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Full Length Research Paper

Biodegradation of PAHs by fungi in contaminated-soil containing cadmium and nickel ions

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The study investigated the degradation of the polycyclic aromatic hydrocarbons (PAH) benzo(a)anthracene, benzo(a) fluoranthene, benzo(a) pyrene, chrysene and phenanthrene in a soil that was sterilized and inoculated with the nonligninolytic fungi, *Fusarium flocciferum* and *Trichoderma* spp. and the ligninolytic fungi, *Trametes versicolor* and *Pleurotus ostreatus* in the presence of cadmium (Cd) and nickel (Ni) during a ten week incubation period. The soil pH was initially 5.3 and after amendment increased to 7.0. The fungi degraded the tested PAHs between 21 and 93% by the end of the tenth week. The fungi degraded the less-soluble PAHs containing five or six aromatic rings more slowly than those containing fewer aromatic rings. Although the presence of cadmium and nickel in the soil affected the activity of the enzymes produced by the fungi, no significant decrease in PAH degradation was found in the contaminated soil containing 50 or 100 mg kg^{-1} of Cd and Ni. However, at 300 and 500 mg kg^{-1}, degradation of the PAHs by the fungi was impaired and the severity of the impairments increased with the increase in the concentrations of Cd and Ni. This was probably due to the lack of the activities of some enzymes such as Mn-dependent peroxidase, which could have resulted from the poor colonization of the fungi at these concentrations.

Key words: Bioremediation, fungi, heavy metals, PAHs, soil.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), produced by incomplete combustion of fossil fuels, and also manufactured for use in the pesticide, pharmaceutical and dye-making industry are ubiquitous and persistent in the environment (Mueller et al., 1996; Clemente et al., 2001; Garon et al., 2004). They are hydrophobic and can readily bio-accumulate in the environment. Some PAHs are harmful and known to be carcinogenic, mutagenic or genotoxic (Baldrian et al., 2000; Sokhn et al., 2001; Baran et al., 2004; Oleszczuk, 2006). Some PAHs have been declared priority pollutants in some countries. Consequently it is important to understand the fate of these compounds in the environment.

The degradation of PAHs in the environment has been extensively studied and documented in the literature and a large diversity of microorganisms, including bacteria and fungi, are capable of oxidizing or mineralizing a range of PAH (Cerniglia and Heitkamp, 1989; Alexander, 1999; Daane et al., 2002; Sasek et al., 2003). The number of benzene rings present in the structure of a PAH affects its physical and chemical properties such as solubility, which is very important for bioavailability. The larger the number of rings, the more recalcitrant the compound becomes.

Some heavy metals for example, Cu, Zn and Fe are essential for the growth of microorganisms in trace amounts but have also been shown to be toxic at high concentrations (Yamamoto et. al., 1985; Baldrian, 2003) and their addition in soil has been known to inhibit soil respiration, nitrogen mineralization and nitrification (Bååth, 1989; McGrath, 1994). Heavy metals have been implicated in the reduction of degradation of vegetable materials (Bååth, 1989) and can potentially limit the biodegradation of organic contaminants in the environment (Sokhn et al., 2001; Riis et al., 2002; Atagana, 2006).

One of the serious problems of contamination biotechnologists is the problem of co-contamination of PAHs and heavy metals, which is a common occurrence in sites of wood treatment plants using creosote and chromated copper arsenate (CCA) and those contaminated with petroleum products and pesticides (Baker and Bryson, 2002). Literature is relatively scarce on the effects of heavy metals on PAHs degradation (Baldrian et al., 2000; Wong et al., 2005) as research in this area is only begin-

ning to attract the attention of bioremediation technologists.

Of the microorganisms identified to have the capability to degrade PAHs in the environment, fungi have been shown to be relatively more successful in breaking down the higher molecular weight compounds (Potin et al., 2004) than bacteria, principally because of the production of non-specific extracellular enzymes such as laccase, lignin peroxidase and manganese dependent peroxidase (MnP), which degrade polyphenolic molecules of wood (Tien and Kirk 1984; Thurston, 1994; Baldrian, 2003; D'Annibale et al., 2005, 2006) and the ability to extend the location of their growth. The presence of cytochrome P450 and ligninolytic enzyme systems in most white rot fungi has lead to the suggestion that the complete degradation of PAHs by white rot fungi may involve these enzyme systems (Levine et al., 2003).

The aim of this study was to investigate the effects of the heavy metals cadmium (Cd) and nickel (Ni) on the biodegradation of selected PAHs by non-ligninolytic and ligninolytic fungi.

MATERIALS AND METHODS

Soil preparation

Seventy two grams of a sandy loam, fresh garden soil (pH 5.3, 20% clay, 60% sand, 17.5% silt, extractable phosphorus 12.3 mg kg-1, total nitrogen 1.35%, total organic carbon 3.5% and C:N:P ratio 27:1:3) was obtained from the University research farm in Pietermaritzburg, South Africa and homogenized before autoclaving at 121°C for 40 min. The soil was amended with 7 g kg^{-1} of agricultural lime and 1g kg^{-1} mono ammonium phosphate (MAP) fertilizer before being amended with 250 mg kg^{-1} phenanthrene, 150 mg kg^{-1} chrysene, 220 mg kg^{-1} benzo(a)anthracene, 200 mg kg^{-1} benzo(a)pyrene and 100 mg kg^{-1} benzo(a)fluoranthene.

Experimental design

Seventy-two 3-litre PVC troughs each with a diameter of 30 cm and a depth of about 12 cm were filled with 1 kg each of the amended soil, as described in the section for soil preparation. The troughs were divided into two groups (A and B) of 36 each. Each group was further subdivided into four units numbered 1, 2, 3 and 4. Units 1-4 of each group were amended with 50, 100, 300 and 500 mg kg^{-1} respectively of either Cd or Ni. Cadmium nitrate and nickel nitrate were used to effect the heavy metal amendment. A set of experiments without the heavy metals was set up as control. All experiments were set up in duplicate and incubated at ambient temperature under laboratory conditions for ten weeks. Samples were taken monthly for residual concentrations of the PAHs and soil respiration measurements. Samples were taken every three days for the first thirty days for enzyme analysis. The data obtained were analyzed by the one-way analysis of variance (ANOVA).

Fungal bulking

The fungi, *Fusarium folciparum, Trichoderma* spp, *Trametes versicolor, Pleurotus ostreatus* used for this study were obtained from old cultures used in an earlier study (Atagana, 2004; Atagana et al., 2005). They were isolated from hydrocarbon-impacted soil and from uncontaminated forest floor in Pietermaritzburg, South Africa by using a modified Czepeks medium described by Atagana (2004). Isolates were purified on PDA containing antibiotics (McGugan, 1997) and identified by microscopic examination of the hyphae and spores (Raper and Thom, 1968; Barnett and Hunter, 1972). Fungal bulking was achieved with barley grains as described by Atagana (2004) by inoculating soaked barley grains in polyethylene bags with 5 mm cubes of colonized PDA. One hundred grams of grains colonized by each fungus were separately inoculated onto 1 kg of contaminated soil in treatment troughs containing the different con-centrations of the tested heavy metals, as described in the section for experimental design.

Measurement of microbial activity in soil

CO_2 evolution was determined by the closed-jar method (Alef, 1995) by placing 30 g soil in a closed glass jar containing 20 ml of 0.1 mol l^{-1} NaOH solutions, which was removed after three days and titrated with 2 mol l^{-1} HCl. Results obtained, were calculated as:

$$\mu g\ CO_2\text{-}C\ g^{-1}\ day^{-1} = (V_{sample} - V_{blank}) \times 2.2 \times 0.27/dwt \times day \times 1000.$$

Where V_{blank} is the volume of HCl for the blank, V_{sample} is the volume of HCl for the sample, 2.2 in the conversion factor (1 mol 0.1 mol l^{-1} NaOH = 2.2 mg CO_2) 0.27 is mg CO_2-C and dwt is dry weight.

Enzyme analysis

Extraction of Mn-dependent peroxidase (MnP) was done by mixing 5 g of soil with 10 ml of phosphate buffer (50 mM, pH 7.0) and incubated on ice for 1 h and centrifuged at 15 000 x g for 15 min at 15°C and then the supernatant was centrifuged at 5 000 x g for 15 min at 15°C (2000). MnP activity was assayed in succinate-lactate buffer (100 mM, pH 4.5) and measured by using a spectrophotometer (Baldrian et al., 2000). All enzyme measurements were done within 30 days of incubation.

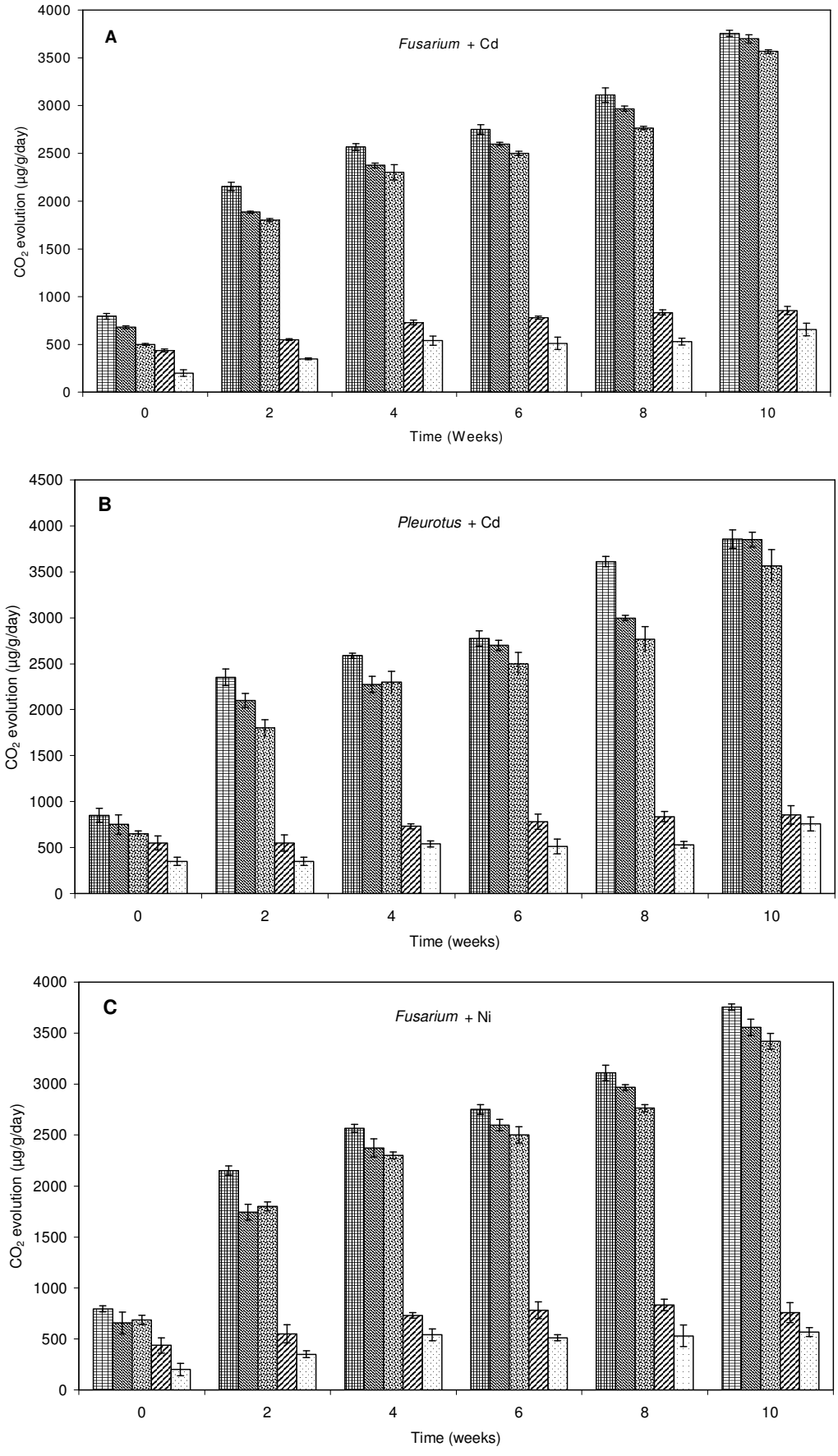
Determination of residual concentrations of PAHs

Five grams of air dried soil sample was soxhlet extracted with acetone-n-hexane mixture (1:4) for 6 h. The extracts were evaporated and resuspended in 20 ml of acetonitrile. High performance liquid chromatography (HPLC) and fluorescence detector (Perkin Elmer) were used to determine the PAHs. Separation was performed at 25°C isocratically with an analytical column as described by Baldrian et al. (2000). Fluorescence detection was at excitation wavelength 270 nm and emission wavelength of 405 nm. Concentrations were determined using calibrations containing all PAHs to be measured.

RESULTS AND DISCUSSION

Soil characteristics

The soil was a sandy loam soil with initial pH of 5.3 (after amendment pH 7), 20% clay, 60% sand and 17.5% silt. The extractable phosphorus was 12.3 mg kg-1, total nitrogen 1.35% and total organic carbon 3.5%. The C:N:P ratio before amendment was 27:1:3 and after amendment was 38:1:2.2. This C:N:P ratio was found not to be significantly different from the recommended ones (25:1:1 to 35:1:1) (Alexander, 1999) for bioremediation.


A
Fusarium + Cd
CO2 evolution (µg/g/day)
Time (Weeks)
B
Pleurotus + Cd
CO2 evolution (µg/g/day)
Time (weeks)
C
Fusarium + Ni
CO2 evolution (µg/g/day)
Time (weeks)

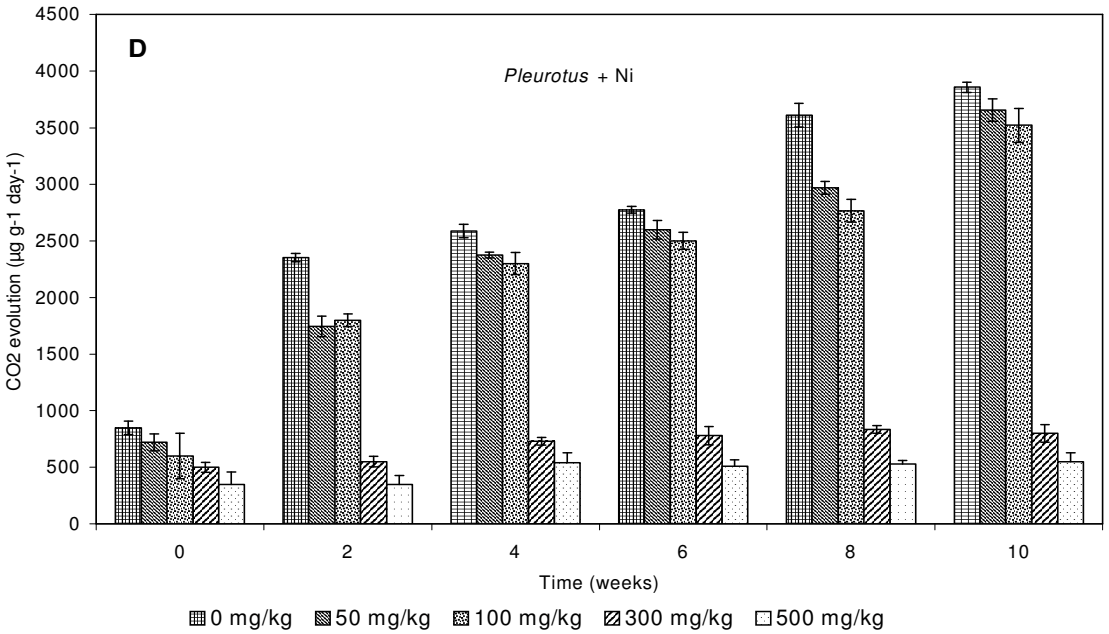


Figure 1a, b, c, d. Changes in the respiration rates of fungi in the *Fusarium* inoculated treatments and the *Pleurotus* inoculated treatments amended with different concentrations of cadmium and nickel. Values are means of two ± 1 standard error.

Microbial activity

CO_2 evolution continued to increase in all treatments except those amended with 300 and 500 mg kg^{-1} of the heavy metals until the end of the experimental period. There was no significant difference at p=0.05 between treatments amended with 0, 50, 100 mg kg^{-1} of the heavy metals in CO_2 evolution (Figure 1). However, there was a significant difference between those containing 300 and 500 mg kg^{-1} of both metals compared to those with lower concentrations.

Enzyme activity increased rapidly within the first two weeks of treatment, thereafter starting to decline before remaining relatively stable for most of the remaining part of the experiment (Figure 2). Enzyme activity was significantly higher in the *Pleurotus* and *Trametes* inoculated treatments (205 and 150 mU g^{-1}) respectively in Cd and Ni amended treatments on day 15 compared to those inoculated with *Fusarium* and *Trichoderma* (120 and 100 mU g^{-1}) (Figure 2). Enzyme activity was also low in the treatments containing 300 and 500 mg kg^{-1}. The highest values obtained were 230 mU g^{-1} on day 9 in 50 mg kg^{-1} Cd treatments inoculated with *Pleurotus* and 205 mU g^{-1} on day 15 in 0 mg kg^{-1} Cd (control) treatment inoculated with *Pleurotus*.

These results are indications that from 300 mg kg^{-1} and above, cadmium and nickel start to exert inhibitory effects on microbial activities in the soil. It also shows that the ligninolytic fungi, *Pleurotus* and *Trametes* were more resistant to the inhibitory effects of the heavy metals than the non-ligninolytic fungi, *Fusarium* and *Trichoderma*.

Reduction in PAH concentrations

After ten weeks of treatment, about 93% of the PAHs were removed from the biological control experiments, which were not amended with heavy metals. About 90% of the PAHs were removed from the treatments amended with 50 and 100 mg kg^{-1} of the heavy metals within the same period (Figures 3 and 4). Earlier reports have shown uninhibited enzyme production at lower concentrations of heavy metals and the subsequent reduction in hydro-carbon concentration (Baldrian, 2003; D'Annibale et al., 2006). There was no significant difference at p=0.05 in the removal of the PAHs in treatments containing 0, 50 and 100 mg/kg of cadmium and nickel.

The inhibitory effects of the heavy metals were significant at 300 and 500 mg kg^{-1} in all the treatments and the effects were more evident in those containing 500 mg kg^{-1} of the heavy metals in some cases. There was, however, no significant difference in the removal of the PAHs at the two concentrations at p=0.05. The least removal (21%) was achieved in the 500 mg kg^{-1} treatment inoculated with *Fusarium.*

The effects of the higher concentrations (300 and 500 mg kg^{-1}) of the heavy metals were more evident in the weekly measurements of the residual concentrations of the PAHs. Removal was relatively slower in these treatments when compared with those with lower concentrations (50 and 100 mg kg^{-1}) (Figure 5). The slow rate of reduction in the 300 and 500 mg amended treatments

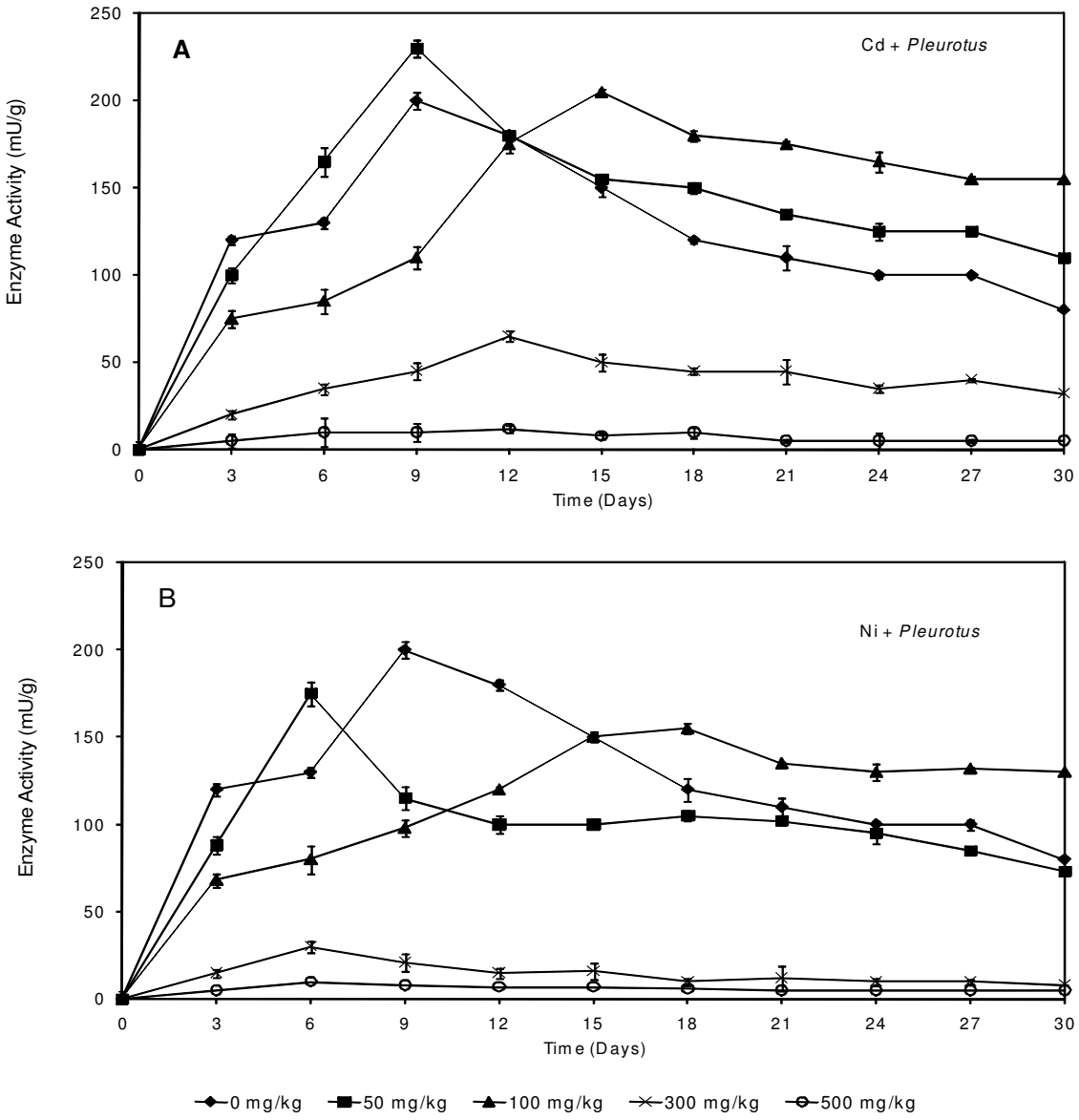


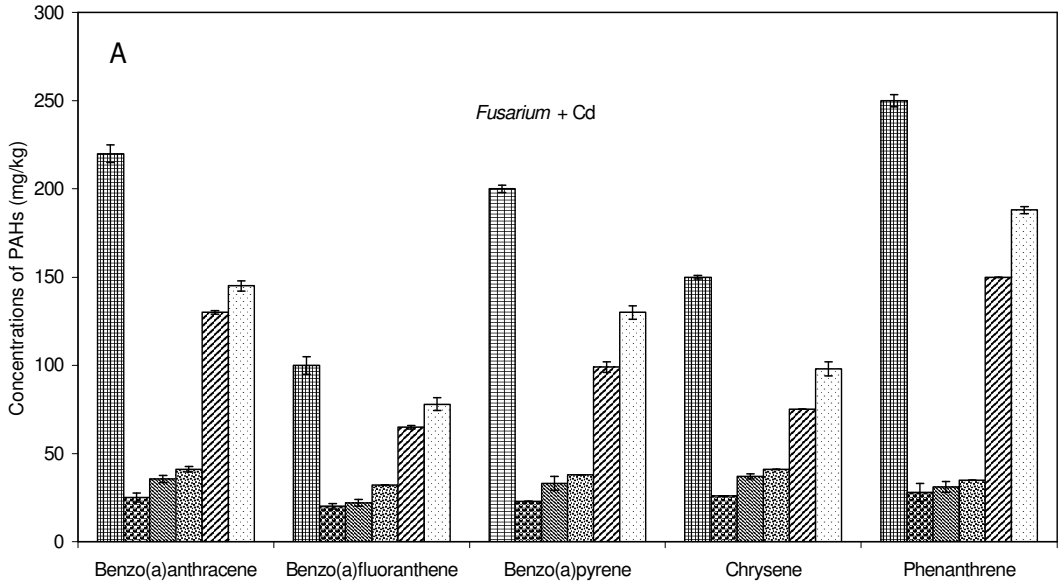
Figure 2a, b. Measurements of enzyme activities in the treatments inoculated with *Pleurotus* and amended with different concentration of cadmium and nickel. Values are means of two ± 1 Standard Error.

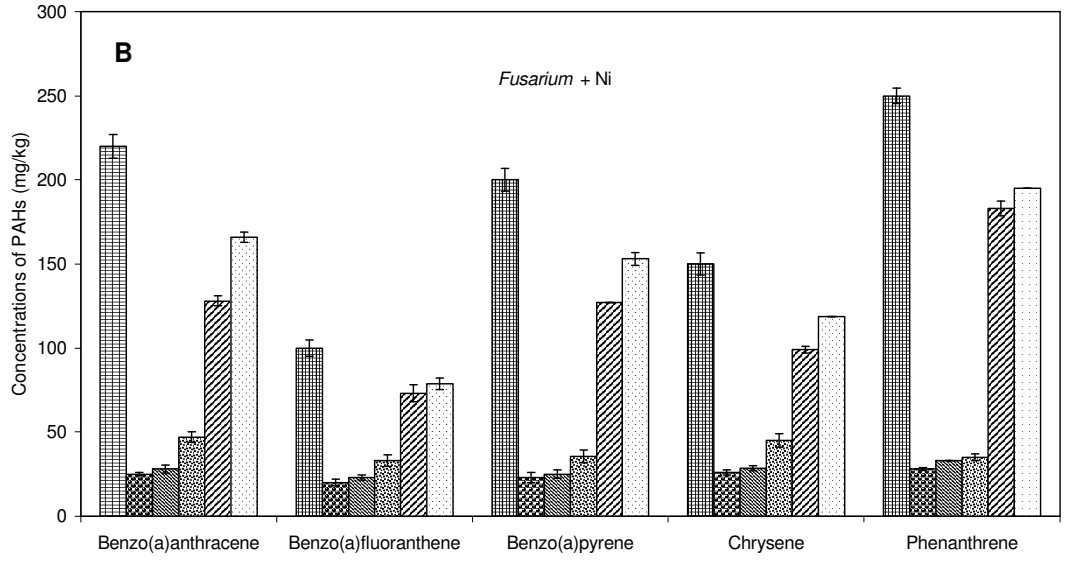
can be attributed to the toxicity of the heavy metals to the fungi at these concentrations, which may have resulted in inhibition of enzyme activities, as is evident from the results of enzyme activities at these concentrations and also the poor colonization of the soil system by the fungi. It is also possible that enzymes may have been denatured in the beginning by the high concentrations of the metals hence causing retardation in the growth of the fungi and consequently reducing its colonization of the soil system. The fungi could also have attempted to assimilate the heavy metals thus leaving the PAHs in the beginning of the treatment.

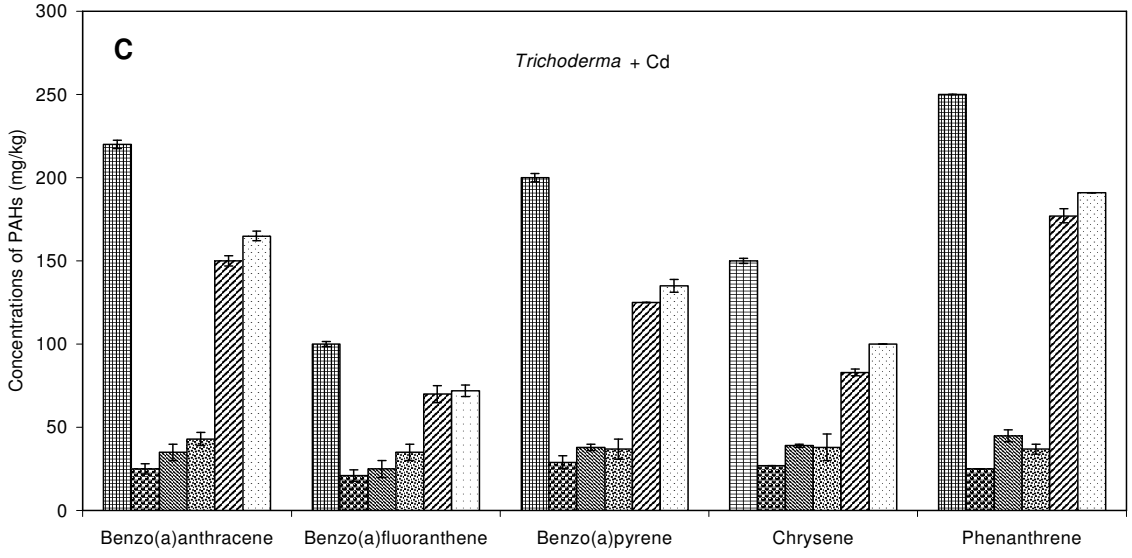
The lower molecular weight PAHs were more susceptible to removal than higher molecular weight ones. This could be due to the physical and chemical properties of the compounds, which is a function of the number of rings in their structure, as was earlier reported by Potin et al. (2004). Phenanthrene was more easily removed than all other compounds. The ligninolytic fungi were able to adapt to the heavy metal amendment more readily and thus were able to remove the PAHs better than the non-ligninolytic ones (Figures 3 and 4).

Conclusions

From the results obtained, it can be concluded that all the fungi tested were capable of removing the PAHs from the soil in the presence of the heavy metals tested at all the


A
Fusarium + Cd
Concentrations of PAHs (mg/kg)
300
250
200
150
100
50
0
Benzo(a)anthracene
Benzo(a)fluoranthene
Benzo(a)pyrene
Chrysene
Phenanthrene


B
Fusarium + Ni
Concentrations of PAHs (mg/kg)
300
250
200
150
100
50
0
Benzo(a)anthracene
Benzo(a)fluoranthene
Benzo(a)pyrene
Chrysene
Phenanthrene


C
Trichoderma + Cd
Concentrations of PAHs (mg/kg)
300
250
200
150
100
50
0
Benzo(a)anthracene
Benzo(a)fluoranthene
Benzo(a)pyrene
Chrysene
Phenanthrene

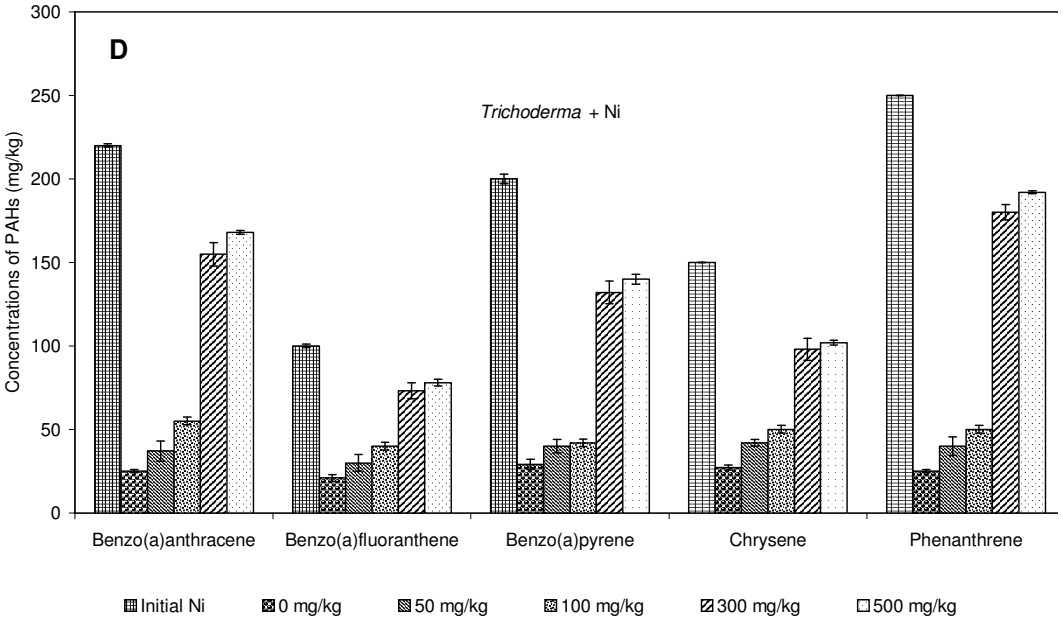
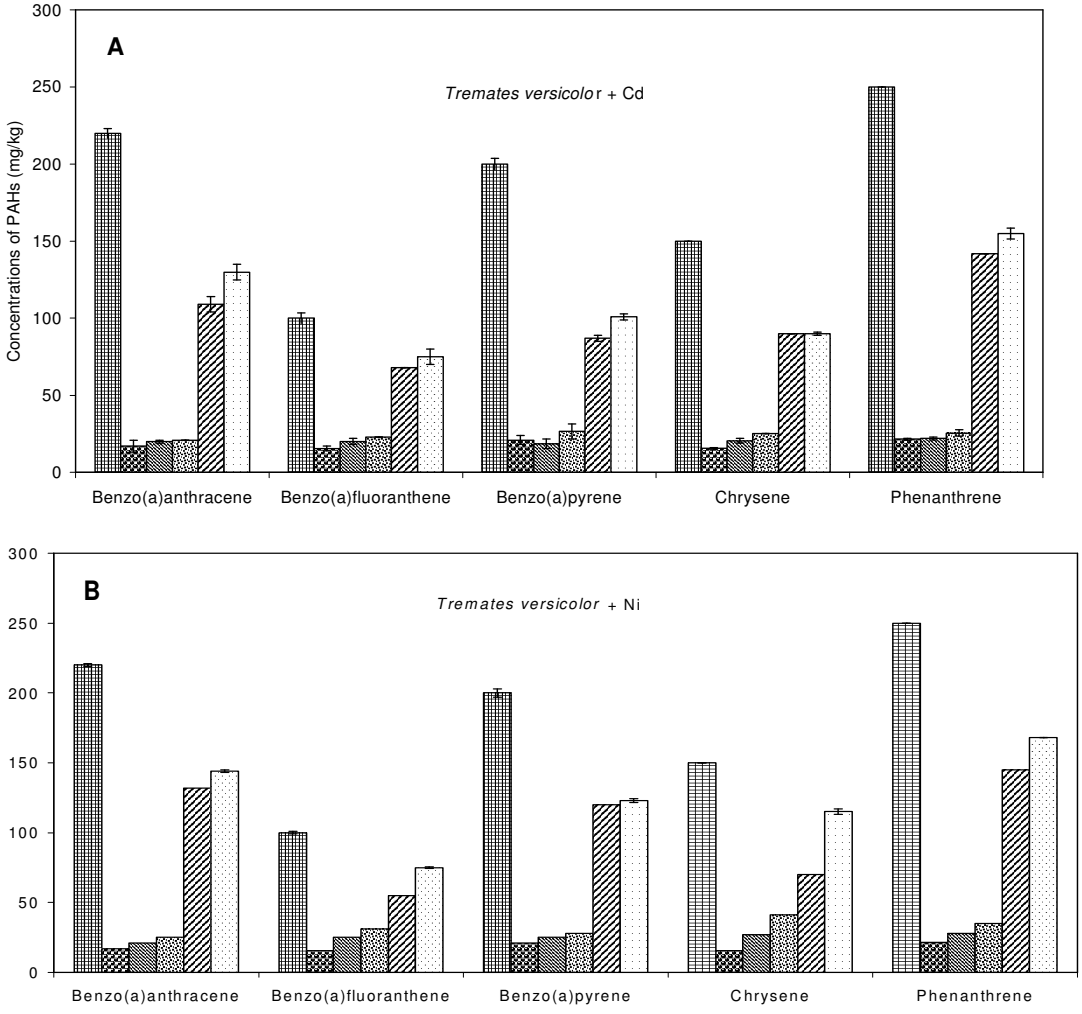
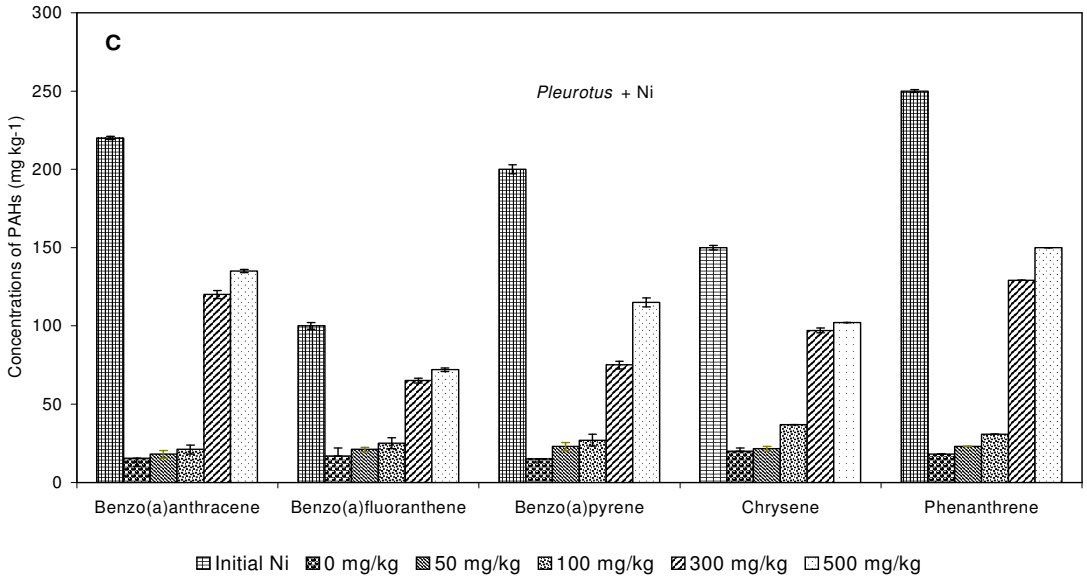


Figure 3a, b, c, d. Final concentrations of PAHs in treatments inoculated with the non-ligninolytic fungi and amended with different concentrations of heavy metals after 10 weeks. Values are means of two ± 1 Standard Error.





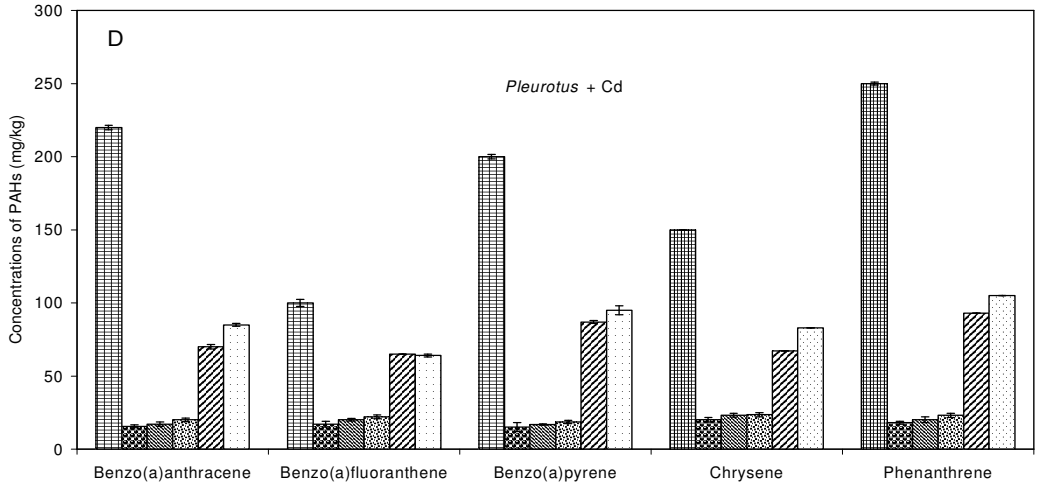
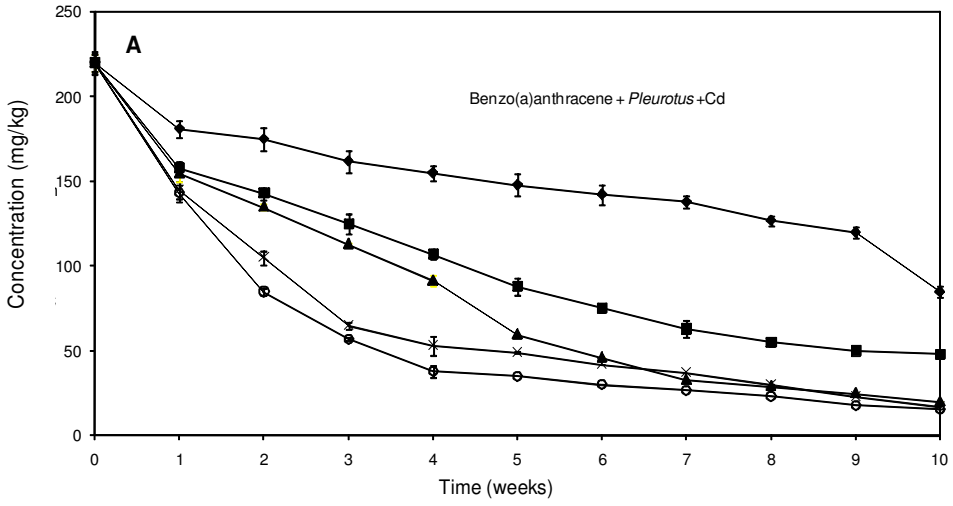


Figure 4a, b, c, d. Final concentrations of PAHs in treatments inoculated with the ligninolytic fungi and amended with different concentrations of the heavy metals after 10 weeks. Values are ± 1 Standard Error.



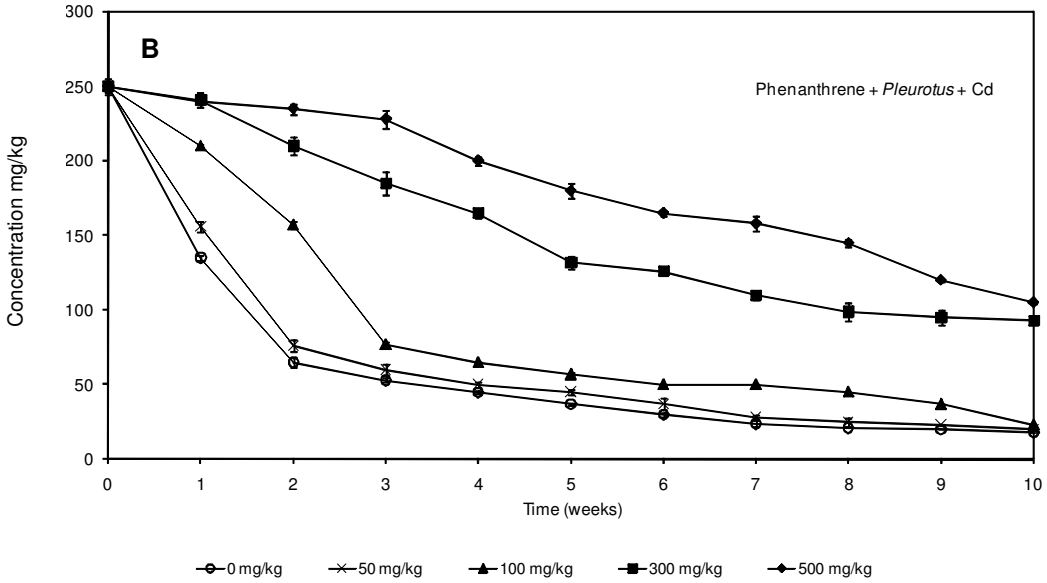


Figure 5a, b. Weekly changes in the concentrations of benzo(a)anthracene and Phenanthrene in the treatments inoculated with *Pleurotus* and amended with different concentrations of cadmium. Values are means of two ± 1 Standard Error.

concentrations applied. There was no significant difference between the effects of cadmium and those of nickel on the degradation of the PAHs by the tested fungi. At 50 and 100 mg kg^{-1}, both heavy metals did not show any significant inhibitory effects on the degradation of the PAHs by the fungi. The effects of the heavy metals at 300 and 500 mg kg^{-1} soil were more visible in the treatments with non-ligninolytic fungi than those with the ligninolytic fungi. The inhibitory effects of the heavy metals at 300 and 500 mg kg^{-1} were more pronounced on the degradation of the higher molecular weight compounds than the lower molecular weight ones.

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