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Influence of nZnO on Enzyme-mediated PAH-removal from Contaminated Soil

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Abstract

Polycyclic aromatic hydrocarbons are persistent and carcinogenic pollutants that can accumulate in the soil. In order to eliminate them, the implementation of enzyme-based bioremediation is a viable solution. Recently, there has been growing interest in nano-bioremediation, the combined use of enzymes and nanoparticles. In this study, we investigated the potential of nZnO at concentrations of 200 and 1000 mg/kg to enhance the efficiency of gentisate dioxygenase and catalase peroxidase enzymes for PAH degradation. Our results showed that nZnO doses had a positive effect on the degradation of PAHs by enzymes. The a-gentisate 1,2-dioxygenase with 200 mg/kg nZnO proved to be the most efficient for PAH removal, especially for naphthalene, as in this case it had 93.5% removal efficiency. Although the complex mechanisms behind the effects remain to be explored, our results indicate the synergistic potential of using nZnO and enzyme-based bioaugmentation in combination to achieve effective removal of specific PAH contaminants.

Keywords

bioremediation, enzymatic biodegradation, nano zinc oxide, polycyclic aromatic hydrocarbons

1 Introduction

The prevalence of polycyclic aromatic hydrocarbons (PAHs) as a result of long-term anthropogenic activities has led to their widespread contamination of the environment on a global scale. These pollutants are produced mainly by the incomplete combustion of fossil fuels [1–3], automobile exhaust [4] and waste treatment [5, 6]. A wide range of PAHs have been shown to have toxic effects, including mutagenicity, carcinogenicity and teratogenicity [7]. PAHs are highly persistent due to their toxicity and biomagnification potential [2, 8]. They can bind to soil particles and have the potential to be transported to the aquatic environment, such as to groundwater, rivers and sediments, where they can accumulate in higher concentration [8–11].

Several techniques can be used to treat soils to reduce contaminant levels, including technologies based on physical, chemical, thermal and biological processes. Soil washing (solvent extraction) [12], phytoremediation, photocatalytic degradation, electrokinetic remediation [13] and chemical oxidation [14] are the most common methods, furthermore PAHs can be eliminated with high efficiency with thermal remediation techniques [14, 15].

In addition, biodegradation-based bioremediation can also be used as an environmentally friendly method to reduce the concentration of PAH pollutants in soil [13]. The biodegradation performance of the contaminated site can be considerably improved by addition of microorganisms and/or enzymes [16], this technique is called bioaugmentation [17]. Genetically engineering bacteria to have high PAH biodegradation efficiency is possible through advanced metagenomics and recombinant DNA technology [18].

The enzyme-based bioremediation is an environmentally friendly, effective and less expensive method to reduce the recalcitrant xenobiotics from the environment [19].

Recently, novel methods using nanomaterials have emerged. These technologies are promising treatments to reduce the risk of various contaminants [20]. Nanomaterials, such as nanofibers and nanoparticles (NPs), have significant potential to improve the efficiency of bioremediation processes [21] by enhancing microbial activity and thus accelerating the degradation process [22]. The combined use of nanomaterials and/or nanotechnology with bioremediation for their synergistic

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effect to remove contaminants is named nano-bioremediation [22]. According to Cecchin et al. [23] nano-bioremediation is a process where NPs and microorganisms or plants are used to eliminate pollutants, while others specified it by the organism applied for the remediation, thus the techniques are called as phyto-nanoremediation, zoo-nanoremediation, and microbial nanoremediation [24, 25]. Metal oxide-based nanoparticles have demonstrated remarkable capabilities by removing pollutants [26]. The use of nanoparticles in the adsorption of PAH compounds has garnered notable interest, owing to its expedited removal rates and treatment efficacy [27]. Moreover, Ramezani et al. [28] interpreted nano-bioremediation as a method to remove environmental pollutants (e.g. heavy metals, organic and inorganic contaminants) from contaminated sites by using nanoparticles and/or nanomaterials produced by bacteria. However, whichever term is used for bio-nanoremediation, it is an environmentally friendly approach, which has a huge potential to reduce the concentration of contaminants economically and with low toxic consequences compared to physicochemical techniques, which are limited by high costs and monitoring difficulties [29–31].

PAH elimination by nanomaterial-assisted bioremediation is a viable option, thus several nanomaterials (including zinc oxide and titanium dioxide), as well as metal-organic frameworks (MOFs) have been reported to enhance the PAH removal efficacy [20, 32–34]. Brindhadevi et al. [35] detected that 79% of pyrene was degraded within 60 min of treatment when applying NiO nanoparticles. McQueen et al. [36] observed relatively rapid PAH removal with 3D printed PLA-TiO_2 treatments achieving degradation half-lives within ~6 to ~24 h. In another study [37], the addition of iron nanoparticles increased both bacterial biomass and PAHs adsorption over their surface. However, the interaction between microbes and nanoparticles in the adsorption of PAH pollutants is complex, mainly related to the molecular structure of PAHs [21]. There are a number of strategies for nano-bioremediation, including NP-enhanced microbial activity, microbial cells and enzymes immobilized by NPs, NP-mediated electron transfer, and integrated nano-biodegradation, which is defined as the combined action of nanomaterials and biodegradation pathways for comprehensive PAH remediation [21].

On the other hand, using nanomaterials in soil remediation may cause controversial effects. Ge et al. [38] reported that TiO_2 and ZnO NPs endangered the soil microbiota, since the microbial abundance and biomass were diminished by them. Therefore, it is inevitable to evaluate the potential negative impacts of nanomaterials on the environment, as on soil organisms, vegetation, and human welfare as well. Ecological and soil-related effects of nanomaterials must be considered when designing remediation technologies. The recyclability of nanomaterials may also be an important parameter. However, the reuse of nanomaterials applied in soil remediation is difficult to achieve. Though, certain nanomaterials (e.g. Fe_3O_4 and iron-containing nanocomposites) have been described as having advantageous properties, such as being recyclable [34].

Given both the promising potential and the possible ecological risks of nanomaterials, it is essential to assess their effects in remediation systems.

The hypothesis of our research was that the presence of nZnO, depending on its concentration in the contaminated medium, may enhance or possibly inhibit the efficiency of a given enzyme-based bioremediation process, as well as the activity and functional diversity of the soil microbial community. This, in turn, may also affect the efficiency of PAH degradation directly and indirectly. In our previous study [39], two novel enzymes (gentisate dioxygenase and catalase peroxidase enzymes) were cloned and applied in short-term soil bioremediation microcosms. Thus, the key objectives of this study were to evaluate the effect of nano zinc oxide (nZnO) on the efficacy of these novel enzymes aiming at degrading PAH pollutants in soil, which have been proven to effectively degrade these contaminants, and to assess the impact of nZnO on the indigenous soil microbiota through microbial activity and diversity.

2 Materials and methods

2.1 Contaminated matrix and analytical method

The contaminated matrix was aleuritic sand with poor mechanical composition obtained from Elgoscar Environmental Technology Plc., Hungary [39], which was further artificially spiked with naphthalene, phenanthrene and pyrene. These pollutants were spiked into the matrix using gasoil as a carrier.

After spiking, the concentrations of the PAH compounds in the soil were determined by gas chromatography (GC) (see Section 2.5.2). These concentrations were 6.31 mg/kg, 24.6 mg/kg and 29.2 mg/kg (dry mass) for naphthalene, phenanthrene, and pyrene, respectively. The spiked gasoil contamination of the soil was 4750 mg/kg.

The aleuritic sand matrix was alkaline (pH = 8.97) and had a low total nitrogen content; Kjeldahl nitrogen was

1.4 mg/L determined from 1:10 aqueous extract. The dissolved organic carbon (DOC) content (determined from 1:10 aqueous extract) of the matrix was 21 mg/L.

2.2 Nano zinc oxide

The examined zinc oxide nanoparticles (CAS 1314-13-2) were purchased from Sigma-Aldrich Inc. (Budapest, Hungary). Characteristics of the ZnO nanoparticles were provided by the manufacturer, which stated that the particle size of nZnO was less than 100 nm, with an average size of ≤40 nm. The nanoparticles were sonicated for 15 min prior to use.

2.3 The applied enzymes

Two genetically engineered novel enzymes were used to evaluate the biodegradation rate and effect of nZnO on their efficiency. In our previous study [39], we applied a metagenomic data search to find effective novel enzymes (two dioxygenases and one peroxidase) for remediation of PAH-degradation in contaminated soils. The novel enzymes were cloned and used in soil remediation microcosms experiments. These enzymes were characterized by Nagy et al. [39], who demonstrated that they have the potential to effectively degrade PAH pollutants. Out of the investigated 3 enzymes (PAH6_39, PAH1_99, PAH1_105) two (PAH6_39 and PAH1_105) greatly enhanced the microbial activity besides successful pollutant degradation. Accordingly, we focused on these two enzymes in our further research. We refer to the PAH6_39 catalase/peroxidase enzyme as 'E39' and the PAH1_105 gentisate 1,2-dioxygenase enzyme (GDO) as 'E105' in the following text.

The enzymes applied in the experiments were provided by Nagy and coworkers (Budapest University of Technology and Economics, Department of Applied Biotechnology and Food Science), and they were maintained in Tris/HCl buffer (140 mM NaCl, 30 mM Tris/HCl, pH 7.5).

2.4 Experimental setup

We used a contaminated matrix described in Section 2.1.

After spiking, the concentrations of the PAH compounds in the soil were determined by GC (see Sections 2.5.2 and 2.1).

The experiment was conducted in 500 mL Schott bottles; 200 g of the contaminated soil was taken into the bottles then they were completed with different additives according to the treatments. Every treatment had 3 replicates.

The investigation was conducted with the objective of ascertaining the effect of the genetically engineered novel enzymes and nZnO on biodegradation. To this end, 1 mg of either E39 or E105 enzyme was added to 5 mL of buffer and applied to the contaminated matrix.

The effect of calcium peroxide on degradation of PAH compounds and microbial activity was tested in several microcosms. To examine the influence of CaO_2, 0.72 g was added to the soil to reach 10 mmol CaO_2 nominal concentration. CaO_2 (CAS 78403-22-2) was purchased from Sigma-Aldrich Merck (Product No: 466271).

Nutrient supplements, such as nitrogen and phosphorus, were not applied.

As the primary aim was to investigate the effect of ZnO nanoparticles on bioremediation efficiency, 588 µL of nZnO suspension was added to the soil to achieve final nZnO concentrations of 200 and 1000 mg/kg. Distilled water and buffer without enzymes were used as control. Their volume in the control microcosms was the same as in the NP- and enzyme containing microcosms.

The microcosms were kept in the dark at room temperature (22 °C) during the 7-day experiment.

The composition of the microcosms is illustrated in Table 1.

2.5 Monitoring

Following a seven-day incubation period, samples were collected from the microcosms for subsequent analysis to determine the microbial activity and the pollutant removal efficiency caused by the various treatments.

2.5.1 Microbiological methods

Microbial activity measurement was performed to evaluate the impact of the applied compounds on microbial community. For this purpose, Biolog EcoPlate™ (Biolog Inc., Hayward, CA, USA) was applied. The EcoPlate™ is a microplate with 96 wells, that contains 31 organic carbon substrates in three replicates, which enables to investigate the microbial communities through physiological community level profiling (CLPP). The microbial community analyses by Biolog EcoPlate™ exhibited excellent results in our previous study and could be suitable for use in assessing and evaluating biodegradation in soil [40]. The characterization of the microbial populations based on their carbon source consumption pattern. The measurement was carried out as described Nagy et al. [39]. 5 g soil from each reactor was transferred into a sterile 100 mL Schott bottle, then 45 mL sterile 0.85% NaCl solution was added, and it was shaken for 30 min at 150 rpm at 22 °C to suspend and homogenize the soil. After 10 min settlement, 1 mL supernatant was diluted in 9 mL sterile 0.85% NaCl

Table 1 Composition of the microcosms

Treatments	Compounds
Control	200 g soil + 5 mL buffer without enzyme + 588 µL DW
E39	200 g soil + 5 mL buffer with E39 enzyme + 588 µL DW
E105	200 g soil + 5 mL buffer with E105 enzyme + 588 µL DW
CaO_2	200 g soil + 5 mL buffer without enzyme + 588 µL DW + 0.72 g CaO_2
E39 + CaO_2	200 g soil + 5 mL buffer with E39 enzyme + 588 µL DW+ 0.72 g CaO_2
E105 + CaO_2	200 g soil + 5 mL buffer with E105 enzyme + 588 µL DW+ 0.72 g CaO_2
nZnO-200	200 g soil + 5 mL buffer without enzyme + 588 µL nZnO suspension
E39 + nZnO-200	200 g soil + 5 mL buffer with E39 enzyme + 588 µL nZnO suspension
E105 + nZnO-200	200 g soil + 5 mL buffer with E105 enzyme + 588 µL nZnO suspension
nZnO-1000	200 g soil + 5 mL buffer without enzyme + 588 µL nZnO suspension
E39 + nZnO-1000	200 g soil + 5 mL buffer with E39 enzyme + 588 µL nZnO suspension
E105 + nZnO-1000	200 g soil + 5 mL buffer with E105 enzyme + 588 µL nZnO suspension

solution. 125 µl of this suspension was pipetted into each well of the EcoPlateTM, then the absorbance of the wells was measured immediately and every 24 h at 490 nm wavelength with DIALAB EL800 Microplate Reader (Dialab GmbH, Wiener Neudorf, Austria). The plate was incubated at 25 °C in the dark for 168 h.

Different endpoints (average well color development (AWCD), substrate richness (SR), McIntosh index (U)) were calculated from the measured data as described by Németh et al. [41], which represent the potential metabolic activity and diversity of the microbial communities.

2.5.2 Analytical methods

The concentration of PAH compounds in the soil were determined by using Agilent 6890 5973 N gas chromatography mass spectrometry coupled with tandem mass spectrometry (GC-MS/MS) according to the MSZ EN 17503:2022 [42].

2.6 Data analysis and statistics

Statistical analysis was performed with StatSoft® TIBCO Statistica 13.5.0 software (TIBCO, Inc., Palo Alto, CA, USA) [43] to investigate, whether the treatments had significant impact on the examined endpoints. One-way analysis of variance (ANOVA) was performed at $p < 0.05$ significance level to determine the significant differences. Cochran's C test was applied to verify the criteria for the homogeneity of variances.

Fisher's least significant difference (LSD) was carried out to compare the effects of the various treatments. The significant effects are indicated with letters on all figures in alphabetical order, where "a" represents the smallest average value. Columns signed with the same letter imply that there was no significant difference between them.

3 Results

Results of the microbial activity measurements and the concentration of the pollutants according to the analytical measurements are discussed below.

3.1 Microbial activity after the treatment

We have measured the microbial activity with Biolog EcoPlate™ through various endpoints.

The AWCD values (Fig. 1) showed that the enzymes applied significantly increased the microbial activity,

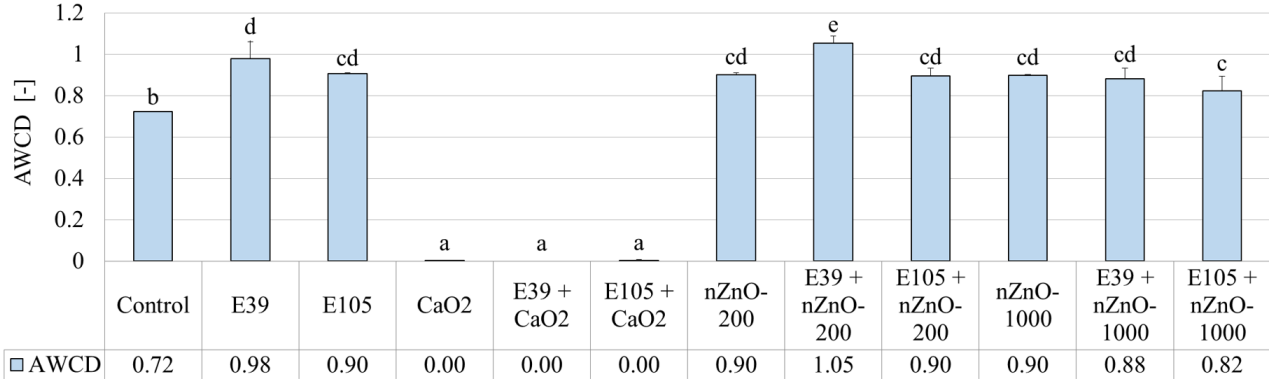


Fig. 1 The effect of ZnO nanoparticles on AWCD value. Letters on the columns indicate significant differences (level of significance: $p < 0.05$).

whereas the added CaO_2 decreased the microbial activity (almost to zero) compared to the control. The increase in microbial activity caused by the addition of enzymes may be attributable to the fact that the enzymes served as nutrients for the soil microbial community.

E39 gave a higher increase in AWCD than E105. In addition, the application of nano-zinc oxide had a positive effect on the microbiome, as each treatment resulted in higher AWCD values than the control. However, both 200 and 1000 mg/kg nZnO increased the microbial activity, applying 200 mg/kg nZnO caused higher activity, and the highest AWCD value (1.05) was observed for the E39+200 mg/kg nZnO treatment; this was significantly higher than the results for E39 and 200 mg/kg nZnO separately.

As demonstrated in Fig. 2, the SR values exhibited analogous outcomes, however in this case the E105 treatment resulted in higher SR value than E39 (Fig. 2).

Applying calcium peroxide resulted in zero SR value, while adding nZnO enhanced the number of the utilized substrates. Consequently, the maximum SR value (24) was recorded in the group that received a combination of E105 and 200 mg/kg nZnO. However, this was not significantly higher than the results of the E39 + 200 mg/kg nZnO treatment, but both were significantly larger than the values of the treatments with only enzymes or only nano zinc oxide.

The U was also determined, as it was identified in our previous study [41] to be the most sensitive diversity index for indicating the effect of nanoparticles on microbial communities. The changes in U values (Fig. 3) showed a similar pattern to the AWCD values. Accordingly, both enzymes significantly increased the U values, whereas CaO_2 significantly decreased the U values. Additionally, nano zinc oxide also enhanced significantly the microbial activity. The largest U (6.96) was measured at E39 + 200 mg/kg nZnO treatment. However, the combined application of E39 and 200 mg/kg nZnO was not significant compared to their enhanced effect separately.

3.2 Pollutant removal efficiency

We used as contaminated matrix aleuritic sand obtained from Elgoscar Environmental Technology Plc., Hungary [39], which was further artificially spiked with naphthalene, phenanthrene and pyrene. These pollutants were applied to a contaminated matrix dissolved in gasoil.

After spiking, the concentrations of the PAH compounds in the soil were determined by GC (see Section 2.5.2). These concentrations were 6.31 mg/kg, 24.6 mg/kg and 29.2 mg/kg (dry mass) for naphthalene, phenanthrene, and pyrene, respectively. The spiked gasoil contamination of the soil was 4750 mg/kg.

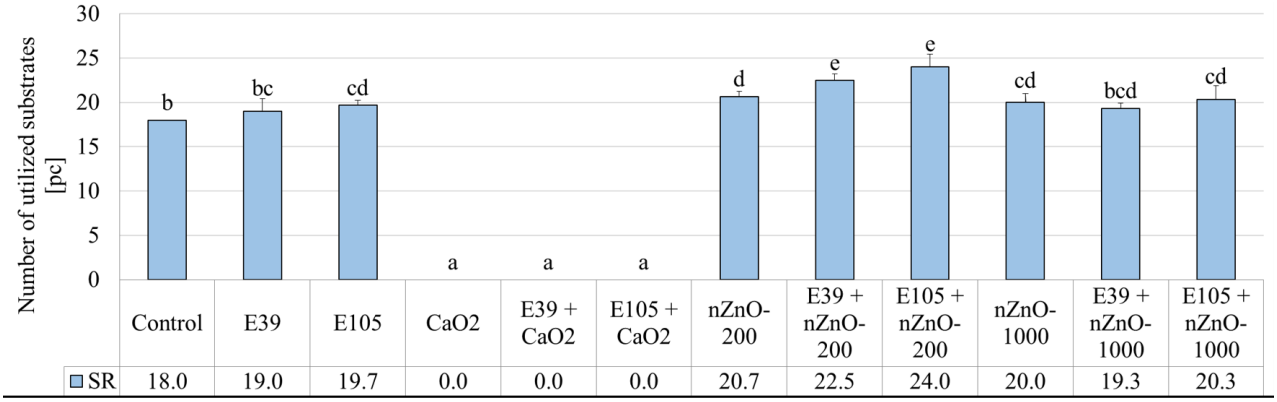


Fig. 2 The effect of ZnO nanoparticles on SR value. Letters on the columns indicate significant differences (level of significance: $p < 0.05$).

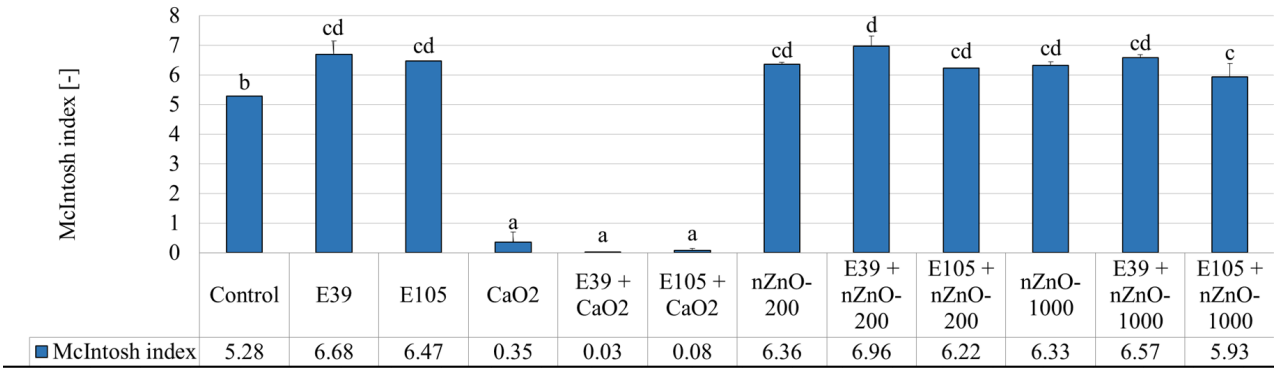


Fig. 3 The effect of ZnO nanoparticles on U value. Letters on the columns indicate significant differences (level of significance: $p < 0.05$).

The concentrations of the contaminants were measured with GC MS.

The results of our microcosm experiments showed no significant differences in extractable petroleum hydrocarbons (EPHs) between treatments (data not shown).

Regarding the PAH compounds, the degradation of naphthalene was not significantly different from the control when the enzymes were applied (Fig. 4). However, the concentration of naphthalene remaining was lower in the case of E39 and slightly higher in the case of E105 compared to the control. But these differences were not significant. Furthermore, the application of CaO_2 caused the lowest naphthalene removal, since the measured concentrations were the highest. Accordingly, the highest residual naphthalene concentration (4.53 mg/kg) was observed in the CaO_2 treatment. In addition, the application of nZnO resulted in a significantly higher removal of naphthalene compared to the control.

The lowest residual naphthalene concentration (0.23 mg/kg) (i.e. the highest removal) was measured with the combined use of E105 and 1000 mg/kg nZnO.

It is interesting to note that the application of nZnO with E105 resulted in lower naphthalene concentrations compared to the combination of E39 and nZnO at both 200 and 1000 mg/kg nZnO doses. Conversely, the application of E105 separately resulted in higher naphthalene concentrations than in the case of E39.

The residual phenanthrene concentration following the treatment process is illustrated in Fig. 5. Similarly to the results of naphthalene, the E105 enzyme exhibited a diminished effect on the degradation process. Conversely, the application of E39 resulted in a considerably diminished phenanthrene concentration in comparison to the control group. The highest observed phenanthrene concentration was 22.90 mg/kg in the case of the CaO_2 treatment. However, the combined use of E105 with 1000 mg/kg nZnO caused

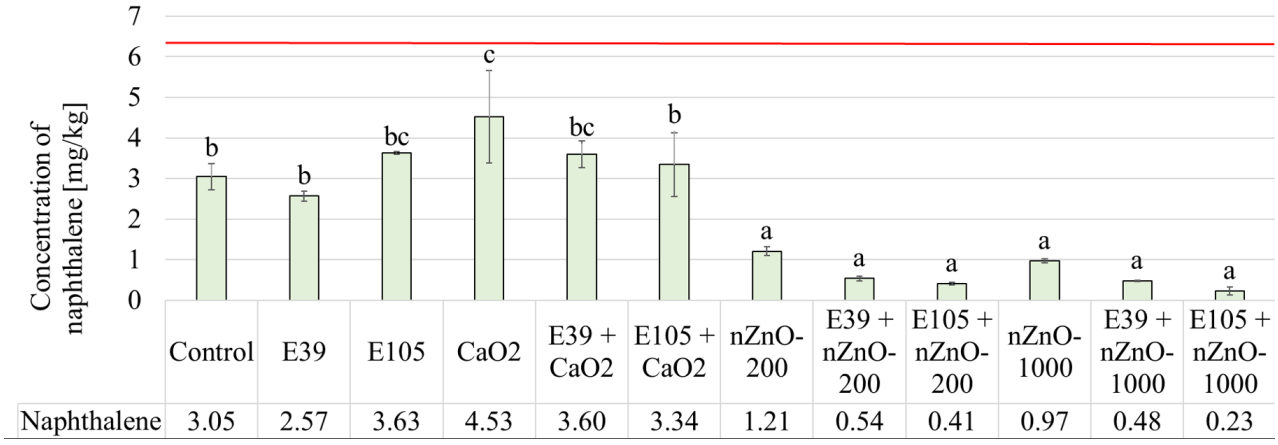


Fig. 4 Concentration of naphthalene after the treatment. Letters on the columns indicate significant differences (level of significance: $p < 0.05$). The red line represents the initial value.

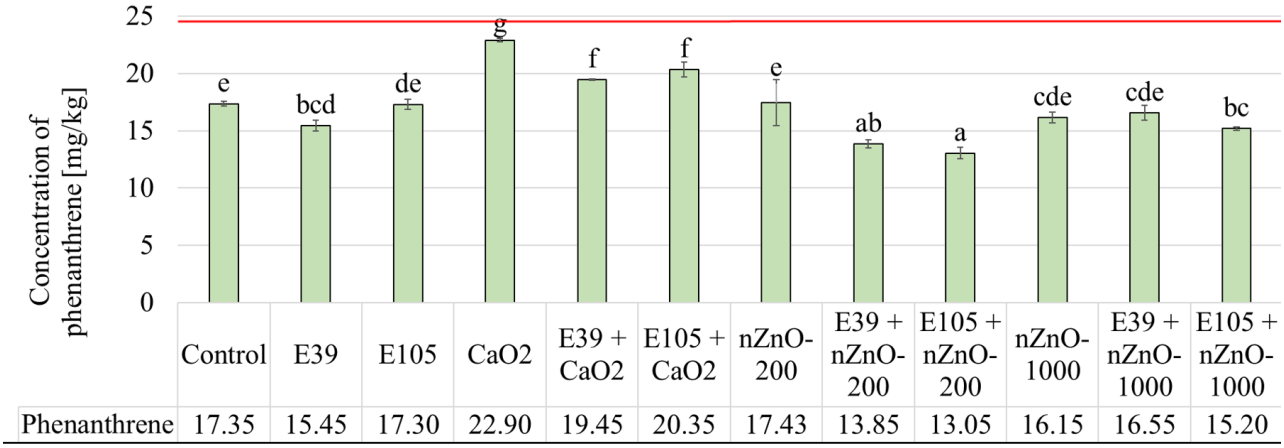


Fig. 5 Concentration of phenanthrene after the treatment. Letters on the columns indicate significant differences (level of significance: $p < 0.05$). The red line represents the initial value.

a significant decrease in phenanthrene concentration compared to the control, the lowest phenanthrene concentration was measured in the case of E105 with 200 mg/kg nZnO dosage. Furthermore, the application of nZnO alone did not cause a significant difference in phenanthrene removal compared to the control, neither at 200 nor at 1000 mg/kg. Furthermore, E39 with 200 mg/kg nZnO did not result in a significantly higher phenanthrene concentration than E105 with 200 mg/kg nZnO, while the application of E105 alone resulted in a higher concentration than E39.

The analytical measurements showed significant differences in the concentration of a persistent PAH compound, chrysene, in response to the several treatments. These results are shown in Fig. 6. The combined application of E39 and CaO_2 (which resulted in the lowest chrysene concentration of 0.03 mg/kg), the combination of E39 with 200 mg/kg nZnO and the application of E105 with 200 and 1000 mg/kg nZnO resulted in significantly lower chrysene concentrations compared to the control.

The measured pyrene concentrations in the contaminated matrices are illustrated in Fig. 7. According to these results, the single use of enzymes did not lead to a higher degradation of this contaminant than the control sample.

On the other hand, the combined use of enzymes with nZnO reduced the concentration of pyrene compared to the separated use of enzymes, but not significantly compared to the control. The lowest pyrene concentration (22.60 mg/kg) was measured at E39 + CaO_2 treatment.

The total PAH concentrations following the treatment are illustrated in Fig. 8. The findings of this study indicated that CaO_2 inhibited the removal process, as evidenced by the observation of elevated PAH concentrations in these treatments in comparison to the control. Furthermore, the maximum total PAH concentration, and consequently the minimum removal, was observed in the single CaO_2 treatment.

However, the E39 enzyme enhanced the degradation process, while the E105 enzyme treatment resulted

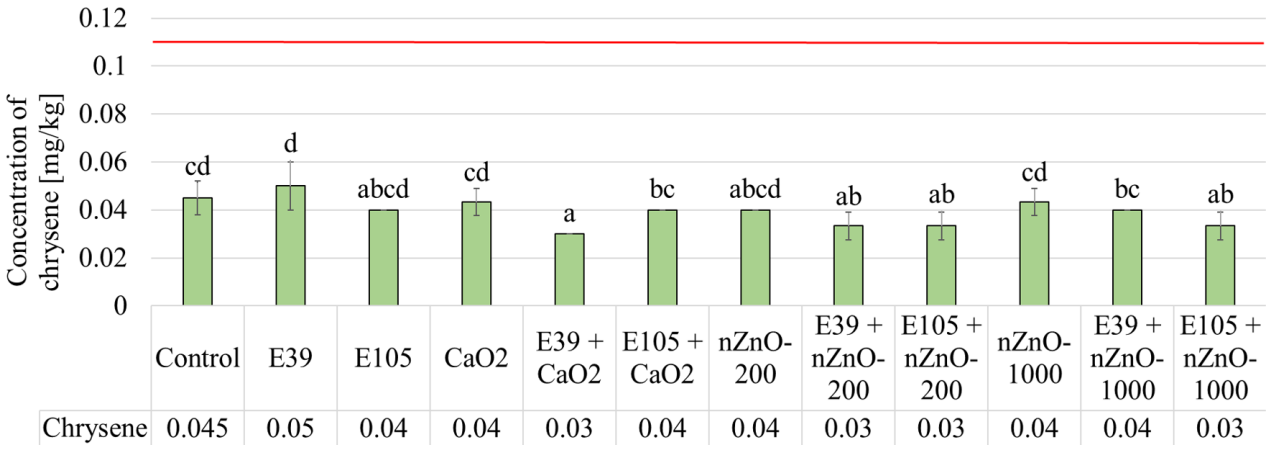


Fig. 6 Concentration of chrysene after the treatment. Letters on the columns indicate significant differences (level of significance: $p < 0.05$). The red line represents the initial value.

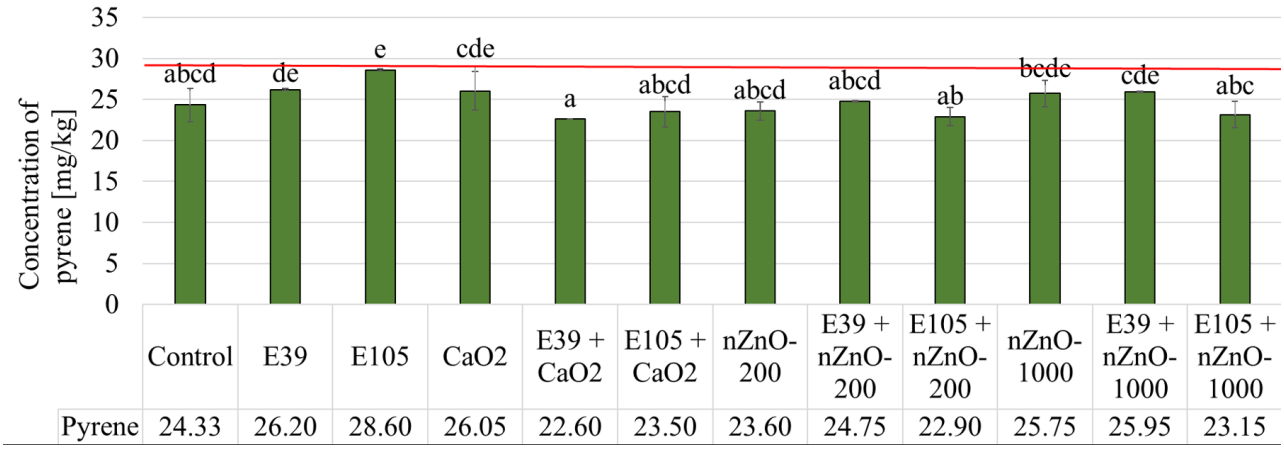


Fig. 7 Concentration of pyrene after the treatment. Letters on the columns indicate significant differences (level of significance: $p < 0.05$). The red line represents the initial value.

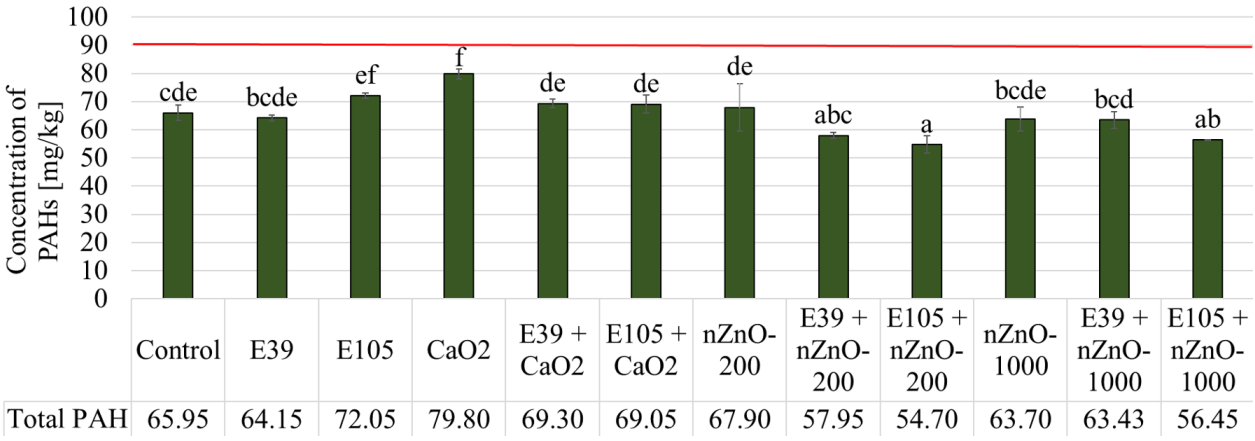


Fig. 8 Concentration of total PAH after the treatment. Letters on the columns indicate significant differences (level of significance: $p < 0.05$). The red line represents the initial value

in a higher PAH concentration compared to the control. However, neither of these results were found to be statistically significant.

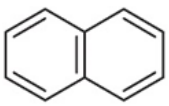
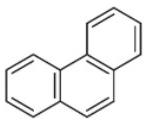
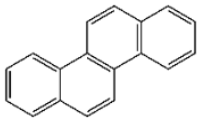
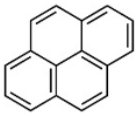
Applying 1000 mg/kg nZnO increased the PAH removal efficiency, nonetheless it was significant compared to control only in case of the combined use with E105 enzyme. Furthermore, the addition of 200 mg/kg nZnO resulted in a higher total PAH concentration compared to the control, but the combined use with enzymes resulted in lower PAH doses. However, similar to 1000 mg/kg nZnO, only the E105 enzyme had a significant effect. Additionally, the lowest total PAH concentration (54.70 mg/kg) and thus the highest removal was observed with this treatment (E105 + 200 mg/kg nZnO).

Removal efficiency rates were calculated compared to the control for the above-mentioned contaminants (results from Figs. 4–8). The calculated removal percentages (%) are presented in Table 2.

The results summarized in Table 2 are in line with the previously discussed results regarding the lowest and highest removal efficiencies of the treatments.

According to our results, CaO_2 strongly inhibited the degradation of the pollutants, although there were some cases where the application of the studied enzymes alone resulted in lower removal efficiencies (E39 enzyme in the case of chrysene and E105 enzyme in the case of pyrene). On the other hand, the combined use of CaO_2 and enzymes increased the degradation rate in these cases.

Table 2 The average pollutant degradation efficiency of the different treatments compared to the initial pollutant concentrations determined by GC–MS. In each column, the low efficiency rates are indicated in red (and its shades), while green (and its shades) show high removal efficiencies. The color depth is proportional to the scale of the values.

Treatments	Average removal [%] with the standard deviation				
	Naphthalene 	Phenanthrene 	Chrysene 	Pyrene 	Total PAH
Control	51.7 ± 5.1	29.5 ± 0.9	59.1 ± 6.4	16.7 ± 6.9	26.6 ± 3.1
E39	59.4 ± 1.9	37.2 ± 2.0	54.6 ± 9.1	10.3 ± 0.5	28.6 ± 1.2
E105	42.5 ± 0.4	29.7 ± 1.7	63.6 ± 0.0	2.1 ± 0.5	19.9 ± 1.0
CaO_2	28.3 ± 18.0	6.9 ± 0.6	60.6 ± 5.3	10.8 ± 8.0	11.2 ± 2.0
E39 + CaO_2	42.9 ± 5.2	20.9 ± 0.3	72.7 ± 0.0	22.6 ± 0.0	22.9 ± 1.7
E105 + CaO_2	47.0 ± 12.4	17.3 ± 2.6	63.6 ± 0.0	19.5 ± 6.3	23.2 ± 3.5
nZnO-200	80.8 ± 1.8	29.1 ± 8.2	63.6 ± 0.0	19.2 ± 3.9	24.5 ± 9.4
E39 + nZnO-200	91.4 ± 0.9	43.7 ± 1.4	69.7 ± 5.3	15.2 ± 0.2	35.5 ± 1.2
E105 + nZnO-200	93.5 ± 0.4	47.0 ± 2.0	69.7 ± 5.3	21.6 ± 3.9	39.2 ± 3.5
nZnO-1000	84.7 ± 0.8	34.3 ± 2.0	60.6 ± 5.3	11.8 ± 5.6	29.1 ± 4.7
E39 + nZnO-1000	92.4 ± 0.2	32.7 ± 2.6	63.6 ± 0.0	11.1 ± 0.2	29.4 ± 3.2
E105 + nZnO-1000	96.4 ± 1.5	38.2 ± 0.6	69.7 ± 5.3	20.7 ± 5.6	37.2 ± 0.1

The application of nZnO increased the degradation rates in most cases compared to the control, both at 200 and 1000 mg/kg. The mixed application with enzymes also increased the degradation, except for pyrene with E39 enzyme. Furthermore, the application of nZnO with E105 enzyme increased the removal efficiency in each treatment.

The addition of 1000 mg/kg nZnO increased the removal efficiency of E105 enzyme compared to E39 enzyme for each pollutant. Similarly, 200 mg/kg nZnO resulted in higher removal rates (except for chrysene) with the E105 enzyme than with the E39 enzyme.

However, the mixed use of nZnO and the enzymes results in effective degradation of PAH pollutants and application of E105 with 1000 mg/kg nZnO resulted in higher naphthalene removal efficiency than with 200 mg/kg nZnO, according to our results, the highest removal rates can be achieved by simultaneous use of E105 enzyme with 200 mg/kg nZnO. In addition, the use of 200 mg/kg nZnO with E39 results in improved removal rates compared to 1000 mg/kg nZnO with E39.

4 Discussion

Designing and developing a bioremediation technology involves considering numerous biotic and abiotic factors. Modifying these factors by adding various substances, such as peroxides, nanoparticles and biosurfactants, has been shown to significantly impact degradation efficiency [39].

Calcium peroxide is frequently used in bioremediation processes since it provides oxygen for microbes thus stimulating them, and the final product of the oxygen release is •OH, which destroys the aromatic structure of the contaminants [44, 45]. CaO_2 has low solubility, therefore it can release oxygen for longer period and more folds of oxygen compared to other additives like magnesium peroxide or hydrogen peroxide. Furthermore, the addition of CaO_2 may result in increased soil pH [44]. Optimization of the environmental factors and the characteristics of soil can lead to enhanced biodegradation efficiency [39, 46, 47]. However, we observed that 10 mmol CaO_2 decreased the microbial activity close to zero, which resulted in the inhibition of pollutants degradation. We observed the highest residual concentration of naphthalene, phenanthrene and total PAH concentration as a consequence of peroxide treatment. These results are in accordance with the findings of Nagy et al. [39], who described that the microbial activity was fully erased upon addition of 20 mmol CaO_2 to the soil, due to destroying the soil-inherent microflora.

Applying enzymes in bioremediation processes has more advantages in comparison with using microbial cells, because they have higher specificity and mobility, they are active in the presence of high doses of toxic contaminants. Additionally, they do not need nutrient supplementation. Moreover, they are biodegradable that inhibits their persistence and recalcitrance [48–53]. On the other hand, the half-life of free enzymes is limited and highly depends on environmental factors. Furthermore, they usually cannot carry out the complete degradation of the contaminants, although generally the first transformation step limits the further degradation of the pollutants [39, 54]. However, these undesirable properties and functional characteristics can be mitigated through the immobilization of enzymes and/or genetic engineering methods. This enables the production of recombinant enzymes that are more stable and active, and have a longer half-life. This approach enables treating contaminated sites with an eco-friendly, cost-effective remediation technique [19, 52, 55].

Our study showed that the enzymes used (E39 and E105) increased the metabolic activity of the soil microbiota, which is in agreement with the results reported by Nagy et al. [39]. However, they described 65% and 73% AWCD enhancement in case of E39 and E105, while we observed only 36% and 25% increase, respectively. This phenomenon may be explained by minor variations in the concentration and composition of PAH pollutants in contaminated soil compared to previous study, which in turn are associated with differences in microbial community. Controversially, we noticed higher impact of E39 than E105 on AWCD values. This phenomenon can be explained by the different composition of the inherent microbial community. The applied enzymes and the degraded intermediates did not serve as proper nutrient as described earlier by Nagy et al. [39]. Nevertheless, the investigation of further endpoints revealed a higher effect on the U for E39, while E105 had a greater impact on SR.

The existing literature on the subject of nZnO and its impact on the environment is inconclusive, with studies reporting both positive and negative effects. However, the results of this study indicate a positive effect of nZnO on microbial activity, observed at both applied doses, with the rate of effect being almost equal at both levels. Nevertheless, the impact of nZnO was found to be less significant than that of the enzymes. However, other studies have yielded both controversial and concordant findings. Liu et al. [56] observed that CuO and ZnO NPs boosted soil microbial communities. It was reflected by the increased

number of extractable bacterial or fungal groups and the enlarged values of different indices. However, the positive impacts of NPs were moderated at concentrations higher than 250 mg/kg. Wu et al. [57] did not observe stronger toxicity at higher (80 mg/L and 120 mg/L) nZnO concentration than at 40 mg/L dose on an intertidal wetland's microbial community activity measured by Biolog EcoPlate™. García-Gómez et al. [58] also applied Biolog EcoPlate™ for microbial community level physiological profiling (CLPP) of two agricultural soils exposed to nZnO. According to their results, 1000 mg/kg nZnO enhanced the bacterial growth in acidic soil, without changing their metabolic profile, while in calcareous soil the functional richness of the bacterial community was highly reduced. Additionally, at lower nZnO concentrations (0–100 mg/kg) a stimulating effect was observed for both types of soil. Shemawar et al. [59] also found that the negative effect of nZnO was temporary, as they observed a decrease in microbial biomass at 100 and 1000 mg/kg nZnO doses after 24 days, but after longer incubation time (64 days) the biomass were similar to that measured in the control.

In contrast, Dinesh et al. [60] found that nZnO reduced the soil's microbial enzyme activities, respiration and microbial biomass, hence the alpha diversity decreased as the ZnO level increased up to 1000 mg/kg Zn. These results are in accordance with the findings of Shen et al. [61], who described that dehydrogenase and fluorescent diacetate hydrolase activity were dose-dependently inhibited even at 1; 5 and 10 mg/kg nZnO concentration. Furthermore, Ge et al. [38] found that both $nTiO_2$ and nZnO reduced the microbial biomass and diversity of the soil and modified the composition of its bacterial community at a concentration of 0.5 mg/kg. Furthermore, the effect of nZnO was higher than that of $nTiO_2$. Ruiz-Leyva et al. [62] investigated the effect of nano zinc peroxide ($nZnO_2$) on soil microbial community and found that the exposure to low $nZnO_2$ doses (<100 mg/kg) increased the taxonomic diversity, while 1000 mg/kg $nZnO_2$ decreased the taxonomic and functional diversity. Strekalovskaya et al. [63] also described a stimulating effect of nZnO on soil microbiota and its activity at relatively low concentrations of up to 250 mg/kg, while if the concentration of nZnO increases above this limit, toxic effects appear.

Enzyme-based bioremediation techniques are widely used, and the application of nanomaterials and nanoparticles for pollutant elimination has recently had a greater impact. However, the simultaneous application of nano zinc oxide with enzymes for PAH biodegradation is a novel approach, indicating that the relevant literature is currently very scarce. Our study highlights the novelty of the combined use of nZnO and PAH-degrading enzymes. Accordingly, the mixed use of enzymes and 200 mg/kg nZnO resulted in the highest observed microbial activities. E39 + nZnO-200 treatment boosted AWCD and U values the most, while E105 + nZnO-200 treatment led to the greatest SR value. On the other hand, compared to the combined use of enzymes and 200 mg/kg nZnO, 1000 mg/kg nZnO with enzymes was less effective. In some cases, it even inhibited microbial activity in contrast to the results of applying only enzymes. Overall, we found that the combined use of enzymes with 200 mg/kg nZnO greatly enhanced microbial activity, and E39 enzyme had a more beneficial effect.

Adding nZnO did not cause a notable change in the pollutant removal efficiency, except in case of naphthalene, where a concentration dependent increase was observed for the degradation efficacy, thus the highest removal rate was noticed in case of applying E105 with 1000 mg/kg nZnO. On the other hand, the combined use of nZnO with the enzymes enhanced the pollutant removal rate. However, we did not observe concentration dependent effect for the degradation efficiency, even in many cases applying 200 mg/kg nZnO caused higher contaminant elimination. In most cases, the highest degradation rate was observed in the E105 + nZnO-200 treatment, despite the fact that the single use of E105 was less advantageous than E39. In some cases, the degradation was even inhibited compared to the control.

GDOs belong to the bicupin family [64]. Most enzymatic cupins contain iron as an active site metal, other members contain either zinc, copper, cobalt, manganese or nickel ions as a cofactor [65]. Huang et al. [66] described that 0.25 mM Zn^{2+} had no impact on the activity of GDO from a halophilic *Martelella* strain, while Feng et al. [67] reported that 5 mM Zn^{2+} inhibited GDOs produced by *Pseudomonas alcaligenes* NCIB 9867 and *Pseudomonas putida* NCIB 9869 strains. These studies reveal that the effect of Zn^{2+} on GDO may be dependent of the microbial source and the structure of the enzyme beyond the concentration of the Zn^{2+}.

According to our results, the positive impact of adding 200 mg/kg nZnO to E105 enzyme was confirmed (Table 2), since the mixed use of them led to the highest removal rate of phenanthrene and total PAHs, which were 47% and 39%, respectively. Furthermore, this treatment was amongst the most efficient ones on other pollutants, although the highest removal efficiencies and the treatments concerned were

the followings: naphthalene: 96% with E105 + nZnO-1000 treatment, chrysene: 72% with E39 + CaO_2 treatment, pyrene: 22% with E39 + CaO_2 treatment.

To the best of the author's knowledge, there are no examples in the extant literature of the combined use of nano Zn and enzymes to promote bioremediation of PAH compounds in soil. However, there has been some research on the use of other nanomaterials and enzymes. Chai et al. [68] reported that they achieved 55.9% removal rate from contaminated soil with immobilized lignin peroxidase which was prepared by using chitosan-modified halloysite nanotubes (HNTs-CTA). Chauhan et al. [69] described that 62.84% of 25 ppm phenanthrene was removed from water by commercial ZnO, while with GO/ZnO nanocomposites they achieved 86% degradation rate. The total PAHs removal rate was 39% with E105 + nZnO-200 treatment in our study which is similar to the results of Xue et al. [70], who described 41% PAH degradation efficiency in soil with the application of 40 mg/kg nano graphene oxide.

In conclusion, the findings of this study demonstrate that the most effective approach to achieving the highest possible degradation rate for PAH pollutants is the combined use of E105 and 200 mg/kg nZnO. However, it is noteworthy that E105 in conjunction with 1000 mg/kg nZnO and E39 with 200 mg/kg nZnO also led to a significant level of pollutant removal, thus indicating that these approaches are highly effective for PAH removal.

To summarize, the findings of microbial activity and analytical measurements indicate that application of nZnO alone to soil is effective in removing more readily degradable naphthalene, probably due to enhanced microbial activity. The combined use of E39 and E105 enzymes with 200 mg/kg nZnO may be beneficial for the biodegradation of PAH-contaminated sites.

The metabolic activity of the indigenous microflora was most significantly enhanced by E39 + nZnO-200, while the greatest PAH degradation was achieved by E105 + nZnO-200 treatment.

However, the application of 1000 mg/kg nZnO would also be favorable in comparison to the processes of the native microbiota (control). Nevertheless, it would result in a worse degradation rate when compared to the combined use of enzymes and 200 mg/kg nZnO.

5 Conclusion

Our study demonstrated that the efficiency of an innovative enzyme-based bioremediation technology developed for the removal of PAHs can be increased in soils with low microbiological activity and unfavorable mechanical composition by the combined use of enzymes (catalase-peroxidase, gentisate 1,2-dioxygenase) and 200 mg/kg nZnO, mainly in the case of naphthalene and the more difficult to break down phenanthrene and chrysene in moderately contaminated media, without observing any potential harmful effects.

Despite the acknowledged efficacy of enzymes as the most efficient bioremediation tools, capable of inducing biodegradation of organic pollutants, the results of the present study demonstrate some limitations of application enzymes in soil bioremediation The application of enzymes had a significant effect on the microbial activity of the contaminated soil, however, our study was not able to demonstrate that the use of enzymes alone would result in the degradation of PAHs to a remarkable extent. Conversely, their combined use with nano zinc oxide would lead to a more efficient elimination of PAH pollutants, which is a novel finding of our study.

We did not observe any negative effect of nZnO even at 1000 mg/kg concentration on the metabolic activity of the soil microbiota, but the degradation efficiency of enzymes was lower at this nZnO concentration compared to 200 mg/kg nZnO dose, which greatly increased the degradation efficiency. Based on these results, the application of nZnO is proposed for PAH polluted environment remediation, which can lead to the development of novel and environmentally friendly methods.

Additionally, the integrated use of E39, E105 and 200 mg/kg nZnO is recommended for the elimination of PAH contaminants from contaminated soils. However, further investigations are needed for more comprehensive studies on the environmental impact of nZnO on a wider range of organisms and environmental matrices (e.g. various types of soils) and to specify a narrow nZnO concentration range that enhances PAH degradation.

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