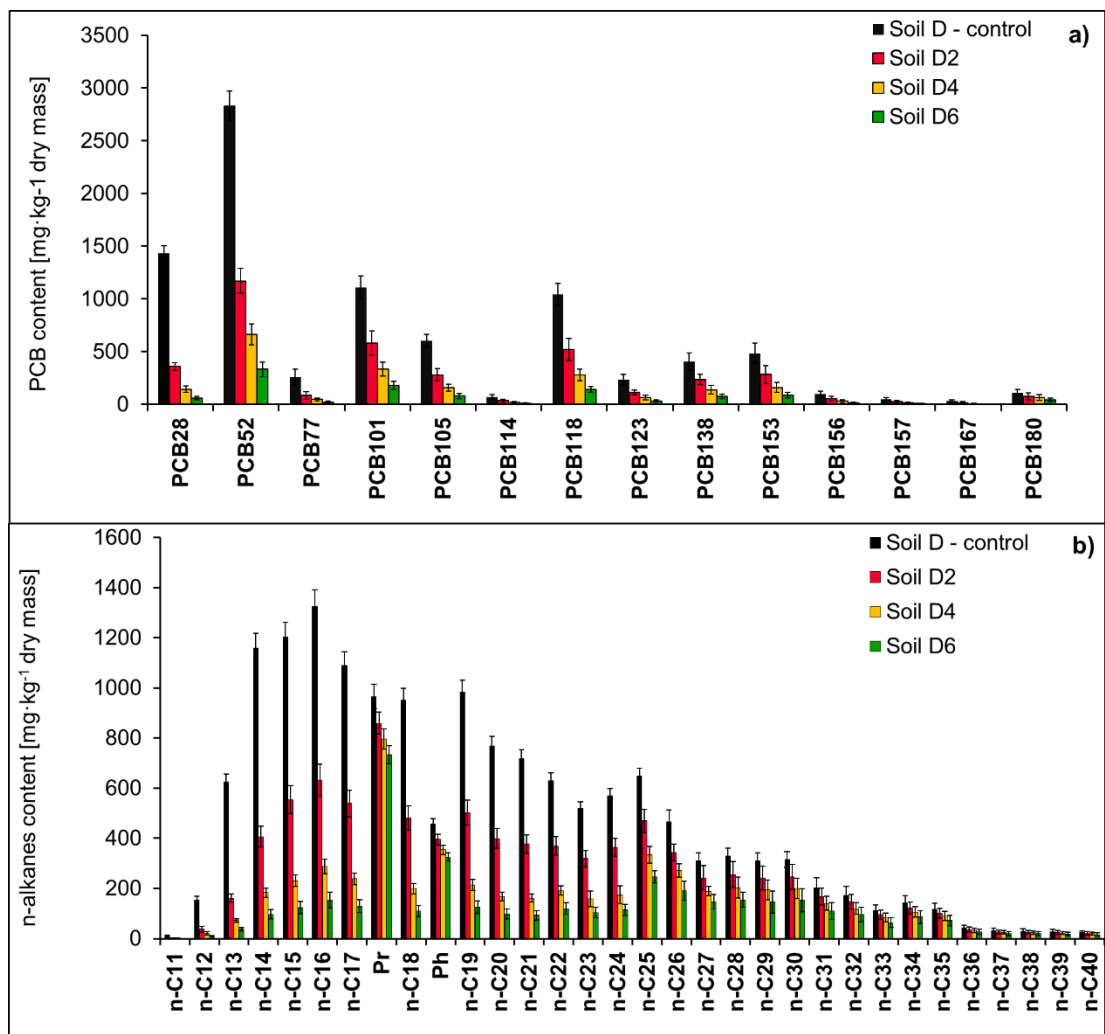
8 Efficiency of petroleum hydrocarbon biodegradation in the presence of co-contamination with polychlorinated biphenyls (PCBs) – Article VII

Literature on the bioremediation of soils historically contaminated as a result of mining activities indicates that the presence of polychlorinated biphenyls (PCBs) in soil may inhibit the activity of petroleum hydrocarbon degrading bacteria due to their low bioavailability and toxic effects on soil microbiota. Therefore, Article VII was designed to experimentally evaluate the effectiveness of bioaugmentation in enhancing the biodegradation of petroleum hydrocarbons in soils co-contaminated with PCBs.

The biodegradation studies presented in Article VII (Steliga et al., 2020) were conducted at laboratory and semi technical scale using an *ex situ* windrow system. Laboratory experiments carried out on three soil types, i.e. soil contaminated exclusively with PCBs, soil contaminated with petroleum hydrocarbons, and soil co-contaminated with petroleum hydrocarbons and PCBs, using respirometric and chromatographic analyses, demonstrated varying effectiveness of the applied inoculation variants depending on the type of contamination. It was shown that in soils co-contaminated with different pollutant classes (in this case TPH and PCBs), the use of a biopreparation M1 based on multiple bacterial strains with a broad metabolic spectrum is recommended. This formulation was selected for further *ex situ* windrow studies.

Monitoring studies conducted at semi technical scale enabled assessment of the biodegradation efficiency of both petroleum hydrocarbons and PCBs at successive stages of the remediation process, as well as evaluation of the impact of the applied bioremediation strategy on the improvement of soil quality (Fig. 9). It was shown that inoculation of soil with biopreparation M1 resulted in a significant reduction in contaminant concentrations, reaching 70.8% for TPH and 84.5% for PCBs after six months. Moreover, calculated biodegradation rate constants for individual contaminant groups confirmed the high effectiveness of the applied biopreparation in supporting the remediation of waste pit soil co-contaminated with petroleum hydrocarbons and PCBs.

Toxicological tests (Phytotoxkit™, Ostracodtoxkit F™, Microtox® SPT, MARA, and Ames) demonstrated a reduction in soil toxicity as a result of the applied bioremediation strategy, with no formation of toxic intermediate metabolites detected under the experimental conditions. These results provide an important complement to the assessment of remediation efficiency, particularly in light of the still limited knowledge regarding the effects of metabolites formed during the degradation of TPH and PCBs on living organisms



Rys. 9 Comparison of concentrations of (a) PCB congeners and (b) n-alkanes determined in non-sterile soil D subjected to inoculation with biopreparation M1 (*Steliga et al., 2020*).

The studies presented in Article VII complement the previously discussed research on the efficiency of bioremediation of soils from waste pit areas, indicating that bioaugmentation using a suitably selected biopreparation based on bacteria with broad degradative potential represents a promising approach for the remediation of soils co-contaminated with different groups of organic compounds.

9 Summary

This doctoral dissertation presents and analyzes various approaches aimed at enhancing the efficiency of petroleum hydrocarbon biodegradation in soils originating from waste pit sites. A key aspect of the work is an interdisciplinary approach to the investigated problem, integrating drilling engineering, environmental engineering, chemical analytics, microbiology, and ecotoxicology. This enabled a comprehensive treatment of the issue and a multi-faceted evaluation of the effectiveness of the proposed bioremediation strategies.

The research results presented in the articles made it possible to achieve all the objectives of the doctoral thesis and to formulate the most important conclusions:

- Increasing the efficiency of petroleum hydrocarbon biodegradation can be achieved both through modification of the bioaugmentation process and through the combination of different bioremediation techniques.
- Modification of the bioaugmentation process using additives that enhance contaminant bioavailability, such as γ -polyglutamic acid (γ -PGA) or biosurfactants, represents an effective way to intensify petroleum hydrocarbon biodegradation in waste pit soils.
- The combination of phytoremediation with bioaugmentation may serve as an effective method for “polishing” petroleum contaminated soils originating from waste pit areas.
- Higher remediation efficiency of waste pit soils can be achieved by using wild plant species naturally occurring in oil contaminated environments, such as *Scirpus sylvaticus* and *Cirsium oleraceum*, compared to cultivated plants such as *Zea mays* and *Echinacea purpurea*.
- Soil enrichment with nanomaterials may represent an effective approach to enhance both bioaugmentation and phytoremediation supported by bioaugmentation, leading to increased efficiency of petroleum hydrocarbon biodegradation in soils.
- Bioremediation of soils co-contaminated with different groups of organic pollutants should employ biopreparations based on microorganisms with broad metabolic capabilities, capable of simultaneous degradation of compounds with different chemical structures.
- Toxicological tests (Phytotoxkit™, Ostracodtoxkit F™, Microtox SPT, MARA, and Ames) confirmed that reductions in contaminant concentrations are accompanied by a decrease in soil toxicity.
- The comprehensive evaluation of the proposed strategies for enhancing petroleum hydrocarbon biodegradation in waste pit soils confirms their application potential and supports further research aimed at field scale implementation of these solutions.

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