

# IMPACT ON METAL BIOAVAILABILITY AND PLANT UPTAKE DURING THE BIOREMEDIATION OF A PHENANTHRENE-CONTAMINATED SOIL

## Impacto en la Biodisponibilidad y Absorción de los Metales en las Plantas durante la Biorremediación de Suelo Contaminado con Fenantreno

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### SUMMARY

The impact on bioavailability (Pb, Ni, Cu) behaviour was assessed prior and subsequent to fungal bioremediation of phenanthrene contaminated soil. Metal fluxes were assessed by diffusive gradients in thin-films (DGT) and metal uptake was quantified in roots and leaves of *Echinochloa polystachya* and *Triticum aestivum*. DGT metal fluxes were found to significantly increase (at the 95% confidence level  $\alpha = 0.05$ ,  $\alpha = 0.001$ ) after the addition of fungus in the presence of plants. Plants mobilized significantly less metals without fungus, although plants did cause the fluxes to increase above background levels in the presence of phenanthrene. Fluxes were increased significantly, approximately  $0.05 \text{ pg cm}^{-1} \text{ s}^{-1}$  for Cu and Pb and  $0.1 \text{ pg cm}^{-1} \text{ s}^{-1}$  for Ni before bioremediation and approximately  $0.5 \text{ pg cm}^{-1} \text{ s}^{-1}$  for Cu and Pb and  $1.2 - 2.0 \text{ pg cm}^{-1} \text{ s}^{-1}$  for Ni after fungal addition. The two plant species showed higher metal uptake in the presence of fungus than in its absence, which corresponds with DGT fluxes. Nevertheless, while DGT responded immediately to uptake, plant uptake does not start immediately. For both Cu and Pb, uptake was fairly low for nine days after fungal addition before increasing rapidly over the last six days, while Ni accumulation was slow over the entire period. However, Ni was freely translocated, whereas Pb was not translocated and Cu was only partially translocated. The results indicate that phenanthrene bioremediation increased the supply of metals to plants but certain inhibitory mechanisms were actively used by the plants to partially reduce metal uptake at high flux levels.

*Index words:* DGT, diffusive gradient in thin-film, PAHs, polycyclic aromatic hydrocarbons, metal uptake.

### RESUMEN

Se estudió el impacto en la biodisponibilidad del Pb, Ni y Cu, antes y después de la biorremediación del fenantreno en suelo empleando un hongo. Los flujos metálicos se establecieron mediante la técnica de diffusive gradients in thin-films (DGT), mientras que el contenido metálico en *Echinochloa polystachya* y *Triticum aestivum* se cuantificó en raíces y hojas. Los flujos metálicos aumentaron significativamente (a 95% de confianza,  $\alpha = 0.05$ ,  $\alpha = 0.001$ ) después de la adición del hongo y en presencia de las plantas. Las plantas movilizaron significativamente menos metal en ausencia del hongo, aunque en presencia del fenantreno, causaron un incremento en el flujo, superior al de las concentraciones de fondo. Los flujos se incrementaron significativamente de  $0.05 \text{ pg cm}^{-1} \text{ s}^{-1}$  para el Cu y Pb y  $0.1 \text{ pg cm}^{-1} \text{ s}^{-1}$  para el Ni antes de la biorremediación a  $0.5 \text{ pg cm}^{-1} \text{ s}^{-1}$  y  $1.2 - 2.0 \text{ pg cm}^{-1} \text{ s}^{-1}$ , respectivamente después de la adición del hongo. Las dos especies de plantas mostraron mayor absorción del metal en presencia del hongo que en su ausencia, lo cual corresponde con los flujos medidos. Sin embargo, mientras que la técnica de DGT respondió inmediatamente a la toma del metal por parte de la planta, no simuló el retraso de la planta al tomar el metal. La toma del Cu y el Pb se realizó nueve días después de la adición del hongo y aumentó rápidamente durante los seis días siguientes, mientras que la acumulación del Ni fue lenta durante todo el período estudiado. Sin embargo, el Ni, a diferencia del Pb, se desplazó a tallo y hojas; por su parte, el Cu fue parcialmente desplazado. Los resultados indican que la biorremediación del fenantreno aumentó la biodisponibilidad de los metales a las plantas, sin embargo, se utilizaron activamente ciertos mecanismos inhibitorios para reducir parcialmente la toma del metal durante los altos flujos metálicos.

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**Palabras clave:** DGT, gradiente de difusión en membrana delgada, PAHs, hidrocarburos poliaromáticos, absorción de metales.

## INTRODUCTION

The contamination of soil by organic pollutants has become an important environmental problem in recent years (UK Environment Agency, 1999) and much time, effort and money has gone into research of this topic. Much of this research has been concerned with the identification of suitable treatments using chemical and biological methods (Merkl *et al.*, 2005; Cunningham *et al.*, 2004) and more recently engineered nanoparticles have been investigated as a possible remediation tool (Tungittiplakorn *et al.*, 2004). Bioremediation using plants, bacteria or fungi have been used extensively to investigate the removal of organic pollutants and there has been a great deal of success in these methods (Riser-Roberts, 1998; Merkl *et al.*, 2005). Indeed, many of the breakdown products from fungal bioremediation have been shown to be less mutagenic compared with the parent compounds for instance (Riser-Roberts, 1998).

Nevertheless, the impact of the bioremediation process is rarely studied on the whole system. Soil contamination with pollutants such as polyaromatic hydrocarbons (PAHs), including phenanthrene, is always accompanied by the presence of naturally occurring metals such as Cu, Ni and Pb in the solid phase. We have previously shown that the fungus *Penicillium frequentans* effectively reduces phenanthrene concentrations in soil (Amezcuá-Allieri *et al.*, 2003). Subsequently, we demonstrated that this bioremediation method increased the concentrations of labile metal species (Amezcuá-Allieri *et al.*, 2005a). Bioavailability is therefore likely to be enhanced by the increase in mobility of naturally occurring metals due to the bioremediation of organic pollutants. However, our previous work did not show this link directly. In this paper, we report some results which show that the bioremediation process changes chemical behaviour of selected metals and subsequently enhances mobility and plant uptake.

## MATERIAL AND METHODS

Soil was sampled from Tabasco, Mexico; the treatment and analysis have been previously documented (Amezcuá-Allieri *et al.*, 2003). In brief, total metal concentrations in the solid phase were 59, 32, and

14 mg kg<sup>-1</sup> for Cu, Ni, and Pb, respectively. Concentrations of PAHs were below the detection limit (0.002 mg kg<sup>-1</sup>) prior to spiking. Soil pH was 5.7, the CEC 5.1 cmol kg<sup>-1</sup> and organic matter was 6.8% and N 0.3%.

The experimental design regarding fungal growth and DGT experiments have been previously documented (Amezcuá-Allieri *et al.*, 2005a). In brief, 0.04 g of *P. frequentans* (vegetative mycelium in pellets) was added to 0.8 g of sugarcane bagasse and incubated in the dark in sealed, sterilised vials for 15 days at 26 °C.

Two types of plants were grown on clean soil (containing low levels of both phenanthrene and metals). The first plant was a grass, *Echinochloa polystachya*, and was propagated vegetatively. The second one was wheat, *Triticum aestivum* L., which was propagated by seeds under shade, using a Saturno S-80 variety certified seeds produced by Productora Nacional de Semillas (PRONASE), Secretaría de Agricultura, Ganadería y Desarrollo Rural, México.

Separately, at day one, soils were prepared in glass tanks, with soil water and C:N:P ratio optimized (moisture content of 40% and a ratio of 60:1:0.2) for DGT and fungal bioremediation. These levels were maintained by the addition of water every 1-2 days, as necessary. After germination/vegetative propagation, the plant material, along with DGT devices, were transplanted into the soil and covered under special cover to prevent atmospheric contamination. The tank contained non-sterilised soil (including soil microflora) under different treatments. The following treatments were used: Treatment 1 contained S + F + P + Pl, Treatment 2 S + F + Pl, Treatment 3 contained S + P + Pl, Treatment 4 contained S + Pl and Treatment 5 S only, where F is the fungus *Penicillium frequentans*, P is phenanthrene, Pl are plants and S is the soil.

Metal behaviour was evaluated by use of DGT and filtration and by analysis of plant root and leaf tissue, all measured as a function of time. DGT has been fully described elsewhere (Davison *et al.*, 2000a). In brief, metal species freely diffuse through a layer of hydrogel and are then immobilized in an underlying layer of binding agent. As the thickness and area of the gel are well characterized, fluxes to the DGT device can be calculated, which can be used as a measure of the resupply of the solid phase, from either the solid phase or from diffusive movement elsewhere in the system (Davison and Zhang, 1994). Total concentration is not measured, but only free metal and dynamic metal

complexes capable of the penetrating the gel (Van Leeuwen, 1999; Van Leeuwen *et al.*, 2005).

The DGT units were placed on the surface of the soil inside a covered glass tank, making sure that each unit was in full contact with the soil. The relevant plants were also grown in these tanks, also covered to prevent atmospheric contamination. Every three days, over the 30-day period in which the experiment was run, DGT units, soil solution and plant root and leaf samples (the latter from day 15 to day 30) were collected (all in triplicate). Since the sampling was periodic, DGT units were retrieved from the soil and rinsed with pure water. As reported in the literature (Davison *et al.*, 2000a), the Chelex resin was retrieved, rinsed and was completely immersed in 1 mL of 1M HNO<sub>3</sub> for 24 h to extract the metal into solution. The solution was diluted as necessary and measured. DGT fluxes were obtained according to Zhang *et al.* (1998). For filtration, soil solution samples were extracted by centrifugation at 28 960 g for 21 min. The resulting soil solutions were filtered through 0.45 µm millipore cellulose nitrate membrane, acidified and analyzed for trace metals. For plant tissues, 0.5 g of dried plant (roots or leaves) was digested in 5 mL of concentrated HNO<sub>3</sub> and 50 mL Milli-Q water for 15 min using a CEM Corporation Mars X microwave. The sample and acid were placed in a fluorine-resin coated vessel, sealed and heated in the microwave system under standard conditions until complete digestion. Dilute nitric acid was added to prepare the samples for analysis.

Metal analysis of all samples was performed using a 280 Perkin Elmer graphite furnace atomic absorption spectrometer. The analysis consists of measuring and dispensing a known volume of the sample into furnace. The sample then was subjected to a multi-step temperature program. Purchased stock standards solutions were used. Suitable standards and blanks (an aliquot of reagent Milli-Q water that was treated exactly as a sample) were prepared to measure each trace metals. To prevent cross-contamination, Milli-Q water was injected between samples and measured in the same manner as the samples. If a sample with high concentration was followed by one with a low concentration, the second sample was re-measured. All samples were analyzed in triplicate. Analytical errors were determined by replication of blanks and standards; the analysis of variance was the main statistical method used in the data analysis. The relative standard deviation was 2%.

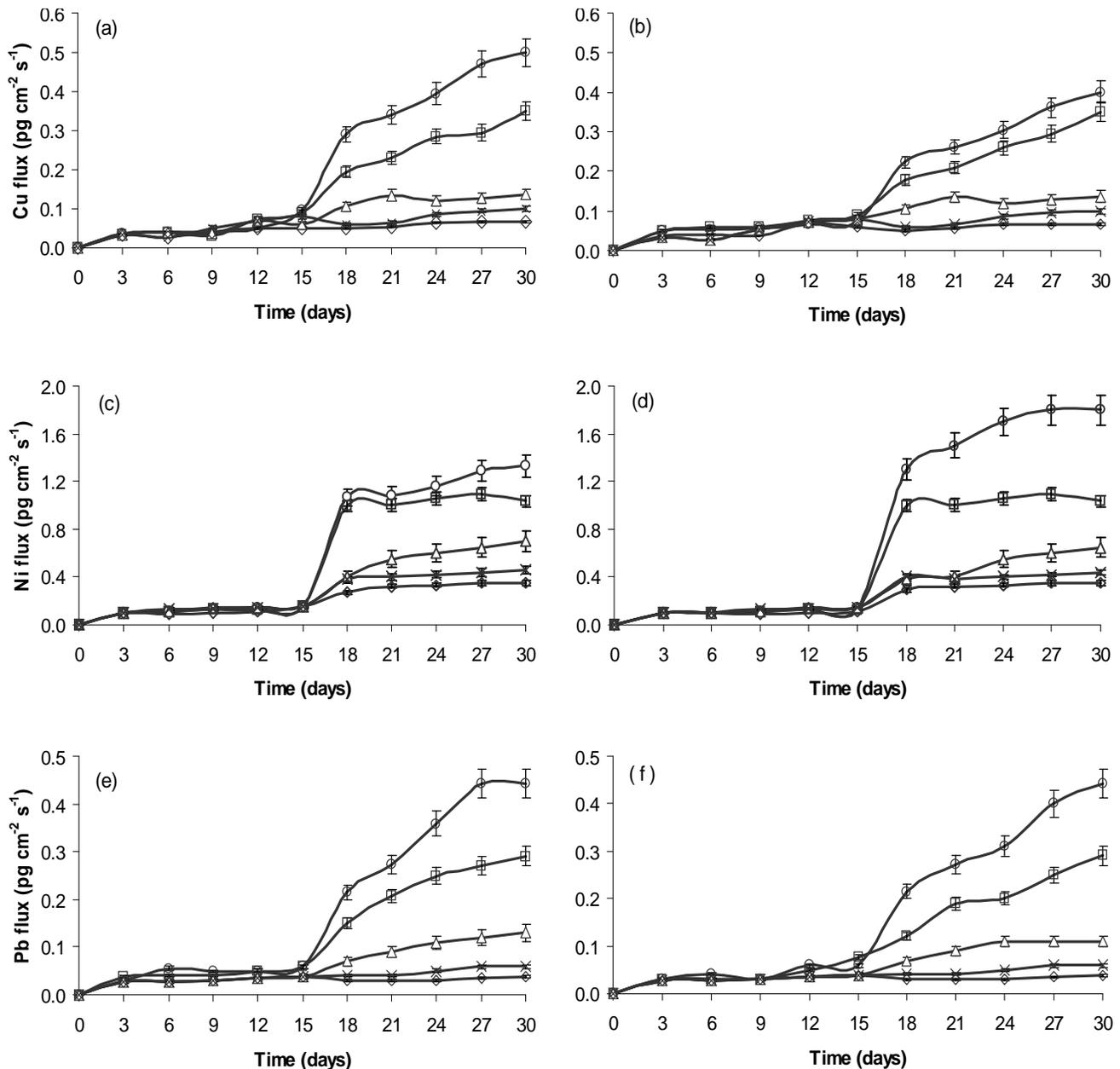
## RESULTS AND DISCUSSION

Previously, we have shown that fungal treatment reduces the concentrations of phenanthrene substantially (Amezcuá-Allieri *et al.*, 2005a,b). In the work reported here, with plants present, fungus and plants, fungus alone and plants alone all are capable of reducing concentrations of phenanthrene by 77, 73 and 67%, respectively (data not shown) from the initial concentrations. We have thus shown that plants and fungi alone or together are capable of bioremediation, although plants show a somewhat lower efficiency for this.

As reported in our previous work (Amezcuá-Allieri *et al.*, 2005a), soil pH values change over a small but significant range from 5.7 at T = 0 to 5.4 after 30 days. The relatively small degree of change is likely because of the natural buffering of the soil and of the added growth media. Nevertheless, at this pH range, we might expect substantial relevant changes in metal chemistry, with alteration in the solid-solution distribution of the metal. However, these small changes were observed in controls also (without fungus and without both fungus and plants). Therefore any changes in metal behaviour cannot simply be explained by changes in the bulk pH of the soil samples.

Trends in Cu, Ni, and Pb DGT fluxes are shown in Figure 1. All graphs show essentially similar trends, with DGT derived metal fluxes (used here as a measure of the resupply of the solution phase after depletion of the solution by the DGT device) all low and fairly constant over the first 15 days in the absence of fungus. The flux values compare well with those previously derived for soils measured by DGT (Davison *et al.*, 2000b). Fluxes were approximately 0.05 pg cm<sup>-1</sup> s<sup>-1</sup> for Cu and Pb and 0.1 pg cm<sup>-1</sup> s<sup>-1</sup> for Ni before bioremediation i.e. up to day 15, and approximately 0.5 pg cm<sup>-1</sup> s<sup>-1</sup> for Cu and Pb and 1.2 – 2.0 pg cm<sup>-1</sup> s<sup>-1</sup> for Ni after fungal addition i.e. from day 15-30, and these changes are significant ( $P < 0.01$ ). The results confirm previous results in the absence of plants (Amezcuá-Allieri *et al.*, 2005a,b) that fungal bioremediation results in a significant mobilisation of metal from the solid phase.

These trends in metal flux are consistent between plants and between different treatments, although some variation in their absolute values can be seen. In all cases (Figure 1), the presence of fungus stimulates the mobilization of significantly more metal compared to the absence of fungus. In addition, the presence of



**Figure 1.** Metal fluxes measured by diffusive gradients in thin-films (DGT) in non-sterilised soil and in presence of: (a, c, e) *Echinochloa polystachya*; (b, d, f) *Triticum aestivum*. Treatment 1 (O) : Soil + Fungus + Phenanthrene + Plant, Treatment 2 (□): Soil + Fungus + Plant, Treatment 3 (Δ): Soil + Phenanthrene + Plant, Treatment 4 (x): Soil + Plant and Treatment 5 (à): Soil. On day 15 soil + fungus mixed together. Error bars represent standard deviations from three replicate treatments.

phenanthrene also appears to stimulate metal mobility, with plants or plants and fungus present. This confirms our previous results (Amezcuca-Allieri *et al.*, 2005b), where the presence of phenanthrene appears to act as an easily available carbon or energy source for the fungus. This result might be expected from the ability of the fungus to remediate phenanthrene.

In the absence of fungus, metal mobility in the presence of plants and phenanthrene is consistently higher than the metal mobility in the presence of plants only. Indeed, in the absence of both fungus and phenanthrene, metal fluxes (when plants are grown on the soil) are equivalent to fluxes of the soil. It appears that both plant types (or more likely rhizosphere microorganisms) are

stimulated by the presence of phenanthrene to release more metal i.e. phenanthrene is a easily utilized carbon and/or energy source for the rhizospheric organisms, again as expected based on the plants ability to degrade phenanthrene.

DGT fluxes have previously been shown to be analogous to plant uptake (Davison *et al.* 2000b), and these results indicate that plant biouptake of metal will be enhanced by fungal bioremediation and to a lesser extent by plant bioremediation. Nevertheless, the exact mechanism of this change awaits further detailed spectroscopic and microscopic investigation of the solution and solid phases. Solution pH can not be the explanation for this effect as the pH change in the controls, where no change in metal behaviour is observed, as discussed. Given the relatively short time scales, it also unlikely that the bulk solid phase is substantially solubilised and indeed no significant changes were observed. We think that metal mobilisation is due to root or fungal exudates chelating surface bound metal and thus releasing those into solution and/or that plant and fungus alter the pH at the surface of the solid phase, again releasing soil into solution and increasing its mobility.

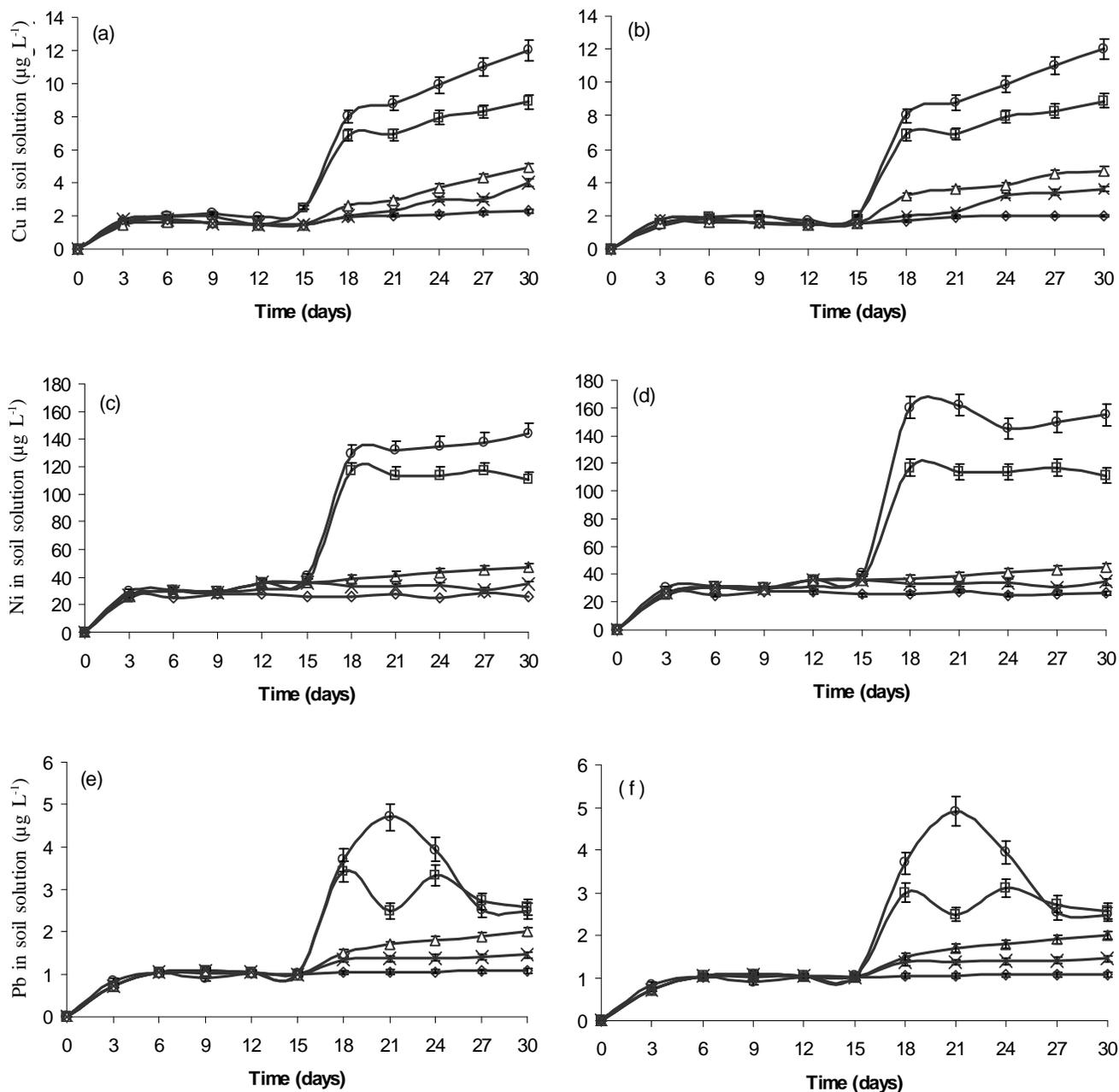
The same experiments were analysed for soil solution concentrations (Figure 2). i.e. the filtrate through a 0.45  $\mu\text{m}$  membrane after syringe filtration. Metal concentrations again increase significantly in all cases after the addition of fungus and phenanthrene. Again both factors may play a role in the increased soil solution concentrations, but fungus is clearly quantitatively more important. Essentially similar trends are observed as with the DGT fluxes, with concentrations increasing by factors of about 4-6 for all metals. Slightly different trends are observed with Pb and this may be due to subsequent re-adsorption of released metal, although higher frequency sampling is needed to investigate this further. Perhaps Cu re-adsorption is expected due to the organic matter value (6.8%). Nevertheless, the results support the DGT results that metal mobility is significantly increased due to bioremediation.

Metal concentrations were measured in roots and leaves from day 15 onwards and results for Cu are shown in Figure 3, for Ni in Figure 4 and for Pb in Figure 5. In all cases, concentrations increased in all treatments over time, with significantly greater uptake from Cu, Pb, and Ni in the treatments containing fungus. Metal uptake in the presence of fungus was about 2-5 times higher than in the absence of fungus, depending on the metal and

plant species ( $P < 0.01$ ). Again the results indicate that bioremediation has a significant and substantial effect on the release and uptake of metals. The relative order of the metal uptake are in agreement with those which might be expected based on the previously observed (Athar and Ahmad, 2002) relative toxicities of these metals to plants. Additionally, there is a significant difference in metal accumulation between species with *E. polystachya* taking up 2-4 times more metal than *T. aestivum*. Further work is needed in order to determine metal behaviour when soil is highly polluted by both metals.

Firstly, the presence of phenanthrene had no measurable effect on metal uptake by plants, although phenanthrene caused a small but significant increase in the DGT measured fluxes. Despite the overall similarities between observed trends, there were differences between DGT fluxes and plant uptake in the response to the addition of fungus and/or phenanthrene. Secondly, while DGT metal response was almost immediate, with significant and substantial increases in metal flux at day 18 (the next measurement period after fungal addition), plant response was delayed several days and, in most cases, remained relatively low. Given that the DGT units and roots operate at similar spatial scales, it seems unlikely that the difference is related to limited diffusional mass transport in this case. This idea is given further weight by the good agreement between DGT and plant response in the literature (Hooda *et al.*, 1999; Davison *et al.*, 2000b), albeit at lower flux values. The difference is presumably due to biological hindrance of metal uptake at higher flux (and concentration) values due to bioremediation, at which the plant may experience deleterious effects. Despite this, metal is subsequently taken up (after day 21-24). For Pb and Cu, flux/biouptake both rapidly increase from day 24 to day 30, while Ni flux/biouptake values appear to be reaching a plateau or only very slightly increasing at the final measurement period. The results indicate that DGT is a reasonable analogue for plant uptake by metal. However, at higher metal flux values which were reaching as a result of the phenanthrene removal, biological processes of defence by plants may be operative against metal toxic effects. These biological processes of uptake resistance cannot be simulated by DGT.

All metals were measured in roots of both types of plants. For Pb, no metal could be measured in leaves, indicating that no Pb translocation had occurred likely due to biological controls as a result of the toxic



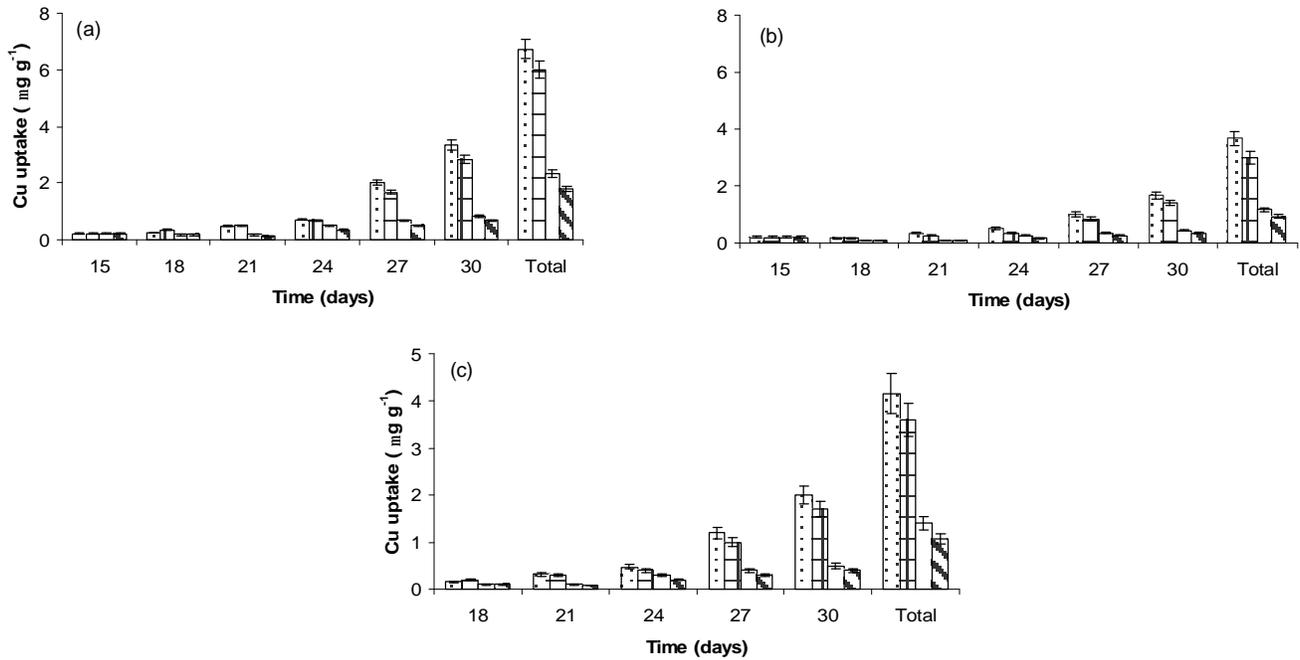
**Figure 2.** Metal concentration measured by filtration in non-sterilised soil and in presence of: (a, c, e) *Echinochloa polystachya*; (b, d, f) *Triticum aestivum*. Treatment 1 (O) : Soil + Fungus + Phenanthrene + Plant, Treatment 2 (□): Soil + Fungus + Plant, Treatment 3 (Δ): Soil + Phenanthrene + Plant, Treatment 4 (x): Soil + Plant and Treatment 5 (◇): Soil. On day 15 soil + fungus mixed together. Error bars represent standard deviations from three replicate treatments.

properties of Pb (Pattee and Pain, 2003). However, Ni was fully translocated and measured in the leaves of both plants. Indeed, in the case of *T. aestivum*, the concentration in the leaves twice as high as in the roots (ca. 6  $\mu\text{g L}^{-1}$  compared with ca. 3  $\mu\text{g L}^{-1}$  in the roots). Cu was an due to intermediate case, with concentrations detectable in the leaves for *E. polystachya* alone. The results again indicate that biological processes may be

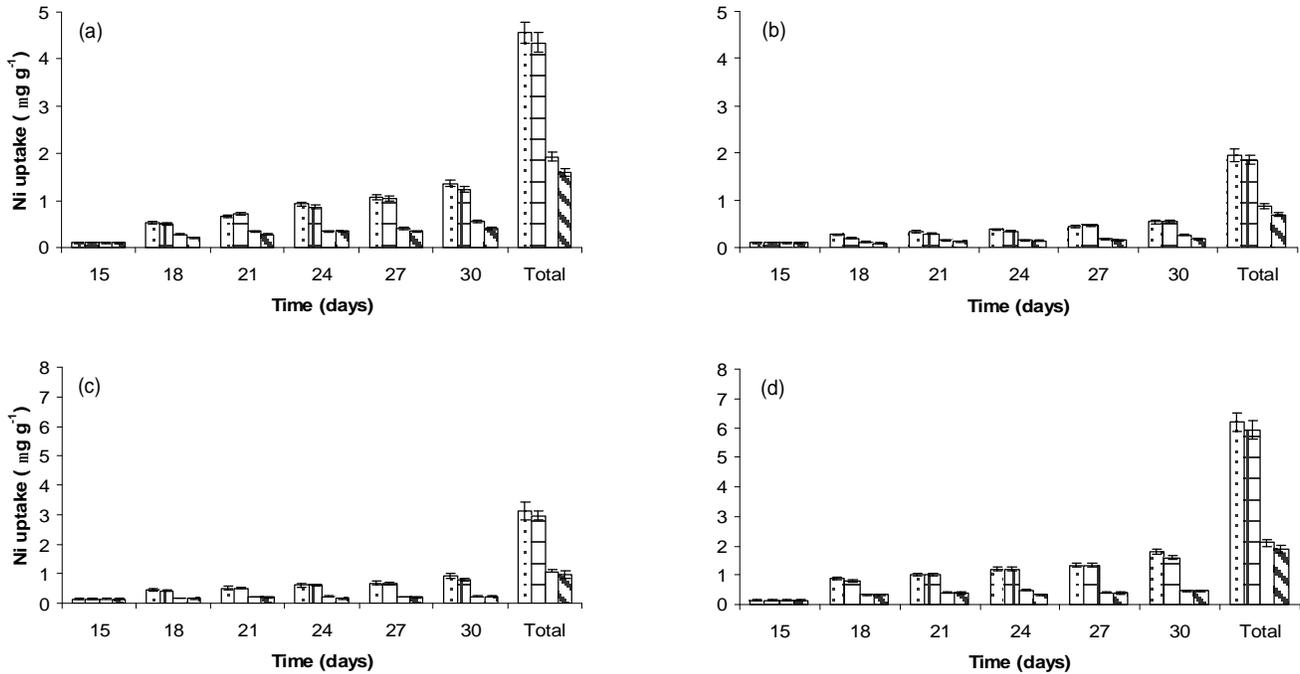
operating somewhat limiting the effects of metals released through the bioremediation process.

## CONCLUSIONS

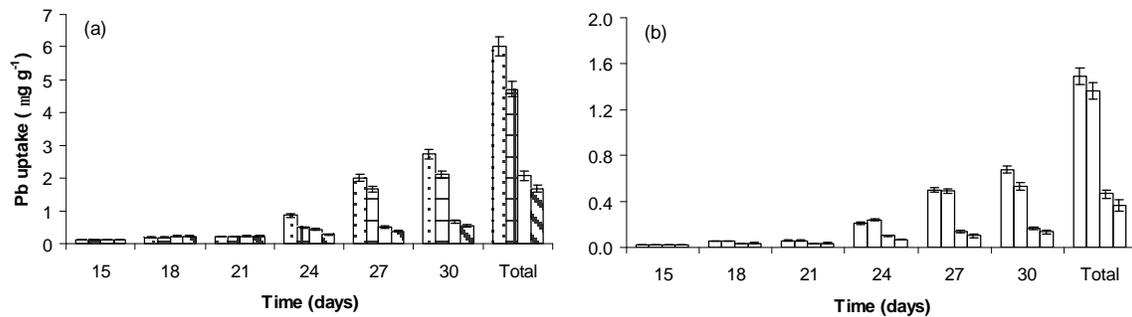
Bioremediation has become an important process in the clean-up of contaminated land and a great deal of research on the optimization of procedures has been



**Figure 3. Copper uptake by *Echinochloa polystachya* in: (a) roots and (b) leaves and *Triticum aestivum* in: (c) roots.** Treatment 1 : Soil + Fungus + Phenanthrene + Plant. Treatment 2 : Soil + Fungus + Plant. Treatment 3 : Soil + Phenanthrene + Plant; and Treatment 4 : Soil + Plant. Treatment 5 is omitted because it only contained soil. Error bars represent standard deviations from three replicate treatments.



**Figure 4. Nickel uptake by *Echinochloa polystachya* in: (a) roots and (b) leaves and *Triticum aestivum* in: (c) roots and (d) leaves.** Treatment 1 : Soil + Fungus + Phenanthrene + Plant. Treatment 2 : Soil + Fungus + Plant. Treatment 3 : Soil + Phenanthrene + Plant; and Treatment 4 : Soil + Plant. Treatment 5 is omitted because it only contained soil. Error bars represent standard deviations from three replicate treatments.



**Figure 5. Lead uptake by *Echinochloa polystachya* in: (a) roots and *Triticum aestivum* in: (b) roots. Treatment 1  $\square$ : Soil + Fungus + Phenanthrene + Plant. Treatment 2  $\blacksquare$ : Soil + Fungus + Plant. Treatment 3  $\square$ : Soil + Phenanthrene + Plant; and Treatment 4  $\blacksquare$ : Soil + Plant. Treatment 5 is omitted because it only contained soil. Error bars represent standard deviations from three replicate treatments.**

completed. Nevertheless, little research has been completed on the wider environmental effects of these remediation processes. Here, it has been shown that fungal bioremediation reduces organic contaminants significantly while it simultaneously increases metal fluxes, soil solution concentrations and plant uptake. Plants alone mobilise less of potentially harmful metals but also show a lower ability to degrade the organic contaminants. This ironic result indicates the importance of considering whole systems, including likely side reactions and side effects when considering the implementation of bioremediation strategies. From these results, phytoremediation looks a more useful strategy than fungal remediation. Plants appear to minimize the effects on toxic metals while removing a large fraction of the phenanthrene. Nevertheless, at least under these conditions, remediation of any recalcitrant organic pollutants may be difficult by plants alone and the effectiveness of the method may be questioned. Further work is needed to discover if this is a general result and to elucidate the mechanisms involved in metal mobility and to investigate the subsequent behaviour of released metal and to perform longer term experiments.

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