THE UNIVERSITY
OF BIRMINGHAM

# IMPACTS OF MICROBIAL ACTIVITY ON TRACE METAL BEHAVIOUR DURING THE BIOREMEDIATION OF PHENANTHRENE-CONTAMINATED SOILS

by

**MYRIAM ADELA AMEZCUA ALLIERI**

A thesis submitted to

The University of Birmingham

for the degree of

**DOCTOR OF PHILOSOPHY**

School of Geography, Earth and Environmental Sciences

The University of Birmingham

2003

Esta tesis es el sueño cumplido de una estudiante mexicana en Reino Unido. Es el resultado de tres años repletos de experiencias, algunas malas, otras muy buenas. Todas ellas me condujeron finalmente a lo más importante… a aprender, no solo desde el punto de vista académico, sino tambien me enseñaron a valorar más a mi país y a crecer como ser humano. Y por supuesto, este logro tiene que estar dedicado a lo mejor que hay en mi vida.

A la adoración de mi vida, a ti mamita adorada. Por tu entrega diaria, tu sacrificio, tu ejemplo de perseverancia y tu tierno cariño que ilumina mi camino cada instante. *Gracias mamá*.

## ACKNOWLEDGEMENTS

This thesis is not just the results of three years of my life, but is something for which I have yearned for all my academic life. Thank you dear God for letting me reach this achievement. Without your help and assistance this crazy dream would have been impossible and now is true.

I owe a debt of gratitude to the ANUIES-SUPERA Mexican program for funding my PhD and IPN for supporting me. My thanks to Dr Jamie R. Lead for your trust, supervision, encouragement and useful discussions through the last three years. Thanks also Dr Refugio Rodríguez-Vázquez, for your advice, support, encouragement and your tender friendship.

I am also grateful to everyone who helped me through my years in Birmingham. People involved are too numerous to list. However, I have been fortunate enough to be assisted in my work, especially by W. Davison and H. Zhang from Lancaster University with DGT methods and analysis, Gael O'Neill for provision of laboratory space and Andy Moss for his helpful advices.

I would like to thank all of those friends who have been encouragement during my PhD in UK. Especially mention is for Dragica and Pero Despinic for all their support, Tim Grapes (who also shared his food with me), AJ and Reyna (for making me realise that dreams can come true), Trena and Thom

Huggins, Sonia (my Paraguayan best friend), Ale, Jo Goodson (always tender and sweet), Pilar (my Colombian best friend), Vicky, Donna, David and Andy for their always-helpful advice and help.

I would like to thank all of those Mexican friends who have encouraged me during my PhD. A special mention for Jorge Meléndez-Estrada and Enrique Molina Franco, without whose help and assistance this dream would have been impossible. Also specially thanks to Jesús Reyes García and Alfonso Aguilar Águila for your help in this academic dream. Also thanks to Lucy, Jaque, Angy, José Luis, Felipa y Edith, Laura, Martha, Toñita, Alfredo Medina, Juan Corona, my dear fellow postgraduates of CINVESTAV, Dr Prieto and Harald for their friendship and encouragement. Thanks to those whose commitment motivate me to reach my objective.

Last, and by no means least, specially thanks go to the most important persons in my life. Thank you dear mother for giving me everything without asking for anything, for your love and your encouragement, for your sacrifice and tender help. Thank you Sadot for your patience and love.

## ABSTRACT

A novel method for the bioremediation of phenanthrene using the fungus *Penicillium frequentans* was utilised to remove phenanthrene (200 mg $kg^{-1}$) from soil containing both metals and phenanthrene, over 29 days. Bioremediation of phenanthrene and its effects on trace metal behaviour has been investigated. Metal behaviour studied includes metal speciation and the kinetics of exchange between solution and solid phase and plant uptake of the more labile and mobile, and potentially more bioavailable metal species. Phenanthrene removal by *P. frequentans* was optimised in terms of both soil water and nutrient composition. Slightly lower removal rates were obtained using *P. frequentans* alone (73%) and plants alone (67%). However, the highest phenanthrene removal (77%) was obtained using both fungus and plant. Assessment of the metal behaviour before and after phenanthrene biodegradation showed that the removal of phenanthrene by either fungal or mixed fungal and native microflora resulted in an increased flux of metal from solid to solution, an increased pool of potentially bioavailable and toxic metal species and increased plant uptake to both *Echinochloa polystachia* and *Triticum aestivum*, by factors of 4-13. In the presence of plants alone, metal mobilisation and uptake increased by smaller factors. In some cases, there was no increase in metal mobilisation and a maximal increase of 2 was found in Ni and Pb. These results highlight the impact of bioremediation process on metal behaviour. In addition, it is suggested that phytoremediation and not bioaugmentation using *P. frequentans* is the best overall option to obtain a considerable phenanthrene removal, reducing the increased pool of potentially bioavailable and toxic metal species.

## NOMENCLATURE

| | |
|---|---|
| AW | Air weight basis of samples (used mainly in relation to plant material) |
| C/N/P | Carbon/Nitrogen/Phosphorous ratio |
| Cd | Cadmium |
| CEC | Cationic Exchange Capacity |
| $CO_2$ | Dioxide carbon |
| Cr | Chromium |
| Cu | Copper |
| DGT | Diffusive Gradients in thin-films |
| DTPA | Diethylene-triamine-pentaacetic acid |
| DW | Air dried or oven dried (up to 70°C) weight basis of samples |
| *E. polystachia* | *Echinochloa polystachia* (alemangrass) |
| EDTA | Ethylene-diamine-tetraacetic acid |
| FIAM | Free-ion activity model |
| FW | Fresh or wet weight basis of samples (used mainly in relation to plant material) |
| GC/MS | Gas chromatography/Mass Spectrometry |
| GFAAS | Graphite Furnace Atomic Absorption Spectrophotometry |
| HS | Humic substances |
| IDM | Initial dry matter |
| $k_d$, $k'_d$ | Rate constants for dissociation of the surface complex |

| | |
|---|---|
| $k_f$, $k'_f$ | Rate constant for formation of the surface complex |
| $k_{int}$ | Rate constant for transport of the metal across the biological membrane |
| M | Metal |
| ML | Metal complex in solution |
| MQ water | Milli Q water |
| M-X- membrane | Surface metal complex |
| $M^{z+}$ | Free ion metal |
| Ni | Nickel |
| NTA | Nitrilotriacetic acid |
| P | Phenanthrene |
| PAHs | Polycyclic aromatic hydrocarbons |
| Pb | Lead |
| PCR | Polymerase chain reaction |
| PDA | Potato Dextrose Agar |
| Pl | Plant |
| *T. aestivum* L. | *Triticum aestivum* (wheat) |
| Treatment 1 | Contained soil, fungus, phenanthrene and plant (S+ F+ P+ Pl) |
| Treatment 2 | Contained soil, fungus, and plant (S+ F+ Pl) |
| Treatment 3 | Contained soil, phenanthrene and plant (S+ P+ Pl) |
| Treatment 4 | Contained soil and plant (S + Pl) |
| Treatment 5 | Contained non-sterilised soil (S) |
| Treatment A | Contained soil, fungus and phenanthrene (S + F + P) |
| Treatment B | Contained soil and fungus (S + F) |

| | |
|---|---|
| Treatment C | Contained soil and phenanthrene (S + P) |
| Treatment D | Contained soil (S) |
| { } | Refers to the concentration of surface species |
| [ ] | Refers to the concentration of dissolved species |

## CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1

# INTRODUCTION

## 1.1 Introductory remarks

Environmental pollution with polycyclic aromatic hydrocarbons (PAHs) has attracted much attention in recent years. PAHs constitute a group of priority pollutants (WHO, 2001), which are present in the soils of many industrially contaminated sites, particularly those associated with the petroleum industry (Samanta *et al.*, 2002). In Mexico soil contamination by the petrochemical industry is the main source of contamination for hydrocarbons in the Southeast part of Mexico (IMP, 1999). Due to the exploitation, refinement, transport, commercialisation and storage of hydrocarbons, both tropical and subtropical soils have been heavily polluted with organic chemicals and certain metals (Battelle, 1998).

Soil contamination with PAHs is often accompanied by the presence of high levels of metals, which are known to be toxic for soil microflora (Ruiz-Duenas *et al.*, 1999). Biological activity is low in soils contaminated with PAHs and heavy metals (Riha *et al.*, 1993). However, the bioremediation of soil contaminated with PAHs has received increasing attention internationally. Bioremediation by bioaugmentation (the supplementation of microorganisms) and biostimulation (the stimulation of microbial degradation by the addition of nutrients) have been used especially for the bioremediation of PAHs.

However, the influence of microbial activity on metal behaviour is still completely unknown.

Phenanthrene was chosen as a model because it is one of the most common PAHs and is considered as a good marker for refined petroleum products (see section 2.2.2). In addition, the behaviour of five different metals will be studied, due to the role and properties of each one. For instance, Ni is one of the most toxic metal to soil microflora and it is often associated with the presence of PAHs, while Cu plays a significant role in several physiological processes (see section 2.5.3 for more detail).

It is worth studying the influence of microbial activity on metal behaviour because if bioremediation affects metal chemistry, it may affect metal biouptake and toxicity. Consideration of such processes emphasises both the complexity of environmental processes and their impacts on pollution problems and remediation procedures. Furthermore, consideration of such mutually dependent processes is necessary, if the most effective *overall* remediation techniques are to be successfully implemented.

## 1.2 Objectives

### 1.2.1 Overall objective

The main objective of this thesis is to study trace metal (cadmium, copper, chromium, nickel and lead) behaviour during the bioremoval of phenanthrene in soils. The behaviour includes metal speciation, the kinetics of exchange between solution and solid phase and bioavailability of metal species to plants.

### 1.2.2 Specific objectives

The specific objectives of this study are:

1) To study phenanthrene removal in soil using a novel bioremediation method.
2) To optimise culture conditions for the removal of phenanthrene in soil.
3) To quantify metal physicochemical behaviour in soil before and after the bioremoval process, considering soil solution fluxes, speciation and concentration.
4) To quantify metal uptake to plants before and after the bioremediation process.

## 1.3 Hypothesis

The bioremediation process increases the concentration of labile and bioavailable trace metals.

## 1.4 Organisation of this thesis

This thesis contains 7 chapters. Chapter 2 contains a literature review on polycyclic aromatic hydrocarbons, biological degradation of phenanthrene and the use of bioaugmentation and biostimulation to enhance phenanthrene bioremoval. There is a discussion of the importance of trace metals, particularly in the study of metal behaviour in soil. Techniques to measure metal speciation, lability and bioavailability are reviewed briefly. The diffusion gradient in thin-films technique (DGT) is discussed in detail, as a speciation measurement of the more labile metal fraction. At the end of Chapter 2, a survey of literature on cadmium, chromium, copper, nickel and lead behaviour in soil is presented.

Chapter 3 describes the experimental procedures used to soil characterisation, phenanthrene bioremediation, metal behaviour in soil during the phenanthrene biodegradation using DGT and filtration. All analytical techniques are fully described.

Chapter 4 contains the results and discussion of the bioremediation process, which include soil characterisation and effects on biodegradation, and the study on phenanthrene removal using the fungi *P. frequentans*. Additionally, the chapter contains the investigation of whether phenanthrene removal could be enhanced by manipulating the carbon-to-nitrogen-to phosphorous

ratio (C:N:P) and moisture content. Once that the main culture conditions for bioremediation process have been established, metal behaviour is studied under these conditions.

Chapter 5 contains the results and discussion of the impact of microbial activity on metal physicochemical behaviour. Heterothrophic activity and phenanthrene removal are discussed in both sterilised and non-sterilised soils and under different treatments. Metal fluxes measured by DGT, metal soil solution concentration measured by DGT and metal in soil solution measured by filtration are presented and discussed for each metal.

Chapter 6 contains the results and discussion of the impact of microbial activity on metal uptake. Phenanthrene removal is discussed in both the presence and absence of *Echinochloa polystachia* and *Triticum aestivum* L. Metal fluxes measured by DGT, metal soil solution concentration measured by DGT, metal in soil solution measured by filtration in addition to metal uptake by both type of plants are presented and discussed for each metal under different treatments.

Chapter 7 presents the conclusions that can be drawn from the work and discusses further possible research needs in this area.

A part of the material in Chapters 3 and 4 has been incorporated into the published paper Amezcua-Allieri, *et al.* (2003). Phenanthrene removal in a selected Mexican soil by the fungus *Penicillium frequentans*: role of C:N ratio

and water content, *J. Soil Sed. Contam. Internat. J.*, **12**, 387-399. This paper has been incorporated at the end of the thesis on Appendix A.

Results from Chapters 3 and 5 have been prepared as two papers and have been submitted to the journals *Environmental Science & Technology* and *Environmental Microbiology*. The details of these two submitted papers are described below.

Amezcua-Allieri, M. A., Lead, J. R. & Rodríguez-Vázquez, R. (2003). Impact of microbial activity on copper, lead and nickel speciation and fluxes during the bioremediation of soil PAHs, *Environ. Sci. Technol.* (submitted).

Amezcua-Allieri, M. A., Lead, J. R. & Rodríguez-Vázquez, R. (2003). Changes of chromium behaviour in soil during phenanthrene removal by *Penicillium frequentans*, *Environ. Microbiol.* (submitted).

Amezcua-Allieri, M. A., Lead, J. R. & Rodríguez-Vázquez, R. (2003). Impact of microbial activity on cadmium speciation and fluxes during the bioremediation of phenanthrene contaminated soils, *Environ. Pollut.* (submitted).

Amezcua-Allieri, M. A., Lead, J. R. & Rodríguez-Vázquez, R. (2003). Phenanthrene biodegradation in the presence of plants (*Echinochloa polystachia* or *Triticum aestivum* L.) and fungi (*Penicillium frequentans*), *Lett. Appl. Microbiol.* (submitted).

# CHAPTER 2

# BACKGROUND

## 2.1 Summary

This chapter reports the main findings found in the literature relevant to the bioremediation of polycyclic aromatic hydrocarbons (PAHs), focussing on phenanthrene. The main pathway of bacterial and fungal biodegradation of phenanthrene is reported.

This chapter also presents a review of the literature related to the importance of trace metals, metal fate and behaviour in soils. Emphasis is put on the problems to measure metal bioavailability and lability in soils. Diffusive Gradient in Thin-Films (DGT) is presented as a suitable technique to assess the potential metal biouptake from soils.

In the last section of this chapter, a review of literature on cadmium, chromium, copper, nickel and lead is presented, mainly related to the metal concentration in soils and plants. Metal speciation and uptake, in addition to metal toxicity and nutrition, is fully described.

## 2.2 Polycyclic aromatic hydrocarbons

### 2.2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of hazardous organic chemicals, made up of two or more fused benzene rings in linear, angular, or cluster arrangements, containing carbon and hydrogen (Cerniglia, 1992). By definition, they contain only C and H atoms, although N, S, and O atoms may readily substitute in the benzene ring to form heterocyclic aromatic compounds, commonly grouped with the PAHs (Wilson and Jones, 1993).

PAHs constitute a group of priority pollutants (WHO, 2001), which are present in the soils of many industrially contaminated sites, particularly those associated with the petroleum, gas-production and wood-preserving industries (Wilson and Jones, 1993). They are formed whenever organic materials are burnt, with temperature influencing the specific mixture of PAHs formed. PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel and mineral production, during the burning of vegetation in forest and bush fires, and also by some plant and bacterial reactions (Riser-Roberts, 1998). However, anthropogenic sources, particularly the burning of fossil fuels are significant sources of PAHs to the environment (Samanta *et al.*, 2002).

Anthropogenic combustion activities are the principal source of PAHs to soils in industrialised countries via atmospheric deposition. As a result, soil concentrations of PAHs have increased over the last 100-150 years, particularly in urban areas (Riser-Roberts, 1998).

Industrial activities associated with PAH production usually involve the processing, combustion, and disposal of fossil fuels, or fossil-fuel-derived products, such as coal tar and carbon black (Nishioka *et al.*, 1986). Fractionating products derived from crude oil, such as diesel, petroleum, fuel oil, lubricating oil, etc. contain PAHs. Sites where refining and distillation have occurred are frequently contaminated with PAHs. PAH contamination on industrial sites is commonly associated with spills and leaks from storage tanks, and disposal of these products. PAH contamination is also associated with wood-treatment activities, since PAHs are major constituents of creosote (Wilson and Jones, 1993).

PAHs are on the U.S. Environmental Protection Agency (EPA) priority pollutants list, since some are known carcinogens and mutagens (WHO, 2001). PAHs are hydrophobic and most are esentially insoluble in water, which contributes to their persistence in the environment (Riser-Roberts, 1998), since they are less easy to degrade than many other organic compounds (Wilson and Jones, 1993).

### 2.2.2 Phenanthrene

Phenanthrene (Figure 2.1) is one of the most common of the class of PAHs. Release of phenanthrene most likely results from the incomplete combustion of a variety of organic compounds including wood and fossil fuels. Phenanthrene is considered as a good marker for refined petroleum products, such as diesel fuel and fuel oil (Douglas *et al.*, 1994).

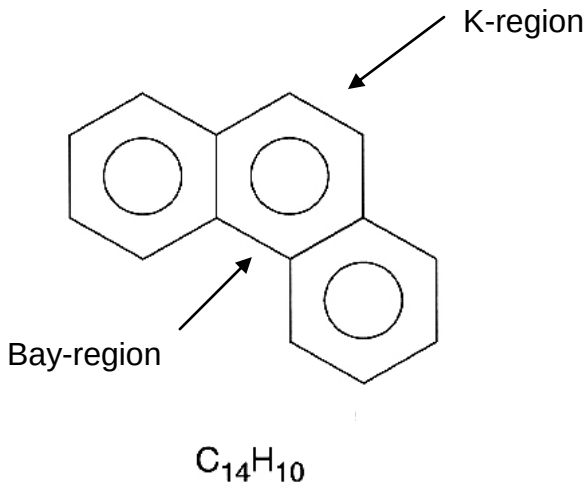



**Figure 2.1** Chemical structure of phenanthrene.

Phenanthrene is a USEPA priority pollutant (WHO, 2001) and is known to be very toxic to aquatic organisms, especially to algae, marine diatoms, gastropods, mussels, crustaceans, and fish. Phenanthrene is considered a mild allergen and mutagenic to bacterial systems (Samanta *et al.*, 2002). The genotoxicity and carcinogenicity for humans of phenanthrene is questionable, according to World Health Organisation (2001). However, phenanthrene is known to be a photosensitizer of human skin (Samanta *et al.*, 2002).

Since phenanthrene is the smallest aromatic hydrocarbon to have a "bay-region" and a "K-region", it is often used as a model substrate for studies on metabolism of carcinogenic PAHs (Riser-Roberts, 1998). In addition, phenanthrene is useful for bioremediation studies (Samanta *et al.*, 2002) because analogues of its structure are found in carcinogenic aromatic hydrocarbons (Müncnerova and Augustin, 1994). The degradation of higher-ring PAHs may also proceed through a phenanthrene intermediate (Cerniglia, 1984). Phenanthrene increases the rate of degradation of highly mutagenic benzo[*a*]pyrene (Cerniglia, 1992).

Phenanthrene and other PAHs are subject to chemical oxidation, photolysis, hydrolysis, volatilisation, bioaccumulation, adsorption to soil particles, and leaching, but microbial degradation is generally the major process in the decontamination of PAHs in the environment (Riser-Roberts, 1998).

## 2.3 Bioremediation

### 2.3.1 Introduction

Bioremediation is a natural or managed process involving biodegradation of environmental contaminants. Biodegradation is the breakdown of organic substances by microorganisms by breaking intramolecular bonds: *e.g.*, involving substituent functional group or mineralisation. As a result, the microorganisms derive energy and may increase in biomass (Riser-Roberts, 1998).

Bioremediation has advantages over thermal and some physico-chemical techniques in terms of cost and because the soil, as a medium available for plant growth, is not destroyed. Costs associated with excavation and disposal of contaminated material are not incurred. Furthermore, the contamination is not transferred elsewhere, as with land filling (Wilson and Jones, 1993; Riser-Roberts, 1998).

The remediation and reclamation of soil contaminated with PAHs has received increasing attention internationally in recent years, with enhanced awareness of the potential adverse effects on human health and the environment. Biological remediation techniques have been used at a number of sites contaminated with organic compounds (Wilson and Jones, 1993).

Microorganisms can totally degrade (mineralise) or partially transform PAHs, through the action of individual microbes or interdependent communities (Gibson and Subramanian, 1984; Cerniglia and Heitkamp, 1989). Bacteria, filamentous fungi, yeasts, cyanobacteria, diatoms, and eukaryotic algae all have the enzymatic capacity to oxidize PAHs that range in size from naphthalene to benzo[*a*]pyrene (Cerniglia, 1984; Atlas and Cerniglia, 1995).

Bacteria employ dioxygenases to incorporate two oxygen atoms into the substrate to form dioxethanes, which are then oxided to cis-dihydrodiols and then dihydroxy products (Wilson and Jones, 1993). In contrast to bacteria, fungi produce cytochrome P-450 monooxygenases to incorporate one oxygen atom into the substrate to form arene oxides, which is followed by

enzymatic addition of water to produce trans-dihydrodiols and phenols (Wilson and Jones, 1993).

Both ligninolytic and non-ligninolytic fungi (with or without the ability to degrade lignocellulose) have the ability to enzymaticaly oxidize PAHs, especially when grown on an alternative carbon source in addition to soil organic matter (Cerniglia, 1992). The biochemical pathway for the degradation of PAHs is of toxicological and environmental significance, since some of the metabolic products have been implicated as carcinogenic, tumorigenic, or mutagenic in higher organisms (Riser-Roberts, 1998).

### 2.3.2 Biological degradation of phenanthrene

Some microorganisms have been exploited for their ability to remove phenanthrene (Table 2.1).

#### 2.3.2.1 Bacteria

Phenanthrene is degraded by some soil bacteria through one of two different routes. In one route (Figure 2.2 a), 1-hydroxy-2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene, which is further degraded via the naphthalene pathway to salicylate, which can be further metabolised. In the other pathway (Figure 2.2 b), the ring of 1-hydroxy-2-naphthoic acid is cleaved and further metabolised via the phthalate pathway. It has been demonstrated that naphthalene and phenanthrene share a common upper metabolic pathway (Kiyohara, *et al.*, 1994).

**Table 2.1** Some microorganisms that remove phenanthrene and other PAHs.

| Microorganisms | Phenanthrene removal rate | Reference |
|---|---|---|
| *Cunninghamella elegans* | 35% | Cerniglia and Heitkamp, 1989 |
| *P. chrysosporium* | 7% in 27 days | Bumpus, 1993 |
| *Penicillium janthinellum* | and benzo[*a*]pyrene pyrene | Launen *et al.*, 1995 |
| *Pleurotus ostreatus* | 3% in 11 days | Bezale *et al.*, 1996 |
| *Aspergillus niger* | and pyrene | Sack *et al.*, 1997 |
| *P. aeruginosa and Penicillium sp* | 69% | Chávez-Gómez *et al.*, 2003 |
| *Pseudomona pickettii and Penicillium sp* | 73% | Chávez-Gómez *et al.*, 2003 |
| *Pseudomona cepacea and Penicillium sp* | 45% | Chávez-Gómez *et al.*, 2003 |
| *P. cepacea and Penicillium sp* | 73% | Chávez-Gómez *et al.*, 2003 |

In addition, the metabolism of phenanthrene by *Streptomyces flavovirens* and the marine cyanobacterium *Agmenellum quadruplicatum* PR-6 is similar to that reported in mammalian and fungal enzyme systems than those catalysed by bacteria (Cerniglia, 1992). Both oxidize phenanthrene to phenanthrene *trans*-9,10-dihydrodiol via a monooxygenase-epoxide hydrolase-catalysed reaction rather than by a dioxygenase. The metabolic formation of 1-methoxyphenanthrene from phenanthrene was first reported in *Synechococcus sp.* PR-6. This organism may detoxify other 1-phenanthrols. The metabolic fate of 1-methoxyphenanthrene in *Synechococcus sp*. PR-6 remains to be elucidated (Ouyang, 2002).

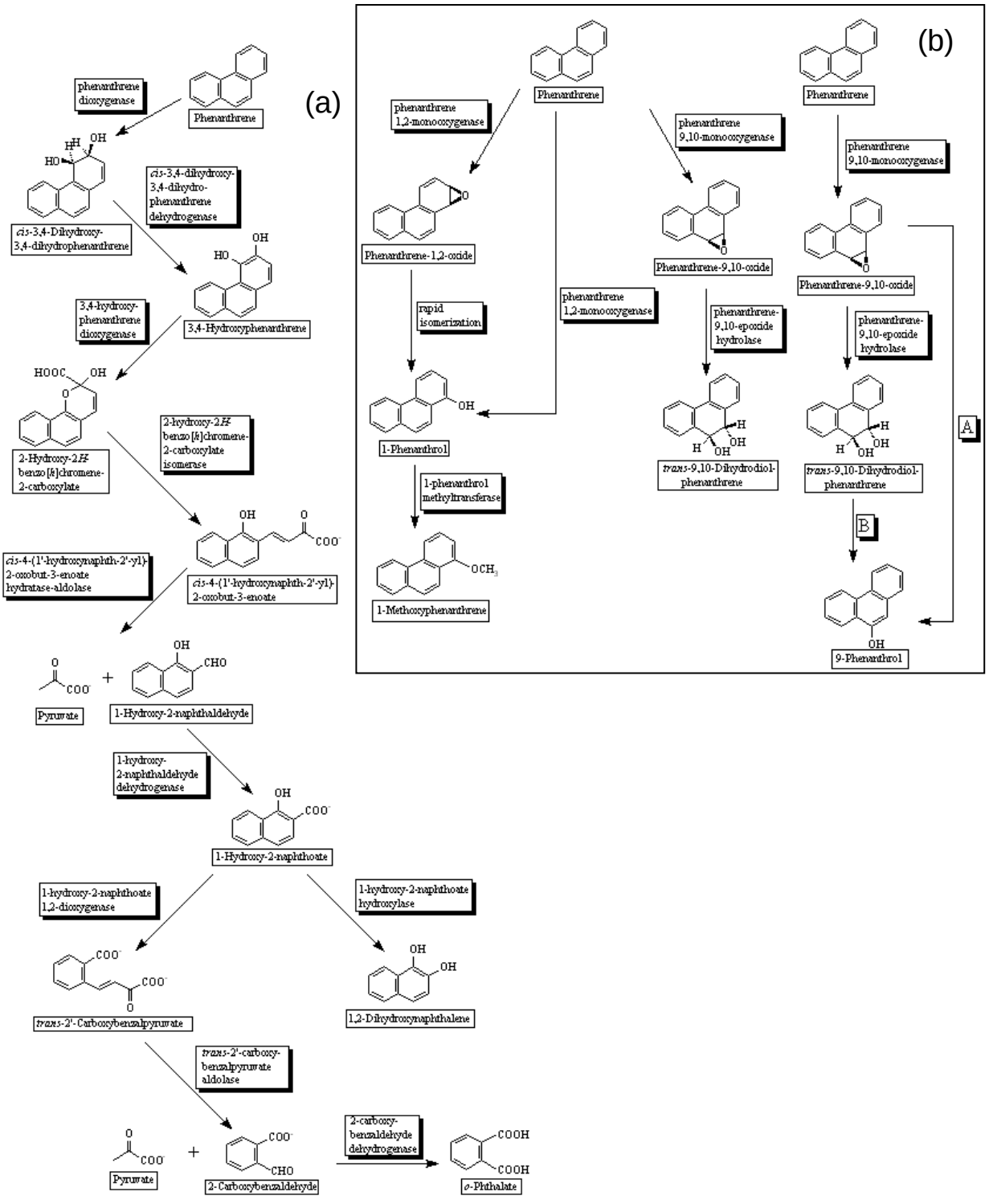



**Figure 2.2** Two different routes (a and b) of phenanthrene degradation by some soil bacteria (The University of Minnesota Biocatalysis/Biodegradation Database, 2002).

**2.3.2.2 Fungi**

Fungi are eukaryotic microorganisms that lack photosynthetic structures and depend upon heterothrophic metabolism (Solanas *et al.*, 1984). Fungi make up a large part of the microbial biomass in soil, especially in acidic conditions, and they contribute to most decomposition processes (Riser-Roberts, 1998).

Fungi play an important role in the hydrocarbon-oxidising activities of the soil, and they seem to be at least as versatile as bacteria in metabolising aromatics (Riser-Roberts, 1998). Several fungi (*Penicillium* and *Cunninghamella*) exhibit greater hydrocarbon biodegradation than bacteria (Atlas and Bartha, 1993). Fungi appear to be predominantly involved in degradation of those xenobiotics of lower water solubility and greater adsorptivity. Soil fungi are generally believed to play a more important role in the formation, metabolism, and interactions of soil-organic matter complexes than bacteria (Cerniglia, 1992). This is because fungi have various bonding mechanisms involved with adsorbed materials (Sutherland, 1992). In fact, most of the fungal transformation products formed by these fungi are less mutagenic than the parent compound (Riser-Roberts, 1998). A wide range of fungi have the enzymatic capacity to oxidise PAHs when grown on an alternative (to soil organic matter) carbon source (Cerniglia, 1992). For instance, *Aspergillus* and *Penicillium* are rich in hydrocarbon-assimilating strains (Sutherland, 1992).

Fungi can also metabolise phenanthrene (Figure 2.3). Non-ligninolytic (*i.e.*, high-N) cultures of *Phanerochaete chrysosporium* fit the typical eukaryotic pattern. They metabolise phenanthrene to *trans*-dihydrodiols and

phenanthrene conjugates. Phenanthrene *trans*-9,10- and *trans*-3,4-dihydrodiols are formed by the successive activities of monooxygenases and epoxide hydrolases. The regiospecificity of *P. chrysosporium* differs from that of *Cunninghamella elegans*, which principally produces the *trans*-1,2-dihydrodiol with smaller amounts of the *trans*-3,4-dihydrodiol. There was no metabolism at 9, 10- positions ("K-region") of phenanthrene as evidenced by the failure to detect *trans*-9,10-dihydrodiols (Cerniglia and Heitkamp, 1989; Müncnerova and Augustin, 1994). The 3-phenanthrols were most likely produced either by dehydration of the *trans*-dihydrodiols or by rearrangement of the postulated arene oxides (reactions: A, B, C and D). Similarly, in *Cunninghamella elegans*, 1-phenanthrol was produced via dehydration of the corresponding *trans*-1,2-dihydrodiols (reaction I). The novel 9-phenanthryl-*beta* -D-glucopyranoside produced by *P. chrysosporium* differs from the 1-phenanthryl-*beta*- D-glucopyranoside produced by *C. elegans*. Those conjugates may be considered to be detoxification products of phenanthrene (Ouyang, 2002).

Unlike non-ligninolytic *Phanerochaete chrysosporium*, ligninolytic *P. chrysosporium* does not accumulate *trans*-dihydrodiols and phenanthrols (Hammel *et al.*, 1992, Müncnerova and Augustin, 1994, Hammel, 1995). It gives 2,2'-diphenic acid (DPA) as a major fate of phenanthrene by 9,10-oxidation and ring cleavage (Figure 2.3, reactions E and F). These two reactions could involve multiple steps. However, no intermediates were reported so far. The oxidation of phenanthrene-9,10-quinone (PQ) to DPA involves both fungal and abiotic mechanisms, and is unaffected by the level

of nitrogen added. Phenanthrene degradation by ligninolytic *P. chrysosporium* (Hammel, 1995) involves both ligninolytic and non-ligninolytic enzymes and is not initiated by a classical microsomal cytochrome P-450. In reaction H, 2,2'-biphenyldimethanol occurred as a minor phenanthrene metabolite which was probably a reduction product of DPA. Both phenanthrene and PQ could be mineralised to similar extents by this kind of fungus (Müncnerova and Augustin, 1994; Ouyang, 2002).

Metabolism of phenanthrene by *Phanerochaete chrysosporium*, *A. quadruplicatum* PR-6, and *S. flavovirens* all produce phenanthrene *trans*-9*S*,10*S*-dihydrodiol as the predominant metabolite. However, the cytochrome P-450 and epoxide hydrolase of *P. ostreatus, C. elegans, and S. racemosum* favour the stereoselective formation of phenanthrene *trans*-9*R*,10*R*-dihydrodiol (Cerniglia and Heitkamp, 1989; Müncnerova and Augustin, 1994; Ouyang, 2002).

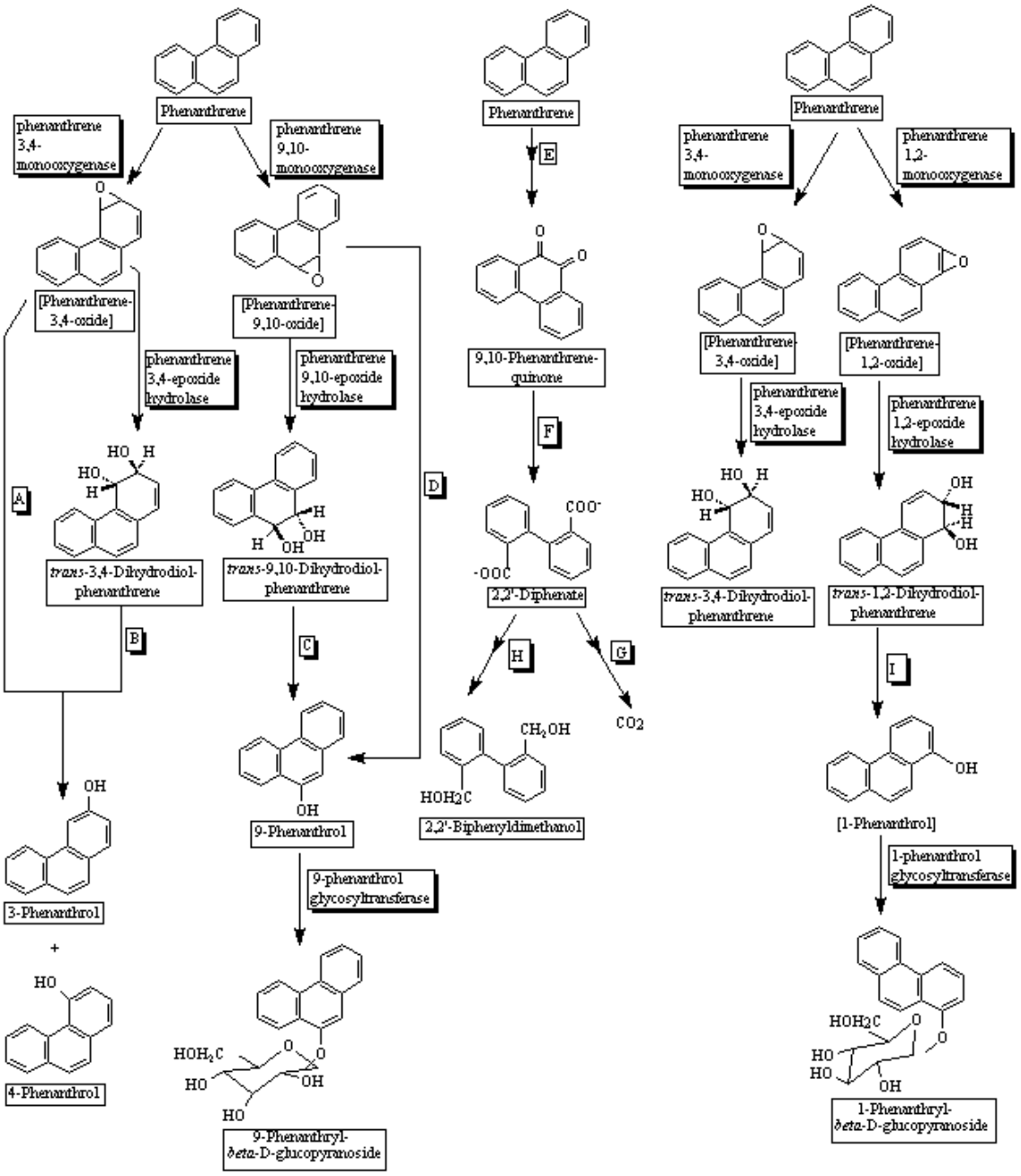



**Figure 2.3** Metabolic pathway of phenanthrene by fungi (Cerniglia and Heitkamp, 1989; Müncnerova and Augustin, 1994; The University of Minnesota Biocatalysis/Biodegradation Database, 2002).

Most of the fungal transformation products formed are less mutagenic than the parent compound and the genus of *Penicillium* is a good hydrocarbon-assimilating strain (Riser-Roberts, 1998). Several fungi (*Penicillium* and

*Cunninghamella*) exhibit greater hydrocarbon biodegradation than bacteria (Atlas and Cerniglia, 1995). Hofrichter and Scheibner (1993) discussed the utilization of aromatic compounds by *Penicillium* strain BI7/2. Hofrichter *et al.* (1995) described the co-metabolic degradation of o-cresol and 2,6-dimethilphenol by *P. frequentans*. Wunderwald (1997) found that the same fungus, using phenol as a sole source of carbon and energy, transformed difluorinated phenol into difluorocatechol. Recently, Pérez-Armendariz *et al.* (2003) isolated *P. frequentans* from bagasse and demonstrated the ability of this fungus to growth in PAHs contaminated soil. The genera most frequently isolated from soils are those producing abundant small conidia, such as *Penicillium* and *Verticillium* spp (Müncnerova, and Augustin, 1994). However, the potential of the species *P. frequentans* to degrade phenanthrene, has received limited attention in the literature (Laborda *et al.*, 1999). For the above reasons the need to study *P. frequentans* for phenanthrene removal in soil is evident, using bioaugmentation and biostimulation.

### 2.3.3 Biostimulation and bioaugmentation

Biostimulation or enhanced biodegradation is the stimulation of microbial degradation of organic contaminants by the addition of microorganisms, nutrients, or optimisation of environmental factors on-site or *in situ*. Bioaugmentation is the supplementation of microorganisms to a contaminated site to enhance bioremediation (Riser-Roberts, 1998).

Addition of an allochtonous microbial population may not be necessary or effective in most cases (Vecchioloi *et al.*, 1990), so before microorganisms

are added to a site, it is necessary to determine if the existing population has the ability to degrade PAHs at the required rate (Andersson and Henrysson, 1996) and if this population is sufficient to be utilised (Maxwell and Baqai, 1995) through biostimulation and bioaugmentation.

During the optimisation of bioremediation process, emphasis is placed on meeting requirements of the microorganisms. Stimulation of indigenous, biodegrading microorganisms does show promise (Atlas and Bartha, 1993). Addition of stimulants, such as electron acceptors, electron donors, and nutrients, should increase biodegradation process (National Research Council, 1993).

Microbial degradation requires the presence of nitrogen, phosphorous, and potassium, in addition to smaller levels of Zn, Ca, Mn, Mg, Fe, and Na, but nitrogen and phosphate are the nutrients most frequently present in limiting concentrations in soils (USEPA, 1985). Addition of nutrient solutions often enhances the ability of microorganisms to degrade PAHs through increased microbial activity (Rogers *et al.*, 1993; Meulenberg *et al.*, 1997; Rodríguez-Vázquez *et al.*, 1999a, 1999b).

There are advantages to relying on indigenous microorganisms rather than added microorganisms to degrade PAHs (USEPA, 1985), as these are most fitted to survival in this environment, available and cheap and also less likely to cause environmental problems. This is particularly true of uncontrolled hazardous waste sites where microorganisms have been exposed to xenobiotic compounds for years (Riser-Roberts, 1998).

## 2.3 Trace metals

### 2.3.1 Importance of metals

Metals are naturally present, but humans have been introducing trace metals into the environment since they first gained knowledge of their many useful properties (Smith, 1992). However, since the Industrial Revolution, metal levels in the environment have increased due to human activity (Nriagu, 1996).

Metals are found throughout the earth, in rocks, soils and sediments and waters. Through natural processes such as weathering and erosion, small amounts of metals are removed from bedrock and are allowed to circulate in water, air and the soil. This process is essential to life because many biochemical processes require a given amount of many of these metals. These metals are therefore essential to life, but may also be toxic at elevated concentrations and fluxes.

With the increase in the living standards and use of technology in the 20th century, large quantities of various metals have been required. Inevitably, as the use of metals increased, so did the pollution associated with it (Smith, 1992). In many cases quantities and fluxes of metals *e.g.* lead, cadmium and chromium introduced into the environment by anthropogenic sources now far exceed natural sources (Han *et al.*, 2001). Mining of ores, smelting and other

purification practices, result in the metal being released from bedrock and sediments into other parts of environment.

Metals are distributed in the atmosphere, water, soil and sediments (Smith, 1992). Atmospheric metal pollution arises mainly from the mining, smelting and refining of metallic ores, the manufacturing and use of metallic products, and the burning of fossil fuels. Atmospheric pollutants are often the largest source of waterborne metals, because of subsequent deposition (Smith, 1992). In rural areas, atmospheric deposition of metals plays a major role. In general, freshwater ecosystems have low natural background metal levels due to natural removal mechanism such as sedimentation (Sigg, 1994) and therefore, tend to be sensitive to even small additions of most trace metals (GPA, 2002). Soil pollution is predominantly derived from decomposition of metal-containing products and the disposal of fly ash. In addition, arable soils receive metal burdens from pesticides, fertilizers and animal waste. Metals tend to accumulate in the biologically active regions of the soil, where crops can take them up (Smith, 1992).

The major problems associated with excessive release of trace metals into the environment are metal toxicity, persistency and accumulation in the environment. In addition, each metal has a specific chemical form (speciation), which help to determine its properties *e.g.* solubility in water and biological availability. Metals have a tendency to indiscriminately bind to enzymatic, electronegative ligands in an organism. These metals, since they are unable to be biodegraded or biotransformed, remain as persistent toxicants within ecosystems and specific food chains (Beeby and Richmond, 2003).

Today, much more is known about the health effects of heavy metals (Edwards, 1996). It is believed that in acute toxicity, binding occurs to the first available ligand. This would imply that the route of administration is critical in determining the effects of acute toxicity. During chronic toxicity, the metal distributes itself throughout the body and preferentially binds to the ligand with the highest binding affinity (Smith, 1992). Cells with high affinity ligands associated with toxicity, are referred to as target cells. Organometallic compounds are able to pass through biological membranes because of their high degree of lipophilicity. Consequently, membranes such as the blood-brain barrier can be permeated (Smith, 1992; Beeby and Richmond, 2003). Thus speciation, biouptake and bioeffects are intimately related.

Metals may act as antimetabolites (eg. substituting for phosphates at sites), may affect permeability of membranes (by binding to or destroying transport sites), or may substitute for an element in some protein compartment (like Li for Na). Some also cause mutagenic effects and reduce the activity of

cytochrome P450, which is involved in the detoxification of foreign compounds (Beeby and Richmond, 2003).

The induction of metallothionien and glutathione in response to exposure to increased levels of trace metals are examples of biochemical process that have evolved to help protect organisms against the problem of high trace metal levels within the body. These proteins contain sulphur groups, allowing them to bind some metals very tightly, thus reducing their toxicity. In cases of acute poisoning, non-specific ligands, known as chelating agents can be administered to help bind free ionic metal (Smith, 1992).

Anthropogenic sources have caused detrimental effects to the natural biogeochemical cycling pattern through excessive processing of mineral ores from the earth's crust (Kabata-Pendias, 2001). Through several processes, stable mineral ores are being mined, thus producing metals that are capable of interfering with biological activities.

### 2.4.2 Metal fate and behaviour in soils

In considering the interactions of trace metals with biota, three areas of concern can be identified (Campbell, 1995): 1) metal speciation in the external environment; 2) metal interactions with the biological membrane separating the organism from its environment, and 3) metal partitioning within the organism and the attendant biological effects.

In order to link speciation of metals in the external environment and their bioavailability, it has been shown that total concentration of a metal is not a good predictor of its 'bioavailability', *i.e.* metal speciation will greatly affect its availability to organisms. This evidence, from a quantitative point of view, has been discussed through the free-ion activity model (FIAM). However, apparent 'exceptions' to the FIAM have been found (Campbell, 1995), perhaps indicating the importance of biological uptake aspects, chemical kinetics and mass transport not include in the model.

Within this general model (Figure 2.4), the interaction of a metal with a microorganism involving the following steps: 1) advection or diffusion of the metal from the bulk solution to the biological surface, 2) diffusion of the metal through the outer 'protective layer', 3) sorption/surface complexation of the metal at passive binding sites within the protective layer, or at sites on the outer surface of the plasma membrane, 4) uptake or 'internalisation' of the metal (Campbell, 1995). Metal take up comprises both metal absorbed at the surface of the organism and metal absorbed, *i.e.*, transported across the

membrane and stored within the organism (Tessier *et al.*, 1994). The biological end points considered include metal bioaccumulation, as well as metal effects. The goal is to predict the bioavailability of a metal as a function of its speciation in the bulk solution (Campbell, 1995).

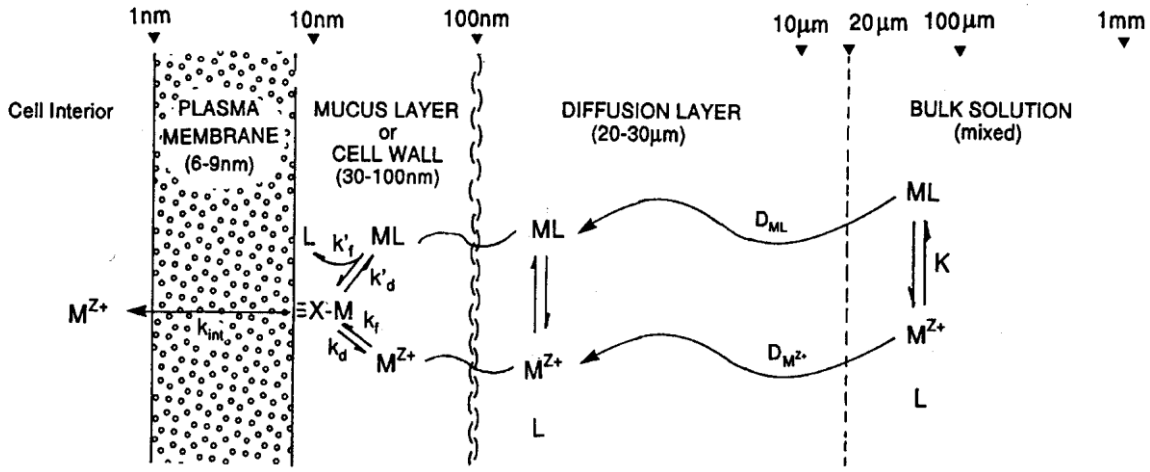



**Figure 2.4** Conceptual model of metal-organism interactions. $M^{Z+}$=free ion metal; ML= metal complex in solution; M-X- membrane= surface metal complex; $k_f$, $k'_f$= rate constant for formation of the suface complex; $k_d$, $k'_d$=rate constants for dissociation of the surface complex; $k_{int}$= rate constant for 'internalisation' or transport of the metal across the biological membrane (taken from Campbell, 1995).

### 2.4.2.1 Free-ion activity model (FIAM)

The interaction of the metal with the cell surface, involving either the free metal ion ($M^{Z+}$) or a metal complex (ML) as the reactive species, can be represented in terms of the formation of M-X cell surface complexes, where $^-$X-cell is a cellular lipid ligand present at the cell surface. In the simplest case,

the free-metal ion is the species reacting at the cell surface, one can envisage the following reaction (Campbell, 1995):

$$\{M\text{-}X\text{-}cell\} = K_2 * K_1 \{^{-}X\text{-}cell\} [M^{z+}] \qquad (2.1)$$

Where $K_1$ and $K_2$ are conditional equilibrium constants, and charges on complexes are omitted for simplicity. { } and [ ] refer to the concentration of surface and dissolved species, respectively. Equation (2.1) indicates that the biological response will vary directly as a function of $[M^{z+}]$.

A considerable body of experimental evidence has accumulated in support of the FIAM in freshwater, but contrasted with those observed in marine organisms. From a close examination of the studies performed, it is clear that the free ion model alone is insufficient to predict the biological response.

Documented examples of experiments, which do not conform to the FIAM, are discussed in detail in Campbell (1995). This lack of conformity may be due to the labile and mobile metal species, which may be important in uptake. Additionally, the chemical kinetics and mass transport properties of the metal-ligand complexes may play a role in metal uptake.

#### 2.4.2.2 Parameters that affect metal speciation, bioavailability and toxicity in soils

Chemical speciation is defined as the distribution of an individual chemical element between different chemical species or groups of species (Tessier *et al.*, 1994). Chemical speciation has been studied in water, but there is a lack in the literature of metal speciation in soil.

Although the modification of properties such as pH may have similar effects on speciation in soil and water, the chemistry of soil can be differentiated from water by slower rates of transport and removal of metals. Hence, build-up of trace metals in soils is of serious concern.

Several factors control the behaviour of metals in soils. Among them pH, cationic exchange capacity (CEC), redox potential, the presence of surfactants and synergism between metals will be described briefly below.

Metal binding to soils and microbial cells increase with increasing pH (Andersen and Engelstad, 1993). Different metals have different solid solution partitioning at different pH values. In acidic soils, metal desorption from soil binding sites into solution is stimulated due to $H^+$ and $M^{z+}$ competition for binding sites (Lasat, 2002). The pH range of 6.5 to 8.0 is the optimum for the formation of insoluble precipitates in many metals and, thus results in the immobilization of certain metals. Contamination by hydrocarbons can change the pH of soil (Amady *et al.*, 1996), from about 4 to 6 in heavy and moderately impacted zones, which is the range of the study soil. Most of the time, pH decreases following soil contamination with PAHs (Riser-Roberts, 1998). Soil pH also contributes to the surface charge on

many particles, affecting metal binding. In addition, the soil pH may affect solubility, mobility, and ionised forms of metals (Amady *et al.*, 1996).

The CEC of a soil is a measure of the quantity of readily exchangeable cations capable of neutralising negative charge in the soil. CEC is an important measure for understanding adsorption and reduction in metal bioavailability. CEC is a measure of sorption sites for metals. Mobile and organic fractions of metals are not traceable in a sandy clay loam soil, probably due to low CEC (Kashem and Singh, 2001).

Redox potential has been found to play an important role in the solubility of Cd (Davies *et al.*, 1987). For instance, a coupled cycle has been established between Cd and Fe solubilization (Forstner, 1987). The concentration of free metal ions in soils will be controlled by a number of processes including exchange of metals with soil binding sites and redox reactions (Ross, 1994). Readily soluble Cr (VI) in soils is extremely toxic to plants and animals. Therefore, the mechanisms of Cr (III)/Cr (VI) redox transformations in soil is of great environmental concern, since the hexavalent state would be easily available to all organisms (Bartlett, 1999). Oxidation or reduction can convert a more toxic form of an element to a less toxic form (Riser-Roberts, 1998).

Surfactants, such as EDTA, NTA and DTPA, have been found to increase the solubility and mobility of metals, carbonates have produced the reverse effect (Alloway and Jackson, 1991), generally by precipitation of solid metal carbonates. The presence of alginic acid and phosphate has been found to

enhance adsorption of metals, possibly due to the formation of ternary complexes on the mineral surface (Ross, 1994).

Synergism and antagonism between metals has been studied and a couple be explored in greater detail (Grahn and Hakanson, 1987). Cd-Zn interactions are commonly observed, but findings appear contradictory, since both depressing and enhancing effects of each have been reported (Kabata-Pendias, 2001). Related chemical and biological effects seems to be similar with Cd and Zn.

Agricultural practice, such as application of chemicals to soil, may play a role in altering soil speciation over time (Miteva *et al.*, 1997).

### 2.4.3 The study of metal behaviour

#### 2.4.3.1 Single and sequential chemical extractions

Single chemical extractions are generally used to arbitrarily determine "available" amounts of soil metals, and usually aim to extract either the water-soluble, easily exchangeable, or the organically bound metals. Other metal fractions may become available over time through chemical weathering or the decomposition of organic matter, but metals occluded by stable secondary minerals are less likely to become available in the short or medium term. Multistep extraction procedures can provide more information about the metals status in soil relative to a single extraction method (Pickering, 1998).

More commonly used to quantify the different fractions of metal retention in soils are sequences of different chemical extractions, usually starting with the weakest and least aggressive chemicals and ending with the strongest and most aggressive. The chemical form determines metal behaviour in the environment and its remobilisation ability (Lena and Gade, 1997). The sequential procedures are based on operationally defined mobile (F1-F3) and immobile (F4-F6) fractions. The reversible fractions where metals are extracted by $H_2O$ or by exchange reactions are defined as a mobile. The immobile fractions refer to the inner-sphere complexes where strong acids or digestion are required to release the fractions of metals (Almås *et al.*, 1999; Salbu *et al.,* 1998).

Methods for assessing the bioavailability of metals in the field often use chemical extraction techniques (Bryan and Langston, 1992). Extraction with EDTA, CDTA and DTPA has been used by a number of studies to measure available metals in soils (Alloway and Jackson, 1991; Hooda and Alloway, 1994; Singh *et al.*, 1998).

Metals removed from chelating agents, such as EDTA and DTPA are thought to represent the plant-available fractions (Mellum *et al.*, 1998). The DTPA extraction data generally supported the results of plant metal concentrations. However, the concentrations of Cd and Pb extracted by DTPA failed to predict the changes in plant metal uptake (Hooda and Alloway, 1993; 1994). Chelating agents form soluble complexes with metals, reducing the activity of

the metals in solution. Therefore, ions desorb from the surface and enter in to the solution. The relative extractability, expressed as the ratio between the chelating agent and aqua-regia extractable contents, has showed contradictory results. Cadmium is reportedly more soluble than other metals (Mellum *et al.*, 1998). The high ratio between the chelating agent and total concentration of metals in contaminated soils indicate that Cd is more available for plant uptake and leaching as the mobile (F1-F3) fraction (Kashem and Singh, 2001). Nickel is the least extractable, which might be due to its high affinity to soil minerals and oxides (Wang *et al.*, 1997). The extractability is higher in contaminated soils than in non-contaminated soils, as was observed in the mobile fractions of sequential extractions.

From the studies above, only the content of soil organic carbon showed a relationship with the metal fractions. However, with many gaps in the understanding of soil processes, it is essential to fully appreciate the role of trace metals in the environment. In addition, it is vital to have a complete description of their chemical speciation and understand the link between metal speciation and bioavailability.

The fractions of single and sequential chemical extractions are operational, and they overlap. In addition, metals solubilised from a fraction may re-sorb to remaining solid phases (Lead *et al.*, 1997). Reason why new techniques to measure metal behaviour have been attracted much attention in recent years.

### 2.4.3.2 Techniques to measure metal bioavailability, fluxes and speciation

The term speciation is used to refer the physicochemical form of a particular element of interest. There are three ways of defining speciation (Lead *et al.*, 1997). Firstly it can be based on the physical attributes of size (or density, electrical charge and shape). Fractionation may be dynamic (*e.g.* filtration) or reflect equilibrium conditions (*e.g.* dialysis). Secondly, it can be based on complex lability and size calculated indirectly from mass transport and diffusion, which are measured by experimental methods involving transport phenomena, such as voltammetry and diffusion gradient in thin-films (DGT). These are defined as a kinetic speciation method. Thirdly, it can be based on free activity (or potential concentration) at equilibrium, which can be measured by ion selective electrodes or batch techniques equilibrating a solution with ion exchange resin. These are defined as equilibrium speciation methods.

There are methods that can incorporate more than one type of speciation. For instance, the gel technique DGT uses the principles of mass transport to an exchange resin (kinetic speciation). However, the gel has a facing filter paper between the resin and the gel and the surrounding water (dynamic fractionation) (Lead *et al.*, 1997). However, the dynamic fractionation is probably less important than the mass transport and diffusion used in DGT. This technique is discussed in detailed at the end of this section.

#### 2.4.3.2.1 Filtration

Filtration is the most commonly used method of fractionation. The separation is based on size after calibration with a standard. However, there are number of complications associated with concentration polarisation and the formation of a gel layer (Lead *et al.*, 1997). For instance, the increase in colloid concentration directly above the membrane surface due to the slow back diffusion of colloids compared to their movement, under pressure, towards the membrane (Buffle *et al.*, 1992). These effects may result in fractionation occurring in a different size range to that expected from the nominal pore size filter (Lead *et al.*, 1997). Furthermore, the actual size range separated may alter over the course of the filtration and is dependent on filter manufacturer and surface area (Horowitz *et al.*, 1996).

Size resolution by filtration can never be entirely satisfactory (Lead *et al.*, 1997). Moreover, only small quantities can be collected because minimization of coagulation at the membrane surface requires low flow rates and the rapid coagulation of colloids in the bulk solution precludes long filtration times (Lead *et al.*, 1997). Despite these problems, or because they are known and be potentially accounted for, filtration is a useful tool for gaining more information on soil solution behaviour. Filtration gives an indication of the solution phase behaviour of metals and performs a different speciation measurement than, for instance, DGT.

### 2.4.3.3 Problems to measure metal lability and bioavailability in soil

The bioavailability and mobility of potentially toxic trace metals largely determines the environmental impact of metal contaminated soils (Davison *et al.*, 2000a). Assessment of the impact of potentially toxic metals in soils is not well known because of poor understanding of both chemical speciation in soil solution and the kinetics of exchange between solution and solid phase (Zhang and Davison, 1995; Zhang *et al.*, 1995; Zhang *et al.*, 1998).

Soil properties, such as CEC and pH, can alter the toxicity of metals by modifying their bioavailability and hence reducing the impact of the total metal loading (Knight and McGrath, 1995). Bioavailability, mobility, and partition through adsorption/desorption and dissolution/precipitation are affected by the variation in solution chemistry in soil. To understand the above processes, it is necessary to consider the chemical forms in solution rather than total dissolved concentrations. Ideally chemical speciation measurements in soil solution should be made *in situ* by procedures, which either minimise disturbance or perturb the solution in a controlled way (Zhang *et al.*, 1998; Davison *et al.*, 2000a).

Metals in soil are either incorporated within discrete phases or exist as surface adsorbed complexes. If metal is removed from solution by, for example, biological uptake, it may be rapidly resupplied from the solid phase. Conventional measurements of bioavailability have been unable to quantify fully this flux from the solid phase to solution (Zhang *et al.*, 1998).

The metal bioavailability has been studied using either ion-exchange resins directly (batch techniques) or resins encapsulated (Apte and Batley, 1995) in,

for example, porous materials or embedded in membranes (Morrison *et al.*, 1987). Results obtained by batch techniques, in which resin and soil are mixed and shaken, are dependent on resin/soil/solution ratios, shaking time and ionic strength, but batch techniques cannot provide detailed information of the transportation of ions from soil to the resin sink, which is important for assessing bioavailability (Zhang and Davison, 1995).

To simulate metal and nutrient movement to plant roots, resin in either bags, capsules and membranes have been used. Although the resin-available fraction correlates with the amount taken up by plants, these procedures are limited (Hooda *et al.*, 1999). Due to the poorly define geometry of the bags and the diffusion layer between solution and resin, the fluxes from soil to solution and from solution to resin cannot be quantified and the results obtained by different workers cannot be compared (Abrams and Jarrell, 1992).

When the measured species depends on the chemical reaction involved and the rate of mass transport from the bulk solution to the reaction site (kinetically based speciation techniques), the measurement disturbs, to some extent, the pseudo-equilibrium that exist between the various chemical species present in the system (Hooda *et al.*, 1999).

Because of the above problems, conventional methods of measuring labile chemical species of trace metals, such as chemical competition following centrifuging, are inadequate if the speciation changes during sampling and

extraction (Hooda *et al.*, 1999). Changes in metal concentration and speciation can occur during sampling, handling and storage prior to analysis. Ideally, trace metals should be measured *in situ*, but few measurements made previously have involved complicated systems and cannot be used routinely (Davison and Zhang, 1994).

*In situ* speciation measurements of trace metal using thin-film gels allow mass transport to be calculated and therefore enable quantitative data on metal fluxes. According to Davison and Zhang (1994), the idea is simply to introduce a known thickness of gel between the solution and the reactive medium (chelex resin).

The relatively new procedure called diffusion gradient in thin-film (DGT) has been developed to measure quantitatively *in-situ* concentration and speciation. DGT is finding wide application in a) determining solute concentrations in waters (Zhang and Davison, 1995), sediments (Zhang *et al.*, 1995) and soils (Zhang *et al.*, 1998; Hooda *et al.*, 1999), b) directly measuring remobilisation fluxes (Zhang *et al.*, 1995; Harper *et al.*, 1998), c) making measurements at high spatial resolution of <1 mm (Davison *et al.*, 1991; 2000a), and d) flux measurement as a surrogate for metal uptake by plants (Davison *et al.*, 2000b; Zhang *et al.,* 2001).

#### 2.4.3.4 Diffusion Gradient in Thin-film (DGT)

The DGT technique separates species kinetically and does not rely on establishment of equilibrium. Solutes freely diffuse through a layer of hydrogel and are then immobilised in an underlying layer of binding agent (*e.g.* chelex). The device is deployed for a fixed period of time and then the mass of accumulated solute in binding layer is measured. Because the diffusion layer thickness during *in situ* deployment is well defined by the known thickness of the gel, the mean concentration and fluxes in solution can be calculated using Fick's laws (Davison *et al.*, 2000a). DGT separation of chemical species occurs based on molecular size and also on the kinetic availability (lability) of species (Davison *et al.*, 2000a). DGT provides a time-averaged measure of the effect of these *in situ* separation processes. In many cases a near steady-state situation is attained during deployment, allowing direct interpretation of the measurement as a mean flux or as a concentration (Davison *et al.*, 2000a).

#### 2.4.3.4.1 Basis of DGT

Ions diffuse into the gel until equilibrium with the pore waters is established (Figure 2.5). Polyacrylamide gel is used which is backed by a further thin film (~150μm thick) of gel containing a cation exchange resin selective for trace metals (Chelex 100) closely packed in a single layer of 75-150 μm spheres (Davison *et al.*, 2000a).

Within the layer of resin, of thickness $\Delta r$, the concentration of the free metal in solution is effectively zero, owing to the complexation by the resin (Davison

and Zhang, 1994). Within the bulk solution the metal has concentration $C_b$. The gel layer is assumed to be separated from the bulk solution by a diffusive boundary layer (DBL), of the thickness $\delta$, where transport is solely by molecular diffusion (Figure 2.5).

According to Davison and Zhang (1994), to be transported from the solution to the resin, ions must diffuse across the DBL and then through the gel, of thickness $\Delta g$. Small ions can diffuse freely through the effectively 2-5 nm pores (Davison *et al.*, 2000a) of polyacrylamide gel with effective diffusion coefficients, *D*, indistinguishable from those in water.

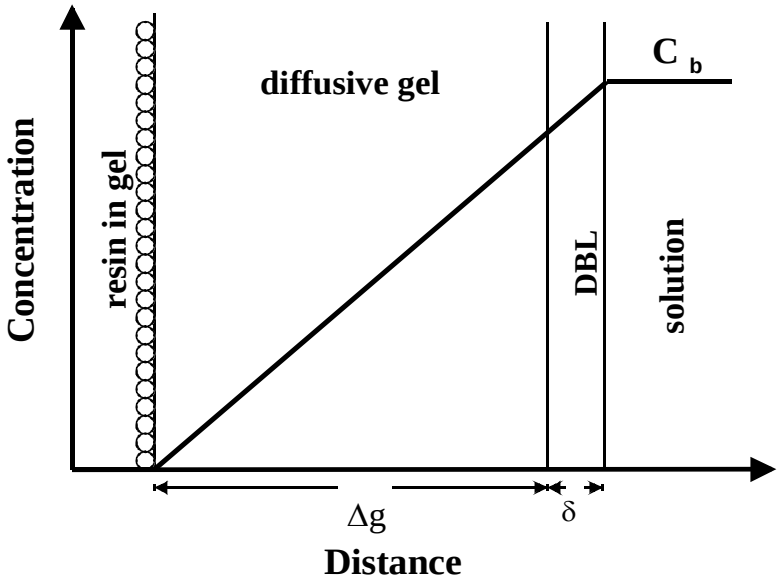



**Figure 2.5** Schematic representation of the free concentration of ionic species in a gel assembly, where the concentration is $C_b$ and DBL is diffusive boundary layer. The diffusive layer is shown as a single layer of gel, but it includes a gel layer and filter (taken from www.dgtresearch.com).

For simplicity, it is assumed that $\delta << \Delta g$, Fick's first law can be used to define the flux of a given metal ion:

$$\text{Flux} = DC_b / \Delta g \qquad (2.2)$$

The mass per unit area of resin, $M_a$, after time t is then:

$$M_a = DC_b t / \Delta g \qquad (2.3)$$

And the concentration in the resin layer, $C_r$, is given by:

$$C_r = M_a / \Delta r \qquad (2.4)$$

After given time, the concentration in the resin layer, $C_r$, can be measured and the concentration in the solution can be quantified by:

$$C_b = C_r \Delta g \Delta r / Dt \qquad (2.5)$$

Providing the resin is not saturated, the longer such a device is immersed, the more metal will be accumulated and its concentration in the resin layer to metal in solution will increase as the thickness of the resin and gel layers are decreased.

Assuming a value for small ions of *D* of $10^{-5}$ $cm^2$ $s^{-1}$, for a 24 h immersion, a gel layer thickness of 1 mm and a resin layer thickness of 0.1 mm, the concentration in the resin layer will be 864 times greater than the

concentration in the bulk solution (Davison *et al.*, 1994). Such a procedure therefore offers a large concentration enhancement for relatively short immersion times (Davison *et al.*, 1994).

The procedure of DGT uses a layer of chelex resin impregnated in a hydrogel to bind the metals. The resin-layer is overlain by a diffusive layer of hydrogel and a filter. Ions have to diffuse through the filter and diffusive layer to reach the resin layer. It is the establishment of a constant concentration gradient in the diffusive layer that forms the basis for measuring metal concentrations in solution quantitatively (Davison *et al.*, 2000a).

Equations (2.2)-(2.5) depend on the assumption that the thickness of the DBL between the gel and the bulk solution is negligibly small. The only available information on DBL thickness in natural waters applies to the sediment-water interface (Davison and Zhang, 1994). If the gel layer is 1 mm thick, variation in $\delta$ of 0.1 mm could at most result in a change in flux to the chelex of 10%. By ensuring that the gel layer is sufficiently thick, the DGT technique can control the mass transfer of metal ions irrespective of changes in the velocity of water in the bulk solution (Davison *et al.*, 2000a).

#### 2.4.3.4.1.1 DGT as a speciation measurement

In principle three factors determine which species are measured by DGT: the binding agent, the diffusion layer thickness and the pore size of the gel (Davison *et al.*, 2000a). Chelex-100 has very strong binding groups, at high

effective concentration, which will out-compete most other ligands for metal ions, especially at the low concentrations of organic components.

Metal is continuously removed from solution to the resin during DGT deployment. If metal-ligand complexes rapidly dissociate they will contribute to this flux, but if they are kinetically inert they will not. The time available for this dissociation is roughly the time taken for the metal to diffuse through the gel layer, which is determined by the gel layer thickness (Zhang and Davison, 1995). For a typical 0.4 mm thick gel it is about 2 min (Davison *et al.*, 2000a). Consequently only labile (dissociation time < 1 min) metal complexes are measured. If inert species can bind directly to the resin they will also be measured by DGT (Davison *et al.*, 2000a).

Recently Scally *et al.* (2003) demonstrated for the first time, the validity of the assumption that only the free metal ion and not the metal complex, reacts with the binding resin of the DGT device. DGT therefore has the potential to distinguish between adjunctive and disjunctive mechanisms of complex dissociation.

Any metal ion or metal complex measured by DGT has to diffuse through the pores of the gel. The agarose-derived cross-linked polyacrylamide gel composition has a very open structure which allows the free diffusion of simple metal ions, the diffusion coefficients of simple cations in the gel are indistinguishable from those in waters (Zhang and Davison, 1995; Davison *et al.*, 1994; Zhang and Davison, 1999). Zhang and Davison (1998) have shown

that humic substances (HS) extracted from soils diffuse through this composition of gel. However, as its molecular diffusion coefficients are appreciably less than of simple metal ions, the transport of the metal humic species to the resin layer in DGT will be retarded compared with simple metal ions. Therefore if DGT with an open gel as the diffusion layer was used to measure metal ions in an unknown soil, the proportional contribution of any humic complexed metal would not be known. Lead *et al.* (2003) demonstrated that diffusion coefficients of humic substances in agarose gel are 10-20% lower than in water. In addition, diffusion in aggregates is completely retarded. DGT is therefore related to both mobility (by diffusion through the gel) and lability (by chelex/ligand competition for the metal) of metal ligand complexes.

A polyacrylamide hydrogel comprising 15 vol % acrylamide (Boehringer) and 0.3 vol % patented agarose-derived cross linker was used throughout this work as the diffusive gel layer. This type of gel let simple metal ions (tested for $Cd^{2+}$ and $Cu^{2+}$) diffuse freely, while retarding the diffusion of fulvic and humic species. In this DGT device, which adopts such a restricted gel, only small, labile species in solution are measured. Simple organic species, such as acetate complexes, would be included in this small labile fraction. In practice, DGT response is likely to be dominated by simple inorganic species (Davison *et al.*, 2000a), but there is not any experimental evidence.

The free ion metal concentration may differ from the total dissolved concentration because there may be metal in solution bound in complexes.

Although metal fulvic acid complexes could be non-labile at extremely low metal concentrations when strong binding sites dominate, they are likely to be labile for most practical situations (Davison *et al.*, 2000a). Therefore, DGT may be expected to bind free metal and metal bound to small inorganic and organic complexes. Metal bound to larger colloids or more strongly bound is likely to be unavailable.

**2.4.3.4.1.2 DGT saturation**

Despite theoretical sophistication in DGT theory, there are a number of practical limitations, which are discussed below.

DGT has been shown to behave in accordance with the theory provided the capacity of the resin to bind ions is not exceeded (Davison *et al.*, 2000a). If a non-selective binding agent is used, other more common ions will quickly accumulate and saturate the binding sites. For instance, in natural waters a linear response between measured metal mass and time was only found for a few hours in seawater (Chang *et al.*, 1998). Subsequently, metal mass levelled off, indicating saturation. Chelex is very selective for trace metals.

DGT devices for trace metals are estimated to have maximum deployment times in the ocean of about two years and several months in more contaminated coastal waters (Zhang and Davison, 1995). Laboratory studies with synthetic lake water showed that the accumulated mass of cadmium increased linearly with time for at least 1 month (Davison and Hutchinson,

1997). Although, saturation in soil has not been studied yet, but is likely to be similar to freshwaters: higher metal concentration, but lower ionic strength.

#### 2.4.3.4.1.3 DGT biofouling

Biofouling of the facing filter is a potentially serious problem for long deployment times and its effects have not been experimentally determined. However, DGT may not in practice be very sensitive to biofouling. The mass of metal accumulated by DGT is very large compared with metal accumulated by biofilms (Davison *et al.*, 2000a) and it is unlikely that a biofilm can significantly affect the steep diffusion gradients created by the device (Davison *et al.*, 2000a).

In a recent study, the need of biofouling quantification is mentioned. Mussels and DGT were used to assess metal levels at four sites situated inside and outside of two enclosed marinas. Mussels accumulated all metals except cadmium. Over one year, DGT showed temporal differences of the same kind as those seen in the mussels, but appeared to have more power to discriminate between sites. Therefore, the effect of fouling on DGT measurements will have to be quantified, or avoided by the use of multiple short-term exposures, if the technique is to become a widespread in situ monitoring tool (Webb and Keough, 2002).

#### 2.4.3.5 Trace metals in soils using DGT

DGT has been investigated in soil, where it accumulates metal in a well-defined geometry that allows quantification of their supply from the solid phase (Davison *et al.*, 2000a). DGT in soil is effectively a simple experimental device that initiates a controlled perturbation *in situ* and automatically monitors any response (Hooda *et al.*, 1999). Using DGT at different soil moisture contents, results showed that for moisture content exceeding the field capacity of soil, the DGT response reflected soil water concentrations. At smaller moisture contents, changes related to tuortosity and dilutions were reflected in the measurements. DGT was therefore defined as a potential technique for measurements in the field, where it should provide quantitative flux data on individual soils (Hooda *et al.*, 1999).

Because DGT is able to measure the most labile metal species, DGT measurement has been showed as a good surrogate for plant uptake (Davison *et al.*, 2000b) and responds to both diffusional transport of solutes and their transfer from solid phase to solution in the vicinity of the roots. Two mechanisms are responsible for metal transport from the bulk soil to plant roots: 1) convection or mass flow, and 2) diffusion. Some ions are absorbed by roots faster than the rate of supply via mass transport (Barber, 1984). Thus, a depleted zone is created in soil immediately adjacent to the root. This generates a concentration gradient direct from the bulk solution and soil particles holding the adsorbed elements, to the solution in contact with the

root surface. This concentration gradient drives the diffusion ions toward the depleted layer surrounding the roots. Davison *et al.* (2000b) and Zhang *et al.* (2001) demonstrated that the kinetically labile solid phase pool of metal is included in the DGT measurement. DGT offers the possibility of a test procedure for soils, because the kinetic perturbation of solute concentrations in the soil system is most likely similar to that occurring during plant uptake.

## 2.5 Selected metals

### 2.5.1 Metal concentration in soil and plants

#### 2.5.1.1 Metal concentration in soils

Average metal concentrations in surface soils on the world scale (mg $kg^{-1}$) are presented in Table 2.2.

There is growing concern about increasing levels of metal contamination of soils, due to their adverse effects on soil biological activity, plant metabolism, and human health. The grand mean for world soils (Table 2.2) for Cd is 0.45 mg $kg^{-1}$, for Cr is 30 mg $kg^{-1}$, ranging from 1.4 to 1100 mg $kg^{-1}$, for Cu varies from 13 to 24 mg $kg^{-1}$, for Ni soils throughout the world contain Ni within the broad range of from 0.2 to 450 mg $kg^{-1}$ and for Pb 25 mg $kg^{-1}$ (Kabata-Pendias, 2001).

**Table 2.2** Average metal concentrations, in a range of common abundance, of surface soils (mg $kg^{-1}$) (Kabata-Pendias, 2001). Arithmetic means are in parenthesis.

| Element | Sandy soils | | Silty and loamy soils | | Histosols (organic soils) | |
|---|---|---|---|---|---|---|
| Cd | 0.01-2.7 | (0.37) | 0.08-1.61 | (0.45) | 0.19-2.2 | (0.78) |
| Cr | 1.4-530 | (47) | 4-1100 | (51) | 1-100 | (12) |
| Cu | 1-70 | (13) | 4-100 | (23) | 1-113 | (16) |
| Ni | 1-110 | (13) | 3-110 | (26) | 0.2-119 | (12) |
| Pb | 2.3-70 | (22) | 1.5-70 | (28) | 1.5-176 | (44) |

### 2.5.1.2 Metal concentrations in plants

#### 2.5.1.2.1 Cadmium

Cadmium concentrations in selected plants are shown in Table 2.3. In general the background levels of Cd in cereal grains, as well as in common feed plants that are reported for various countries, are fairly low and surprisingly similar (*e.g.* U.S., wheat 0.07-0.13 mg $kg^{-1}$, DW; Great Britain, wheat 0.03-0.06 mg $kg^{-1}$, DW). When plants are grown on contaminated soil, Cd is very likely to also be concentrated in roots, confirming the statement that root vegetables such as turnips, carrots, and potatoes, should be considered the main routes of Cd supply to man (Wagner, 1993). At present, Cd levels in cereal grains are of special interest as a potential significant source of this metal in the human diet (Kabata-Pendias, 2001).

**Table 2.3** Levels of Cd (mg $kg^{-1}$) in plants grown in contaminated sites (Kabata-Pendias, 2001).

| Site | Plant and part | Mean or range | Country |
|---|---|---|---|
| Ancient mining area | Grass tops | 1.0-1.6 | Belgium |
| | Grass tops | 1.1-2.0 | Great Britain |
| | Clover tops | 4.9 | Great Britain |
| Metal-processing industry | Lettuce leaves | 45 | Australia |
| | Silver beet leaves | 0.04-0.49 | Australia |
| | Grass, tops | 8.2 | Great Britain |
| | Brown rice | 0.72-4.17 | Japan |
| | Lettuce leaves | 5.2-14.1 | Poland |
| | Spinach leaves | 6.4 | Zambia |
| | Wheat grain | 0.22-0.47 | Poland |
| | Mushrooms | 3-56 | Finland |
| | Grass tops | 1.1 | Czech Republic |
| Urban garden | Cabbage leaves | 1.1-3.8 | Great Britain |
| | Lettuce leaves | 0.9-7.0 | U.S. |
| Sludge, irrigated, or fertilised farmland | Brown rice | 5.2 (max) | Japan |
| | Sudan grass, tops | 0.3-2.9 | Hungary |
| | Lettuce | 70 | U.S. |
| | Lettuce leaves | 8-37 | Germany |
| | Lettuce leaves | 0.5-22.8 | U.S. |
| | Carrot roots | 0.2-3.3 | U.S. |
| | Wheat grains | 5.5-14.2 | Russia |
| | Wheat leaves | 19-47 | Russia |
| | Wheat roots | 397-898 | Russia |
| Airborne contamination | Lettuce leaves | 5.2 | Denmark |
| | Spinach leaves | 3.9 | Denmark |

#### 2.5.1.2.2 Chromium

Common levels of Cr found in plant material are usually in the order of 0.02 to 0.2 mg $kg^{-1}$ (DW), however, a large variation is observed in the Cr content of food plants (Kabata-Pendias, 2001, Table 2.4). Concentrations of Cr in plants vary widely for different types of tissues and stages of growth (Bartlett, 1999).

**Table 2.4** Chromium content (mg $kg^{-1}$) of food and forage plants (Kabata-Pendias, 2001).

| Country | Plant | Tissue sample | FW basis | DW basis |
|---|---|---|---|---|
| Finland | Grass | Tops | | 0.11-0.35 |
| Poland | Oats | Grains | | 0.55 |
| | Wheat | Grains | | 0.2 |
| | Grass | Tops | | 0.6-3.4 |
| USA | Wheat | Grains | | 0.014 |
| | Potato | Tubers | | 0.021 |
| | Beans | Pods | 0.018 | 0.15 |
| | Lettuce | Leaves | 0.009 | |
| | Onion | Bulbs | 0.008 | 0.021 |
| | Carrot | Roots | 0.002 | |
| | Apple | Fruits | 0.004 | 0.013 |
| | Orange | Fruits | 0.008 | 0.029 |

FW basis: Fresh or wet weight basis of samples, DW basis: Dry weight basis of samples.

### 2.5.1.2.3 Copper

The concentration of Cu in plant tissues seems to be a function of its level in the nutrient solution or in soils, but the pattern of this relationship, however, differs among plant species and plant parts (Krishnamurti and Naidu, 2002). However, under both natural and man-induced conditions, the majority of plant species can accumulate much more Cu, especially in root, storage tissues (Table 2.5) (Kabata-Pendias, 2001).

**Table 2.5** Copper content (mg $kg^{-1}$ DW) of plants grown in contaminated sites (Kabata-Pendias, 2001).

| Site and pollution source | Plant and part | Mean or range of content | Country |
|---|---|---|---|
| Metal process industry | Lettuce, leaves | 64 | Australia |
| | Blueberry, leaves | 75 | Canada |
| | Grass, tops | 21 | Great Britain |
| | Grass, tops | 20-70[a] | Canada |
| | Horsetail, tops | 70-250[a] | Canada |
| | Sugar beet, leaves | 79-590 | Poland |
| | Dandelion, tops | 73-274b | Poland |
| | Dandelion, roots | 22-199b | Poland |
| | Wheat, grains | 21 | Poland |

| | | | |
|---|---|---|---|
| Urban garden | Radish, roots | 2-14 | Great Britain |
| | Leafy vegetables | 4-19 | U.S. |
| Sludged or | Grass, tops | 14-38 | Holland |
| irrigated farmland | Rice, grains | 4 | Japan |
| | Rice, roots | 560 | Japan |
| | Potato, tubers | 5 | Germany |
| Fungicide treatment | Coffee[c] | | Kenya |
| | Medium roots | 21.6 | |
| | Fine roots | 154.0 | |
| | Trunk wood | 6.7 | |
| | Trunk bark | 1122.5 | |
| | Whole stems | 9.4 | |
| | Foliage | 409.4 | |
| | Beans | 17.6 | |

[a] For 1.6 to 5.8 Km distance from a smelter, [b] For 0.5 to 2.5 Km distance from a smelter, [c] 68-year-old coffee bushes, 236 mg $kg^{-1}$ Cu in surface soil.

#### 2.5.1.2.4 Nickel

The mean levels of Ni in grasses ranged from around 0.1 to 1.7 mg $kg^{-1}$ (Kabata-Pendias and Pendias, 1999). The easy phytoavailability of Ni to plants has been demonstrated with $^{63}Ni$, where the isotopic composition in soil solution was the same as in plants (Echevarria *et al.*, 1998). As with the other metals, environmental Ni pollution greatly influences the concentrations of this metal in plants, such as lettuce and grass (Table 2.6).

**Table 2.6** Nickel content (mg $kg^{-1}$) of plants grown in contaminated sites (Kabata-Pendias, 2001).

| Site | Plant | Content | Country |
|---|---|---|---|
| Metal-processing | Blueberry | 4.8-6.2 | Sweden |
| industry | Blueberry, leaves | 92 | Canada |
| | Lettuce, leaves | 2.7 | Australia |
| | Lettuce, leaves | 84 | Canada |
| | Lettuce, leaves | 11 | Germany |
| | Grass | 3.9-9.0[a] | Germany |
| | Cereal, leaves | 230-250 | Great Britain |
| | Grass, tops | 1700-32,000 | Great Britain |

| | | | |
|---|---|---|---|
| | Spruce, twigs | 6-14[b] | Sweden |
| Oil refinery | Grass, tops | 2.4-13.3 | Poland |
| Sludged soils or | Grass, tops | 15-19[c] | Holland |
| urban gardens | Collard, leaves | 0.1-11.6 | U.S. |
| | Parsley, leaves | 3.7 | Poland |
| | Lettuce | 1.8-5.8[d] | Australia |
| | Lettuce | 2.4-40.3[e] | Great Britain |
| | Grass | 10-24 | Great Britain |

[a] Industrial emission and urban waste waters, [b] Five and one-year old, respectively, [c] Non-washed and washed leaves, respectively, [d] For field and greenhouse experiments, respectively, [e] Pot experiment, [f] AW basis.

#### 2.5.1.2.5 Lead

Although mean Pb contents calculated for cereal grains of various countries seem to vary considerably (from 0.01 to 2.28 mg $kg^{-1}$ DW), the grand mean, when calculated with the exclusion of two extremes values, is 0.47 and is quite close to the commonly reported concentrations (Table 2.7) (Kabata-Pendias and Pendias, 1999). There is still great attention oriented toward the Pb levels in plant foodstuffs, especially bread and potatoes as a significant source of this metal in human diets (Brüggemann *et al.*, 1996).

**Table 2.7** Lead content (mg $kg^{-1}$) of cereal grains from different countries (Brüggemann *et al.*, 1996, Kabata-Pendias and Pendias, 1999).

| Country | Cereal | Range | Mean |
|---|---|---|---|
| Great Britain | Barley | <1.25-1.50 | - |
| Canada | Barley | 0.1-0.2 | - |
| Egypt | Wheat | 0.10-0.92 | 0.51 |
| Finland | Barley | 0.29-0.56 | 0.40[a] |
| | Oats | 0.33-1.08 | - |
| | Wheat | 0.13-0.28 | 0.18[a] |

| | | | |
|---|---|---|---|
| Japan | Brown rice | - | 0.19 |
| | Buckwheat (flour) | - | 0.36[b] |
| Poland | Wheat | 0.2-0.8 | 0.32 |
| | Oats | 0.05-2.0 | 0.34 |
| | Rye | 0.06-1.3 | 0.34 |
| | Mixed cereals | 0.01-36 | 0.23 |
| Sweden | Wheat | 0.4-0.7 | 0.57 |
| U.S. | Wheat | 0.42-1.0 | 0.64 |
| | Rice | <0.002-0.07 | 0.007[b] |
| Russia | Wheat | 0.4-0.6 | 0.5 |

[a] After $PbNO_3$ addition to soil, [b] FW basis

### 2.5.2 Metal speciation and uptake

Soil minerals are capable of adsorbing metal ions from solution and these properties depend on the surface charge carried by the adsorbents. For instance, Cu can be adsorbed by minerals within the range from 0.001 to 1 $\mu M\ dm^{-3}$ or from 30 to 1000 $\mu M\ g^{-1}$. The greatest amount of adsorbed Cu has always been found for Fe and Mn oxides, amorphous Fe and Al hydroxides, and clay (Krishnamaturi and Naidu, 2002). Cd and Ni bound to residual fraction may be those bound with detrital silicate minerals, resistant sulphides and refractory organic materials. Therefore, metals in this fraction are expected to be chemically stable and biologically inactive (Kashem and Singh, 2001). However, the carbonate form of metal is susceptible to pH changes, mainly in the rhizosphere during plant growth, so it may be regarded as potentially bioavailable (Ramos *et al.*, 1994).

In general, the solubility of metals appears to be highly dependent on the pH. For instance, high pH favours sorption and precipitation of metals as oxides, hydroxides and carbonates (Kashem and Singh, 2001). For instance, the solubility of Pb can be greatly decreased by liming, which increased the pH value. A high soil pH may precipitate Pb as hydroxide, phosphate, or carbonate, as well as promote the formation of Pb-organic complexes, which are rather stable. Increasing acidity may increase the Pb solubility, but this mobilisation is usually slower than the accumulation in the organic-rich layer of soils (Ponizovsky and Mironenko, 2001).

In addition, the nature of the sorbing surfaces and of the solution-phase organic ligands is also of importance (Boekhold *et al.*, 1993). Metal adsorption to organic matter has been studied and all the findings lead to some generalisations: in all soil, metal activity is strongly affected by pH. In acid soils, the organic matter may largely control metal solubility, while in alkaline soil, precipitation of metal compounds is likely to control metal equilibria (Kabata-Pendias and Pendias, 1999). For instance, organic matter is likely to form a stable complex with Cu, at low Cu concentrations. Additionally, organic matter can modify several Cu reactions with inorganic soil components (Studenikina, 1999). Soil organic matter reveals a strong ability to absorb Ni (Bodek *et al.*, 1988).

Soil microbial activity is also believed to play a significant role in the metal speciation and bioavailability in soils. Binding and subsequent release of metal from soil depends on soil microorganisms. The rate and degree of

these processes depends upon soil parameters, of which the Eh-pH system is of most importance (Christensen *et al.*, 1991). For instance, the fast microbial conversion of Cr (VI) to insoluble Cr (III) is responsible for the low Cr availability to plants. Although the reduction of Cr (VI) is commonly reported, the oxidation of Cr (III) in soils has also been observed (Bartlett, 1999). Readily soluble Cr (VI) in soils is extremely toxic to plants and animals. Therefore, the variability in the oxidation states of Cr in soils is of great environmental concern (Pacha *et al.*, 1988), as the hexavalent state would be easily bioavailable.

Above pH 7.5, metal sorbed in soils is not easily mobile (Kabata-Pendias and Pendias, 1999). The higher proportion of mobile Cd in contaminated soils is generally at low clay content and low pH values (Lorenz *et al.*, 1994; Lombi *et al.*, 2001). The solubility of metal is closely related to the acidity of the soil solution. Cd is more mobile in acidic soils within the range of pH 4.5 to 5.5, whereas in alkaline soil Cd is rather immobile (*e.g.* $Cd(OH)_2$). However, as the pH is increased in the alkaline range, monovalent hydroxy ion species are likely to occur (*e.g.* $CdOH^+$).

### 2.5.3 Metal toxicity/nutrition

Metals can be either nutrition elements, and when its concentration increased, metal nutrient become toxic. Table 2.8 shows the approximate concentrations of metals for various plant species.

Particular role in the toxicity and nutrition for each metal is presented below.

**Table 2.8** Approximate concentrations of trace elements in mature leaf tissue generalised for various species (mg $kg^{-1}$ DW) (Macnicol and Beckett, 1985; Kabata-Pendias and Pendias, 1999; Kabata-Pendias, 2001).

| M | Deficient (if less than the stated amounts of essential elements) | Sufficient or Normal | Excessive or toxic | Tolerable in agronomic crops |
|---|---|---|---|---|
| Cd | | 0.05-0.2 | 5-30 | 0.05-0.5 |
| Cr | | 0.1-0.5 | 5-30 | 2 |
| Cu | 2-5 | 5-30 | 20-100 | 5-20 |
| Ni | | 0.1-5 | 10-100 | 1-10 |
| Pb | | 5-10 | 30-300 | 0.5-10 |

### 2.5.3.1 Cadmium

The most important biochemical characteristic of Cd ions is their strong affinity for sulfhydryl groups of several compounds (Cd complexes with metallothionein-like proteins are well documented). In addition, Cd also shows an affinity for other protein functional groups and for phosphate groups (Kabata-Pendias and Pendias, 1999).

There are no known enzymes that depend on Cd for their normal activity (Lombi *et al.*, 2001). Cd is considered to be toxic to plants, mainly via disruption of enzyme and other metabolic activities. In fact, the amount of chlorophyll was found to be a function of the Cd concentration in plant tissues and has been proposed as an indicator of the upper critical Cd level in plants (Kabata-Pendias and Pendias, 1999). Kloke *et al.* (1984) estimated the

phytotoxicity concentrations of Cd to be 5 to 10 mg $kg^{-1}$ (DW) in sensitive plant species, while Macnicol and Beckett (1985) gave the range of 10 to 20 mg $kg^{-1}$ (DW) as critical Cd levels.

In general, overt symptoms induced by elevated Cd contents of plants are growth retardation and root damage, chlorosis of leaves, and red-brown coloration of leaf margins or veins (McLaughlin *et al.*, 1994). The phytotoxicity of Cd, beyond interfering with normal metabolism of some micronutrients, shows inhibitory effects on photosynthesis, disturbs transpiration and $CO_2$ fixation, and alters the permeability of cell membranes (Kabata-Pendias and Pendias, 1999). Excess Cd has a complex inhibitory impact on the Calvin cycle, and especially disturbs a function of the key enzyme, ribulose diphosphate carboxyhydrazine. Cd is also known to increase plant predisposition to fungal invasion (Kabata-Pendias, 2001).

#### 2.5.3.2 Chromium

Although stimulating effects of Cr on plants have been observed, the phytotoxicity of Cr has been often reported, especially in plants on soils developed from ultrabasic rocks (Kabata-Pendias, 2001). The antagonistic interaction between Cr and Mn, Cu, and B has been reported and this can be related to interference both within the soil medium and in the plant tissues. In some cases, however, synergistic interactions between Cr and Fe are also observed (Zayed *et al.*, 1998).

The toxicity of Cr depends on its oxidation state, but is also related to readily available forms of chromate. While a $Cr_2O_7$ addition at the 1 X $10^{-5}$ M concentration level decreased plant growth by about 25%, the same level of $Cr_2(SO_4)_3$ was without any effect on growth, but the Cr additions resulted in 2.2 and 1.3 mg $kg^{-1}$ Cr, respectively, in bush bean leaves (Wallace *et al.*, 1990). The phytotoxicity concentrations of Cr were reported as follows (DW basis): 18 to 24 mg $kg^{-1}$ in tobacco, 4 to 8 mg $kg^{-1}$ in corn, 10 mg $kg^{-1}$ in barley seedlings, and 10 to 100 mg $kg^{-1}$ in rice. At doses of 750 mg $kg^{-1}$ of Cr (VI) and Cr (III), an increased uptake of Cr was observed. However, the harmful effect and mobility were significantly greater in the case of Cr (VI) addition (McGrath, 1998).

Symptoms of Cr phytotoxicity appear as wilting of tops and root injury; also, chlorosis in young leaves, chlorotic bands on cereals, and brownish-red leaves are typical features. Increased levels of Cr in the nutrient solution (up to 104 μM) are reported to disorganise the fine structure of chloroplasts and the chloroplast membranes of *Lemna minor* (Simon *et al.*, 1998).

**2.5.3.3 Copper**

Cu plays a significant role in several physiological processes including photosynthesis, respiration, carbohydrate distribution, N reduction and fixation, protein metabolism, and cell wall metabolism. In addition, Cu influences water permeability of xylem vessels and thus controls water relationships (Kabata-Pendias and Pendias, 1999). Cu controls the

production of DNA and RNA and its deficiency greatly inhibits the reproduction of plants (reduced seed production, pollen sterility). Cu is involved in the mechanisms of disease resistance. This resistance of plants to fungal diseases is likely to be related to an adequate Cu supply. There is also evidence that plants with enriched Cu concentrations are susceptible to some diseases. These phenomena may indicate that the role of Cu in disease resistance is an indirect one (Kabata-Pendias, 2001).

The most important practical implications are related to deficiency and toxicity of Cu, which affects physiological processes and therefore plant production. Cu levels less than 2 mg $kg^{-1}$ are likely to be inadequate for most plants (Macksimiec and Baszynski, 1996).

Despite the general Cu tolerance of plant species and genotypes, this metal is considered to be highly toxic. General symptoms included Cu-induced chlorosis and root malformation. Decrease of photosynthetic efficiency resulting from Cu-induced destabilisation of the photosystem is connected with an inhibition of its donor side and the primary photochemical processes (Macksimiec and Baszynski, 1996). The growth depression of sensitive plants was observed at 15 to 20 mg $kg^{-1}$ (DW) Cu in tissue, and 10% yield decrease is most likely at 10 to 30 mg $kg^{-1}$ (DW) Cu (Macnicol and Beckett, 1985).

Prediction of the Cu content of soil that results in toxic effects on plants is extremely complex. Before toxic symptoms and yield reductions are evident,

the nutritive value of the crops having increased Cu levels seems to create the most significant health risk. McGrath (1998) described that some 24 Cu hyperaccumulating species have been recorded in various families, but as yet no data are available on their use in phytoextraction of this metal.

#### 2.5.3.4 Nickel

The studies of the uptake and chemical behaviour of Ni in plants are related mainly to its toxicity having possible implications with respect to animals and man. There is no evidence of an essential role of Ni in plant metabolism, although the reported beneficial effects of Ni on plant growth have stimulated speculation that this metal may have some function in plant (Kabata-Pendias, 2001). Microorganisms that metabolise $H_2$ and urea are highly sensitive to the Ni nutrition (Kabata-Pendias and Pendias, 1999).

The mechanism of Ni toxicity to plants is not well understood, although the restricted growth of plants and injuries caused by an excess of this metal have been observed for quite a long time. Molas (1997) indicated that Ni fixed with glutamic acid was the most toxic to cabbage, and the least toxic was the Ni-EDTA complex. With plants under Ni stress, the absorption of nutrients, root development, and metabolism are strongly retarded. Before the acute Ni toxicity symptoms are evident, elevated concentrations of this metal in plant tissues are known to inhibit photosynthesis and transpiration. Low $N_2$ fixation by soybean plants was reported to be caused by Ni excesses (Molas, 1997).

The phytotoxic Ni concentrations range of excessive or toxic amounts of Ni in most plant species varies from 10 to 100 mg $kg^{-1}$. Much sensitive species are affected by much lower Ni concentrations, ranging from 10 to 30 mg $kg^{-1}$. The pronounced ability of some plant species to accumulate Ni when grown in soil over Ni ore bodies make them useful biogeochemical indicators (Kloke *et al.*, 1984; Macnicol and Becket, 1985).

### 2.5.3.5 Lead

Although there is no evidence that Pb is essential for the growth of any plant species, there are many reports on the stimulating effects on plant growth of some Pb salts (mainly $PbNO_3$) at low concentrations (Kabata-Pendias and Pendias, 1999). Moreover, other reports have described inhibitory effects of low Pb levels on plant metabolism. Due to the interactions of Pb with other elements and with many environmental factors, it has not been simple to establish Pb concentrations that are toxic to vital plant processes (Table 2.8). Several reports describe the toxic effects of Pb on processes such as photosynthesis, mitosis, and water absorption (Ponizovsky and Mironenko, 2001). However, the toxic symptoms in plants are not very specific. The levels of Pb in soils that are toxic to plants are not easy to evaluate, however, several authors have given quite similar concentrations, ranging from 100 to 500 mg $kg^{-1}$ (Ponizovsky and Mironenko, 2001).

Subcellular effects of Pb on plant tissues are related to the inhibition of respiration and photosynthesis due to the disturbance of electron transfer reaction. These reactions have been found to be inhibited by Pb

concentrations as low as 1 mg $kg^{-1}$ in corn mitochondria (Zimdahl and Koeppe, 1987). Pb is likely to be accumulated in various parts of cells, thus affecting their structures. The most deteriorating is the destruction of the plasma lemma, which in effect disturbs the permeability for water and leads to impaired plant growth (Woźny, 1998).

## 2.6 Conclusions

Environmental pollution with PAHs has attracted much attention in recent years. However, the potential of some common species found in soil to degrade PAHs have not fully been studied yet. This is the case of the fungi *P. frequentans* to remove phenanthrene.

It is clear that bioaugmentation and biostimulation are suitable tools to enhance the bioremediation process. However, when both PAHs and metals are present in a polluted soil, microbial activity could have an impact not only on PAH degradation, but also on metal behaviour. With the recognition that microbial transformations of PAH can result in some problems of soil fertility as well as detoxification processes, the study of microbiota in trace metal behaviour is required.

Sequential fractionation has been mostly used in most of the studies related to metal speciation in soils. However, fractionation has many difficulties and artefacts (Lead *et al.*, 1998). In order to assess metal behaviour in soil is

necessary to consider the solution speciation and the kinetics of exchange between solution and solid phase. These parameters are not quantified using sequential fractionation. Therefore, DGT is a suitable tool for the study of metal speciation of the most labile, and potentially, the most bioavailable metal species in soil.

# CHAPTER 3

# METHOD

## 3.1 Summary

This chapter described the methods used to study metal behaviour before and after the biological removal of phenanthrene from soil-contained metals. Soil from the state of Tabasco, Mexico was selected, as it is representative of contaminated soil with both hydrocarbons and trace metals present. Since experimental controls are needed, the site for sampling was chosen far away from the refinement industry, in order to get soil samples of the same type of soil with metals, but without hydrocarbons. Soil was spiked with phenanthrene and the final concentration in the soil was 200 mg $kg^{-1}$.

To optimise the bioremediation process and to study metal behaviour during phenanthrene bioremoval, three main experiments were performed:

1. The purpose of this experiment was to determine the ability of the non-basidiomycete, filamentous fungi *Penicillium frequentans* to remove phenanthrene in a solid-state culture. Additionally, the study investigated whether phenanthrene removal could be enhanced by manipulating the carbon-to-nitrogen-to-phosphorous ratio (C:N:P) and moisture content. To evaluate the combined effect of moisture content and the C:N:P ratio, on $CO_2$ evolution and phenanthrene removal efficiency, a combined experimental

design, composed by $2^2$ factorial with both central and axial points, was used. Once the culture conditions were established in a solid-state culture, a second experiment was performed.

2. To compare changes on metal behaviour (metal fluxes measured by DGT, metal concentration in pore waters measured by DGT and metal in soil solution by filtration), different experimental conditions were investigated with the culture conditions previously established. Five different metals were studied: cadmium, chromium, copper, nickel and lead (see reasons to choose them in section 2.5.3). All treatments were carried out on both previously sterilised (prior to inoculation with the added fungus) and non-sterilised soils (which include native soil microflora and *P. frequentans* inoculum). The effect of the presence/absence of phenanthrene was also established.

3. To assess metal uptake by plants before and after phenanthrene bioremoval, the DGT technique was used as a surrogate for plant uptake. Changes on metal fluxes, metal DGT concentrations and soil solution concentration by filtration in non-sterilised soil were performed. In addition, metal uptake by plants was studied using two different types of plants: *Echinochloa polystachia* and *Triticum aestivum* L.

### 3.2 Soil selection

In Mexico soil contamination by the petrochemical industry is the main source of contamination with hydrocarbons in the Southeast part of Mexico (IMP, 1999). Due to the exploitation, refinement, transport, commercialisation and storage of hydrocarbons, both tropical and subtropical soils have been heavily polluted with organic chemicals and certain metals (Battelle, 1998).

A soil from the state of Tabasco, Mexico was selected as it is contaminated with both hydrocarbons and trace metals (IMP, 1999). This soil is representative of pollution with both polyaromatic hydrocarbons and heavy metals (Battelle, 1998). Since experimental controls are needed, the site for sampling was chosen far away (1.5 Km) from the refinement industry, in order to get soil samples of the same type of soil with metals, but without hydrocarbons.

#### 3.2.1 Study area

The study area is located in the town of Samaria, in the state of Tabasco, in the southeast of Mexico (Figure 3.1). The geographical location is between 18° 00´ 38´´ and 93° 05´ 02´´ latitude north-longitude west. The climate is warm and humid (García, 1988).

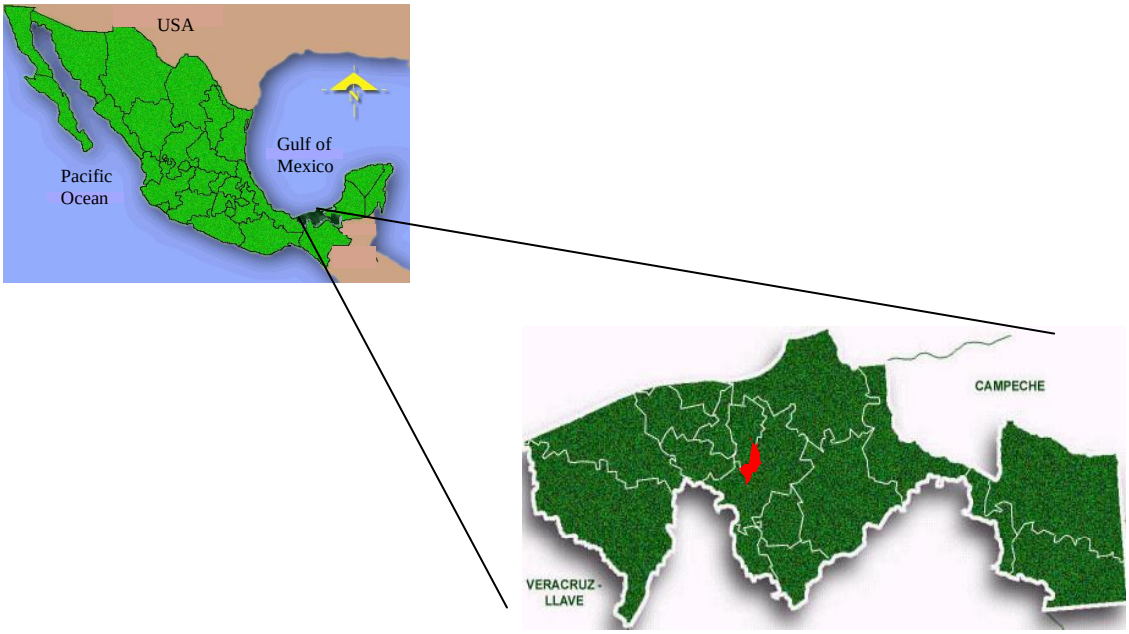



**Figure 3.1** Location of the study area, in the town of Samaria (in red), state of Tabasco, Mexico (taken from http://tab.inegi.gob.mx/).

### 3.2.1.1 Type of soil

The soil of the study area contains unconsolidated terrigenous deposits, primarily composed of sedimentary rocks (calcareous, conglomerates and gritty). Soil grain size is mainly composed of material in the sand range. However, the primary component changes from fine sands at the foot of the mountains, to silts and clays in the centre of the plain (CNA, 1989). The soils, which cover the biggest surface in the study area, are the gleysols (GL), 48%, while the fluvisols (FL) are distributed in 30.4% of the area. The other group of soils is the alisols (AL) occupying 13.9% of the total area (IMP, 1999).

### 3.2.1.2 Soil uses

About 84.6% of the study area has an agricultural use, with corn cultivated in 7.1% of the area. Bean cultivation is carried out in 30%, rice in 30% and Yucca in 20% of the area. Bananas, papayas and zapote are also cultivated. The type of trees that can be found are coconut, citrus, avocado, chinín, coffee, mango, guanábana, cedar, maculís, tatúan, chipilcate, mahogany and pepper (IMP, 1999).

### 3.2.1.3 Hydrology

The study area is located in an extensive flood plain, being part of the hydrological region Grijalva-Usumacinta (RH-30) in the basin of the River Grijalva-Villahermosa, which is divided in the main tributaries: the Samaria River, the Carrizal River and Mecalapa River.

There are 23 micro basins registered in the Tabasco area (Figure 3.2), all with defence borders against floods. In the basin of the Samaria River most of Samarias petroleum industry (68.4%) is located (IMP, 1999).

Soils from Samaria are inundated regularly according to the annual precipitation rate of 2000 to 2500 mm per year (Figure 3.3).

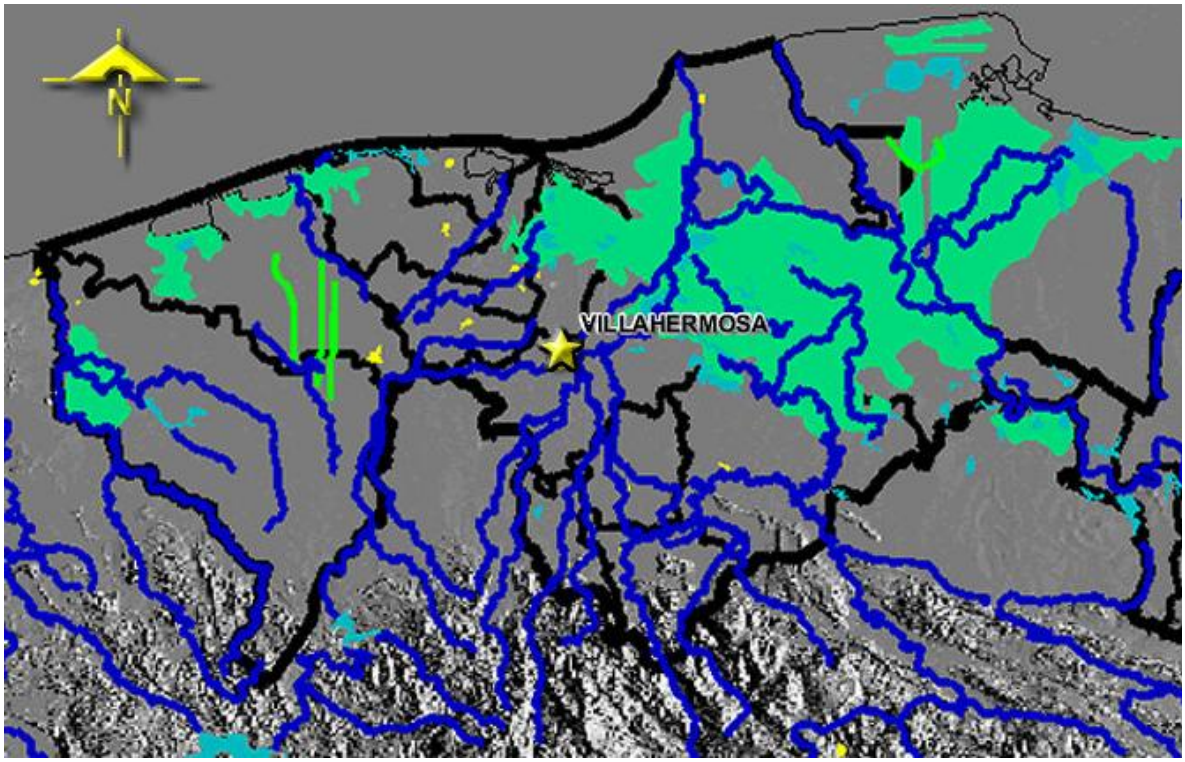




**Figure 3.3** Annual precipitation in the state of Tabasco, Mexico (taken from http://tab.inegi.gob.mx/).

### 3.2.2 Soil collection and pre-treatment

Soil samples were taken from the top-50 cm soil layer, as hydrophobic compounds are usually absorbed (Riser-Roberts, 1998). Samples were taken with stainless steel or chrome-plated sampling tools and plastic buckets to avoid contaminating the samples with traces of chemical elements (micronutrients) from the sampling tools. When sampling, small areas where the soil conditions were obviously different from those in the rest of the field — for example, wet spots, fencerows, and spoil banks— were avoided.

Every soil sample consisted of about 15 to 20 cores taken at random locations throughout the study area. Individual soil cores from a minimum of 20 locations were mixed thoroughly in clean plastic containers. A subsample of the soil mixture was removed and placed in a soil sample bag (lined with plastic). This sample, referred to as the composite sample, consists of a mixture of the individual cores and was considered as a representative soil sample of the study area. The soil was air dried, passed through a 0.86 mm sieve (No. 20 mesh), and stored in clean plastic bags at room temperature.

### 3.2.3 Soil characterisation

The soil was characterised to establish its main physical, chemical and biological characteristics. Table 3.1 shows the characteristics and analytical methods. These methods are discussed on section 3.7.

**Table 3.1** Characteristics evaluated and methods of soil characterisation.

| Characteristic | Method |
|---|---|
| **Physical** | |
| Texture | Bouyoucos |
| Moisture | Gravimetric |
| Water holding capacity | Weight |
| Porosity | Density ratio |
| Real density | Picnometric |
| Apparent density | Paraffin |
| **Chemical** | |
| pH | Potentiometric |
| CEC | Exchange against 1M $NH_4Cl$ |
| Total nitrogen | Micro-Kjeldhal (AOAC 42.014) |
| Total phosphorous | APHA, 1995 |
| Organic matter | Walkley - Black |
| Total organic carbon | TOC Shimadzu S-5000A |
| Polycyclic aromatic hydrocarbons | Extraction clean up (EPA 3611) GC/MS analysis (EPA 8270C) |
| Total metal concentration | Digestion (EPA 3050) GFAAS analysis (EPA 7000) |
| **Biological** | |
| Microbial population | Most Probable Number |
| Hydrocarbonoclastic | Most Probable Number |
| Total fungi | Soil dilution plate method |

#### 3.2.3.1 Sterilised and non-sterilised soil

In the case of sterilised soil, 12.000 ± 0.001 g of soil was placed in 120 ml glass vials and sterilised by autoclaving at 121°C/15 psi (George and Neufeld, 1989) every other day for 6 days, this soil will be called *sterilised soil*. Sterility of soil was checked by adding 2 ml of soil suspension to agar

plates, incubated for 7 days in darkness at room temperature. Three different culture media: nutrient agar, potato-dextrose agar, and Czapek agar were used to stimulate bacteria, fungi and Actinomycetes growth, respectively. Some of the soil was not autoclaved to compare changes in metal behaviour due to the autochthonous soil microflora (called *non-sterilised soil*).

Subsequent to this, soil was divided into appropriate vial glass container. On day 14 of fungal propagation (see section 3.3.1), sterilised and non-sterilised soil was spiked with phenanthrene (Aldrich-Sigma®, HPLC grade) in acetone 24 hrs before inoculation, in order to let the solvent evaporate. The final phenanthrene soil concentration was 200 mg $kg^{-1}$. The Regional Association of oil and natural gas companies in Latin America and the Caribbean (ARPEL, 2002) set a limit of 50 mg $kg^{-1}$ for "contaminated soil". The final phenanthrene concentration was set in order to exceed this limit, so that the soil in this study could be considered as contaminated and will be called *contaminated soil*. Although spiked phenanthrene could be less tightly bound to the soil than phenanthrene "naturally" incorporated, the main purpose of this study was to study the metal behaviour during the phenanthrene removal. In addition, with a mixture of PAHs the experimental controls in this study would not be accurate and enough for the study of metal behaviour.

### 3.3 Phenanthrene bioremoval and optimisation of culture conditions

#### 3.3.1. Propagation

The isolation of the fungal strain was carried out from sugarcane bagasse pith (Pérez-Armendariz *et al.*, 2003). The identification of the strain as

*Penicillium frequentans* (Figure 3.4a) was performed by DNA extraction and PCR (polymerase chain reaction) amplification (Cortés-Espinoza *et al.*, 2003). Stock cultures were maintained at -20°C on Potato Dextrose Agar (PDA) and glycerol. Before each experiment the strains were reactivated on PDA at 30°C for 3 days. The propagation of *P. frequentans* strain was carried out during 7 days in darkness at 26°C in PDA media (Figure 3.4b).

#### 3.3.1.1 Liquid culture

In order to obtain the fungal mycelium in pellets (Figure 3.4c), fungus was grown in modified liquid Wunder media (Table 3.2) (Wunder *et al.*, 1994) over 4 days.

**Table 3.2** Modified Wunder media* composition.

| Composition | g $l^{-1}$ |
|---|---|
| Glucose $H_2O$ | 10.0 |
| $(NH_4)_2SO_4$ | 1.0 |
| $MgSO_4 7H_2O$ | 0.5 |
| $KH_2PO_4$ | 0.875 |
| $K_2HPO_4$ | 0.125 |
| $CaCl_2.2H_2O$ | 0.1 |
| NaCl | 0.1 |
| $FeSO_4$ | 0.001 |

*Media is prepared with MQ water; Ω=18.2 MΩ$cm^{-1}$; final pH= 5.5.

### 3.3.2 Inoculation

When propagation of the pelleted fungal strain was performed, 0.04 g of wet fungal biomass was added to sterile vials that contained 0.8 g sugarcane bagasse pith (using either sterilised or non-sterilised bagasse), in addition to an aliquot of Wunder media. The vials (Figure 3.4d) were incubated in the darkness for 15 days at 26°C. On day 15, sub samples of 12.000 g of sterile and non-sterilised contaminated soil (homogenised for DGT deployment, see

3.3.1 section) and fungus were mixed. To optimise conditions, modified Wunder media was added in addition to $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ (both Aldrich-Fisher ®, 98% purity) to adjust C/N/P ratio. Moisture content was maintained by adding MQ water every 1-2 days (see section 3.3.3 Effect of C/N ratio and moisture content). The vials were sealed and incubated at 26°C in the dark. During this period of time, vials were opened up every 3 days to allow gas exchange with the atmosphere.

Every three days, fungal activity was evaluated by measuring $CO_2$ production (see section 3.5.2). Immediately after, the content of the vials was collected to quantify phenanthrene concentration (at day 15, 18, 21, 24, 27 and 30) by GC/MS (see section 3.5.3). At the same time, DGT and metals in soil solution (by filtration) analyses were performed (see section 3.4.2 DGT and 3.4.3 soil solution sections). The period of time over which the experiment was run (30 days) was fixed due to the length of microbial activity. After 30 days, $CO_2$ production levelled off and the maximal phenanthrene removal had already occurred.

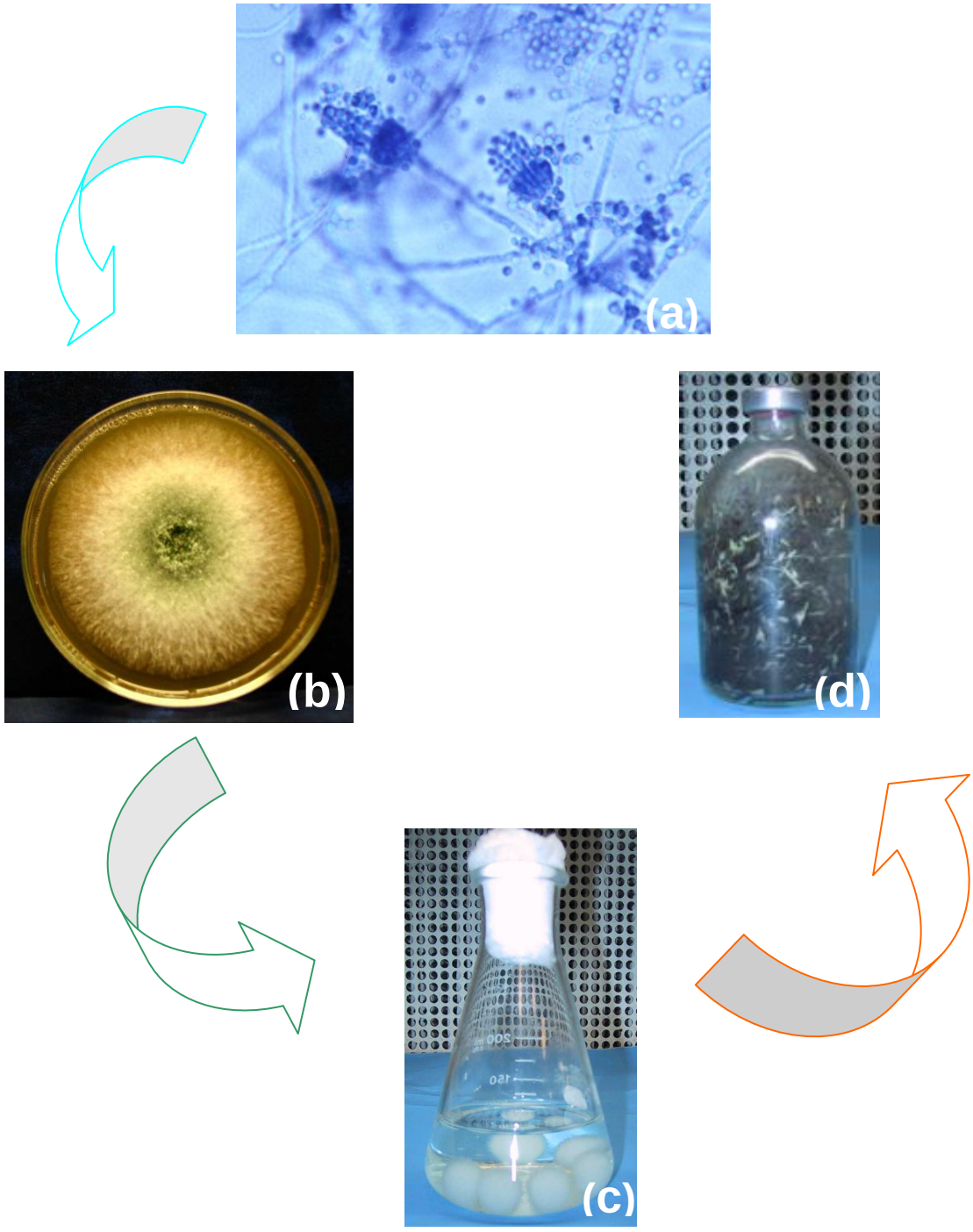



**Figure 3.4** Microbial procedure: (a) *Penicillium frequentans* strain, 200X; (b) *P. frequentans* propagate in PDA media; (c) *P. frequentans* propagated in liquid Wunder media to obtained fungal pellets; (d) Vial contains sugar cane bagasse pith, polluted soil and *P. frequentans* inoculum.

Different variables were evaluated, according with the type of microcosms, following the same inoculation procedure with both sterilised and non-sterilised soil (Table 3.3). The same procedure was followed with non-sterilised soil. All analyses were performed in triplicate.

**Table 3.3** Microcosms type and variables evaluated. All treatments were separated in sterilised and non-sterilised soil.

| Microcosms | Variables measured | Controls* |
|---|---|---|
| Soil + Bagasse + Fungus | $CO_2$, DGT, metals in soil solution | 1, 2, 3 and 4 |
| Soil + Bagasse + Phenanthrene | $CO_2$, Phenanthrene adsorption, DGT, metals in soil solution | 1, 2 and 5 |
| Soil + Bagasse | $CO_2$, DGT, metals in soil solution | 1 and 2 |
| Soil | DGT, metals in soil solution | 1 |

*Controls: 1) Sterilised soil and 2) sterilised bagasse, to assure the lack of microbiological activity (sterilisation efficiency was confirmed by the absence of $CO_2$ production), 3) sterilised soil with *P. frequentans* inoculum, to attribute the removal process to the fungus, 4) sterilised soil and fungi in order to evaluate $\%CO_2$ production attributed to the use of soil organic matter as an auxiliary carbon source and 5) sterilised soil with phenanthrene to quantify the phenanthrene abiotic removal.

### 3.3.3 Effect of moisture content and the C:N ratio

To evaluate the combined effect of moisture content and the C:N ratio on $CO_2$ evolution and phenanthrene removal efficiency, a combined experimental design, composed by $2^2$ factorial with both central and axial points, was used. The experimental error was calculated from central points

of the composite experimental design (Montgomery, 2000). Table 3.4 shows the independent variables with axial and central points.

**Table 3.4** Factorial experimental design with axial and central points, including independent variables (moisture content and C:N ratio), observed and predicted phenanthrene removal.

| **Coded variables** | | **Natural variables** | |
|---|---|---|---|
| **Moisture (%)** | **C:N ratio** | **Moisture (%)** | **C:N ratio** |
| 0 | -1.414 | 30 | 47.58 |
| 0 | 0 | 30 | 90 |
| 0 | 0 | 30 | 90 |
| 0 | 0 | 30 | 90 |
| 0 | 0 | 30 | 90 |
| 0 | 0 | 30 | 90 |
| -1 | 1 | 20 | 120 |
| 0 | 1.414 | 30 | 132 |
| 1.414 | 0 | 44.1 | 90 |
| -1 | -1 | 20 | 60 |
| -1.414 | 0 | 15.9 | 90 |
| 1 | -1 | 40 | 60 |
| 1 | 1 | 40 | 120 |

$\alpha$=1.414. Coded variables were fixed according to the factorial experimental design.

### 3.4 Metal behaviour

In order to compare changes on metal behaviour (DGT fluxes, DGT concentrations, metal in soil solution by filtration), experimental samples using different treatments were prepared as previously described. All treatments were performed on both previously sterilised (prior to inoculation with the added fungus) and non-sterilised soils (which include native soil microflora and *P. frequentans* inoculum).

#### 3.4.1 Treatments and controls

Treatment 1 contained soil, fungus and phenanthrene, Treatment 2 contained soil and fungus, Treatment 3 contained soil and phenanthrene, and Treatment 4 contained soil only. Treatments 3 and 4 contained no fungus and were controls to allow calculation of the net impact of the fungus only.

#### 3.4.2 DGT

##### 3.4.2.1 Soil pre-treatment

A sub sample of dried and sieved sterilised or non-sterilised contaminated soil (12 g) was placed in individual acid-washed glass vials (microcosm system). Appropriate amounts of MQ water were added to the contaminated soil to obtain 44.5% moisture content (referred to as field capacity). The soil samples were then equilibrated at 26°C in an incubator for 24 hours prior to

DGT deployment. Moisture content was constantly maintained by adding MQ water.

#### 3.4.2.1.1 Gel preparation and DGT assembly

A polyacrylamide hydrogel comprising 15 vol % acrylamide (Boehringer) and 0.3 vol % patented agarose-derived cross linker (DGT Research Ltd. UK) was used throughout this work as the diffusive gel layer. The gel layer was prepared by DGT Research and Environmental Science, Lancaster University, and purchased from Hao Zhang and Bill Davison without further pre-treatment. The following procedure was used: Freshly prepared ammonium persulfate initiator (70 μL of 10%) and TEMED catalyst (20 μL) were added to each 10 ml of gel solution. The solution was immediately cast between two glass plates separated by plastic spacers and allowed to set at a temperature of 42 °C for about 45 min. The resin gel consisted of 2 g of ion-exchange resin Chelex 100 (Na form, 100-200 wet mesh) in 10 ml of gel solution.

All gels were hydrated in Milli Q water for at least 24 h before use. During this hydration step, the gel expanded to a new stable dimension. The volume of gel increased by a factor of 3.2 (Zhang *et al.*, 1998) so that the water content was about 95%. The diffusive gels were stored in 0.01 M $NaNO_3$ solution and the resin gels were stored in MQ water.

DGT units were assembled as shown in Figure 3.5. The DGT device was based on a simple tight-fitting piston design (Davison and Zhang, 1994). It

consisted of a backing cylinder and a front cap with a 2.0 cm diameter window. A layer of resin gel was placed on the base with the side containing the resin facing upward. A layer of diffusive gel was placed directly on top of it. A 0.8 mm thick diffusive gel disk was used along with a 0.4 mm thick resin gel disk. To prevent soil particles adhering to the gel surface, a 100 μm thick 0.45 μm pore size Millipore cellulose nitrate membrane was placed on top of the diffusive gel. The filter has been shown to behave like an extension of the gel layer (Davison and Zhang, 1994; Zhang *et al.*, 1998).

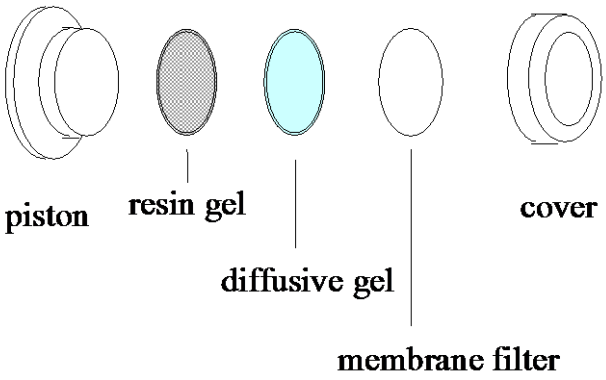



**Figure 3.5** Schematic representation of the DGT assembly.

### 3.4.2.2 Deployment and retrieval

DGT units were placed on the surface of the soil slurry at an angle and pushed gently into the surface, making sure that there are no air bubbles between the soil solution and the DGT device. The DGT units were then pushed slightly into the soil, and the container was loosely covered with a lid. After maintaining at incubator temperature (26 ± 0.1 °C) for different periods of time (0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days), the DGT units were

retrieved from the soil and rinsed with MQ water to wash away all the soil particles adhered on the filter membrane.

The resin gel was retrieved and placed into a clean plastic sample vial. To elute metals, the resin gel was completely immersed in 1 ml of 1M $HNO_3$ for 24 h. The extraction efficiency was 87± 3%.

### 3.4.2.3 Sample analysis

After at least 10-fold dilution to reduce acid concentration, samples were analysed by Graphite Furnace Atomic Absorption (see 3.6.3 GFAAS section).

### 3.4.2.4 Calculations

The theory for DGT calculations is described in Chapter 2, section 2.4.3.4.1. Within the layer of resin, of thickness $\Delta$r, the concentration of the free metal at the resin is effectively zero, owing to the complexation by the resin, which reacts to only a small fraction of its capacity (Davison and Zhang, 1994). Within the bulk solution the metal has concentration $C_b$. The gel layer is assumed to be separated from the bulk solution by a diffusive boundary layer (DBL) of the thickness $\delta$, where transport is solely by molecular diffusion.

Ions transported from the solution to the resin must diffuse across the DBL and then through the gel, of thickness $\Delta$g. Small ions can diffuse freely through the effectively 2-5 nm pores (Zhang and Davison, 1999) of

polyacrylamide gel. These ions have effective diffusion coefficients, *D*, indistinguishable from those in water (Li and Gregory, 1974).

If, for simplicity, it is assumed that $\delta << \Delta g$, Fick's first law can be used to define the flux of a given metal ion:

$$\text{Flux} = DC_b / \Delta g \qquad (3.1)$$

The mass per unit area of resin, $M_a$, after time t is then:

$$M_a = DC_b t / \Delta g \qquad (3.2)$$

And the concentration in the resin layer, $C_r$, is given by:

$$C_r = M_a / \Delta r \qquad (3.3)$$

After a period of time, the concentration in the resin layer, $C_r$, can be measured and the concentration in the solution can be quantified by:

$$C_b = C_r \Delta g \Delta r / Dt \qquad (3.4)$$

Equations (3.1)-(3.4) depend on the assumption that the thickness of the DBL between the gel and the bulk solution is negligibly small (Davison and Zhang, 1994).

### 3.4.3 Soil solution

Soil solutions were extracted from the microcosm system by centrifuging at 13 000 rpm for 21 min, using a IEC 245 centrifuge. Equation (3.5) was used to choose the centrifugal condition necessary to remove particles of given sizes and densities with a certain time (Chen, 1993). For a given spherical and rigid particle,

$$\upsilon=\delta x/\delta t= (\rho_p-\rho_m/18\ \eta_m)\ (d_p^2)\ (\omega^2)\ (x), \text{ where:} \qquad (3.5)$$

$\upsilon$ Is the migration velocity
$\rho_p$ is the density of particle
$\rho_m$ is the density of the medium
$\eta_m$ is the viscosity of the medium
$d_p$ is the particle diameter
$\omega$ is the angular velocity of rotation
x is the radial distance of a suspended particle from the axis of the rotor
$\omega^2(x)$ is the centrifugal force (G), 1G=980 cm $s^{-2}$

The samples were subsequently filtered and acidified with Aristar $HNO_3$. Trace metals in soil solutions were analysed by Graphite Furnace Atomic Absorption (see section 3.6.3).

### 3.4.4 Metal availability to plants

In order to assess metal uptake by plants before and after the bioremediation process, changes in metal flux and mass during phenanthrene bioremoval procedure, an experiment was performed on two types of plants (*Echinochloa polystachia* and *Triticum aestivum* L.) (Figure 3.6).

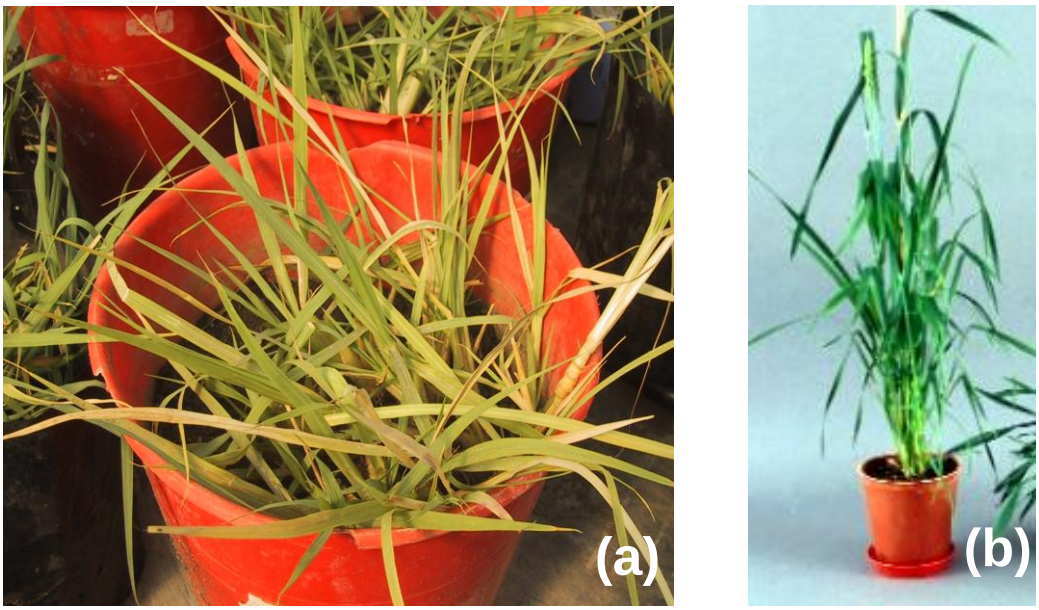



**Figure 3.6** Plants used to assess metal uptake: (a) *Echinochloa polystachia* (alemangrass) and (b) *Triticum aestivum* L. (wheat).

**3.4.4.1 Plant growth.** Two types of plants were grown on clean soil (with not detected metals and hydrocarbons). The first plant, *Echinochloa polystachia*, was propagated using vegetative reproduction (Figure 3.7 a). The second one was wheat (*Triticum aestivum* L.), which was propagated by seeds under shade (Figure 3.7 b), using Saturno S-80 variety, certified seeds proportionate by Productora Nacional de Semillas (PRONASE), SAGAR, Mexico.

**3.4.4.2 Treatments.** After germination/vegetative propagation, the above plant material was transplanted into a fish glass tank (Figure 3.7c) covered under special cover to prevent atmospheric contamination. The tank contained non-sterilised soil under different treatments chosen through a factorial experimental design composed by $2^{4-1}$ ($2^3$ = 8 experimental units), including the following variables: plant, fungi, phenanthrene and time. Each variable had 2 levels, that is, presence (+) and absence (-) (Table 3.5).

Treatments were designed through a factorial experimental design ($2^3$), including plant (Pl), fungi (F), phenanthrene (P) and time (Pl*F*P) as independent variables, and metal uptake by plants (Pl) as dependent variable. Treatment 1 contained S + F + P + Pl, Treatment 2 S + F + P l, Treatment 3 contained S+ P+ Pl, Treatment 4 contained S + Pl and Treatment 5 S (non-sterilised soil).

**Table 3.5** Factorial experimental design ($2^3$), including independent variables, for the experiment with plants.

| Experimental unit | Plant (Pl) | Fungi (F) | Phenanthrene (P) | Time (Pl*F*P) |
|---|---|---|---|---|
| 1 | - | - | - | - |
| 2 | + | - | - | + |
| 3 | - | + | - | + |
| 4 | + | + | - | - |
| 5 | - | - | + | + |
| 6 | + | - | + | - |
| 7 | - | + | + | - |
| 8 | + | + | + | + |

+ presence; - absence.

Soil moisture was maintained at around 44.5% field water capacity with additions of deionised water. Plants were maintained in a glasshouse, with the following conditions: 14 h/10 h day/night, 26 ± 2°C day and night temperatures. The pots were randomly arranged and rotated regularly to ensure that all plots receive equal intensity of natural light. During the 30-day experiment, DGT deployments were made consecutively, each lasting 72 h. Plant samples were collected every three days (on days 15, 18, 21, 24, 27 and 30) after transplanting them to the studied soil. Mean DGT metal fluxes were obtained during this 30-day period, which were compared with the metal concentration in the whole plants harvested.

**3.4.4.3 Analysis.** Roots and leaves of each plant were rinsed with deionised water, and dried at 80°C for 24 h. Then, dried plants were digested (see section 3.5.4.2) and metal analysis was carried by GFAAS.



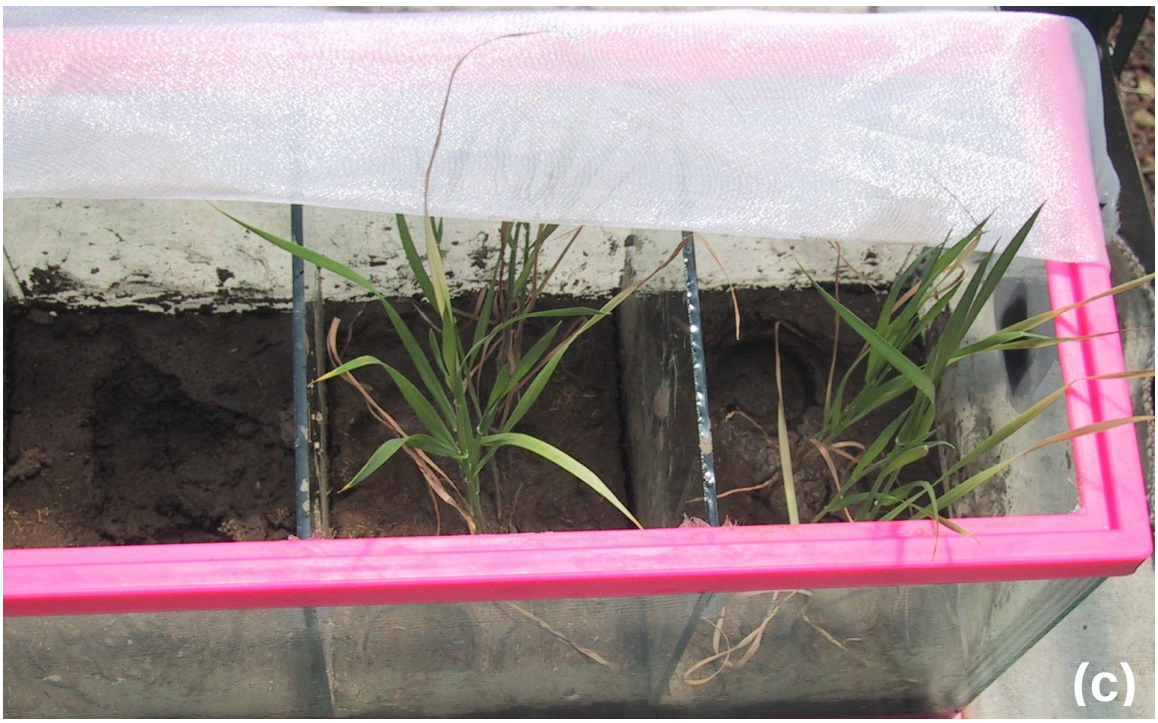



**Figure 3.7** Plant propagation: (a) *Echinochloa polystachia* (vegetative reproduction), (b) *Triticum aestivum* (seeds), (c) Fish glass tank, with three replicates.

### 3.5 Analytical techniques

#### 3.5.1 Soil characterisation

##### 3.5.1.1 Texture

In order to determine grain size, soil was sieved through a >2 mm sieve. A hydrometer was used to perform particles size analysis of the soil (Bouyoucos, 1962). Soil was classified using the Sheppard triangle (Folk, 1969).

##### 3.5.1.2 Moisture

Soil moisture content was determined by difference on weights before and after drying soil (at 105-110 °C for 48 Hrs) (Helalia, 1993).

##### 3.5.1.3 Water holding capacity

The water holding capacity (WHC) represents the maximal water available for plants and included the capillary moisture (around soil particles) and the hygroscopic moisture (adsorbed water into the colloidal particles) (Helalia, 1993). WHC was measured using a 0.3 g soil sample that was pressed at 3,000 psi for 3 minutes on a 125-mm filter paper. Two distinct areas are produced and include a meat area and a water area. The WHC is the ratio of the water to meat areas. Lower ratios are desired, indicating the ability of the sample to bind more water.

### 3.5.1.4 Porosity

Porosity is the part of soil that is occupied by air and water. Porosity is determined by the soil structure. To calculate porosity the apparent and real density was used (Equation 3.6) (SSDS-USDA, 1993).

P= (Rd-Ad/Rd) (100), where: (3.6)

P= Porosity
Rd= Real density
Ad= Apparent density

### 3.5.1.5 Real and apparent density

Density depends on soil composition. The real density includes the solids mass per volume unit. A picnometer was used to determine the real density of soil particles (Donahue *et al.*, 1962).

The apparent density includes both the particles and soil space. This explains why the apparent density is lower than real density. The paraffin method was used to determine the apparent density (Hausenbuiller, 1981). The procedure was as follows.

A representative air-dried soil sample was selected and weighed. Carefully a length of thread was tied around the solid soil, leaving about 20 cm of thread free. The soil suspended in air was weighed. Holding onto the end of the free thread, the soil was dipped momentarily in the melted paraffin and allowed to drain the excess. When the adhering paraffin solidified, the soil and paraffin

were weighed together. The soil with paraffin coating in water was suspended and weighed again. The water temperature was recorded. To calculate the density, equation (3.7) was used (Hausenbuiller, 1981):

Bulk density= DW x ODS/SA-SPV+PA-(PA-DW/DP), where: (3.7)

DW = Density of water at temperature of determination (mg $m^{-3}$)
ODS = Oven dry weight of soil sample (g)
SA = Net weight of soil sample in air (g)
SPW = Net weight of soil sample plus paraffin in water (g)
PA = Weight of paraffin coating in air (g)
DP = Density of paraffin (mg $m^{-3}$)

**3.5.1.6 pH**

The pH was measured using a Fisher brand Hydrus 300 pH meter, with a combination of pH electrode. Buffers of 4, 7 and 9 were used for electrode calibration. The pH electrode was calibrated over the appropriate range using the standard buffers. Then 5 g of sieved, air-dried soil was added to 5 ml deionised water to the sample. The mixture was stirred vigorously for 15 seconds to homogenise and stand for 30 minutes. The electrodes were placed in the slurry, swirled gently, and the pH reading taken.

For comparison, pH in 1: soil and 0.01 M $CaCl_2$ was also measured. The mixture was stirred vigorously and let to stand for 30 minutes, stirring occasionally. The pH was recorded as described previously.

### 3.5.1.7 Cation exchange capacity

Cation exchange capacity (CEC), usually expressed in milliequivalents per 100 g of soil, is a measure of the total negative charge density and hence a measure of cation retention capacity. These exchangeable cations may be viewed as being balanced by either 1) an excess of ions of opposite charge and a deficit (or negative adsorption) of ions of like charge, or 2) the excess of ions of opposite charge over those of like charge. The method follows three main steps: 1) saturation of cation exchange sites with a specific cation, 2) removal of excess saturating solution, and 3) replacement of saturating cations (Rhoades, 1982). The procedure was as follows.

10 g of air-dried soil was put into a 125 ml Erlenmeyer flask and 50 ml 1 M $NH_4Cl$, the flask was covered with a piece of parafilm, and shake for 30 minutes. Then the sample was filtered through Whatman No. 2 filter paper in a plastic funnel to filter the solution into a 250 ml volumetric flask. The remaining soil from the Erlenmeyer flask was rinsed with a small amount of 1M $NH_4Cl$ from a squeeze bottle. Then filtering was repeat with ~10 ml aliquots of $NH_4Cl$ solution. The solution was made up to 250 ml with $NH_4Cl$ solution. The flask was covered with parafilm and the solution mixed thoroughly. Then, 10 ml of the mixed extract was put into a glass test tube. The ions $Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$ were analysed by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). The ICP-OES results were reported in units of μg cation per ml of filtrate (μg $ml^{-1}$). To calculate the total amount of each cation, the values obtained from ICP-OES was

multiplied by the volume of the filtrate (250 ml) and divide by the oven-dry weight of the soil sample in kg. Next, this value was multiplied by the cmol($^{+}$)$\mu g^{-1}$ of each cation to express results in units of cmol(+) $kg^{-1}$. The cationic exchange capacity was calculated as the sum of exchangeable $Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$ cations.

### 3.5.1.8 Total nitrogen

The Kjeldahl method is essentially a wet oxidation procedure. In this method, organic N in the sample under analysis is converted to $NH_4^{+}$-N by digestion with concentrate $H_2SO_4$ containing substances that promote this conversion and the $NH_4^{+}$-N is determined from the amount of $NH_3$ liberated by distillation of the digest with alkali (Bremner and Mulvaney, 1982). The procedure followed is described below.

Approximately 5 g of soil sample was oxidised in 10 ml of hot concentrated sulphuric acid, then cooled, diluted with water, cooled again, and finally made alkaline with a NaOH solution to yield ammonia ($NH_3$). Concentrated NaOH solution was added carefully, so that it forms a separate layer on top of the sulphuric acid. The $NH_3$ formed is separated by distillation, and then it undergoes reaction with a selected acid (boric or hydrochloric acid) in the collection flask distillation. The Kjeldahl flask containing the product of reaction was connected to a distillation apparatus (Lamark DAS-450) and was agitated until the layers mixed. Then the flask was heated so that ammonia, together with some water, was distilled. The distillate ($NH^{3+}$ $H_2O$) is

collected in a receiving flask that contains boric acid (or hydrochloric acid) in excess to neutralize and prevent loss by volatilisation. As the $NH_3$ is collected, it reacts with the boric acid to form the borate ion ($H_2BO^{3-}$) or reacts with HCl acid to form the salt of $NH_4Cl$.

The $NH_4^+$ $H_2BO^{3-}$ was titrated with HCl solution used methyl red as indicator or the remaining (excess). HCl acid was titrated with NaOH solution using methyl red as indicator. The calculation was perform using the followed equation (3.8):

$$\%N=(a*M*14*100)/1000*\ 5g, \text{ where:} \qquad (3.8)$$

a = Volume of HCl solution consumed (ml)
M = Molarity of HCl solution

The method has a detection limit of approximately 0.001% N and is in 8% reproducibility.

### 3.7.1.9 Total phosphorus

Total phosphorous analysis of soils requires the conversion of insoluble materials to soluble forms suitable for colorimetric procedures. The procedure involves $Na_2CO_3$ fusion followed by a colorimetric P determination in water-soluble extract (Olsen and Sommers, 1982; APHA *et al.*, 1995). In summary, approximately 2.5 g of air-dried, sieved soil was added to 25 ml of the Mehlich 3 extracting solution (0.2 *N* $CH_3COOH$ + 0.25 *N* $NH_4NO_3$ + 0.015

*N* $NH_4F$ + 0.013 *N* $HNO_3$ + 0.001 *M* EDTA). The solution was shaken at 200 oscillations per minute for 5 minutes on a reciprocating shaker and filtered through a medium-porosity filter paper (Whatman No. 2). The filtrate was analysed calorimetrically at 882 nm of absorbance.

### 3.5.1.10 Organic matter

The organic matter content influences many soil properties, including 1) the capacity of a soil to supply N, P, S, and trace metals to plants, 2) infiltration and retention of water, 3) degree of aggregation and overall structure that affect air and water relationships, 4) cation exchange capacity, 5) soil colour, which in turn affects temperature relationships and 6) adsorption or deactivation (or both) of agricultural chemicals. To achieve a direct determination of organic matter, one must separate it from inorganic material. The alternative is to destroy the organic matter, after which the loss in weight of the soil is taken as a measure of the organic content. The method for achieving the destruction is the oxidation with ignition of the soil at high temperature (Nelson and Sommers, 1982). The procedure used here is described below.

2.00 g of dried soil (ground to <60 mesh) were transferred to a 500-ml Erlenmeyer flask. Then 10 ml of 0.167 *M* $K_2Cr_2O_7$ were added, in addition to 20 ml of concentrated $H_2SO_4$ (swirled gently to mix). After 30 minutes, the suspension was diluted with about 200 ml of water to provide a clearer suspension for viewing the endpoint. Then, 10 ml of 85% $H_3PO_4$, using a

suitable dispenser, and 0.2 g of NaF were added. 10 drops of ferroin indicator were added and titrate with 0.5 *M* $Fe^{2+}$ to a burgundy endpoint. A reagent blank was run using the above procedure without soil. The blank was used to standardize the $Fe^{2+}$ solution daily. The calculation of %C and % organic matter (Nelson and Sommers, 1982) was performed as follows (equation 3.9).

a) % Easily oxidable organic C

%C = (B-S) x M of $Fe_2$+ x 12 x 100 g of soil x 4000*, where: (3.9)

B = ml of $Fe^{2+}$ solution used to titrate blank
S = ml of $Fe^{2+}$ solution used to titrate sample
*12/4000 = milliequivalent weight of C in g

To convert total organic C to organic matter equation (3.9.1) was used:

b) % Organic Matter =(% total C x 1.72)/0.58 (3.9.1)

**3.5.1.11 Total organic carbon**

Carbon is the chief element present in soil organic matter. Organic carbon was determined using a TOC-5000A Shimadzu 2000 total organic carbon analyser.

### 3.5.1.12 Polycyclic aromatic hydrocarbons

A soil sample was extracted for PAHs in a Soxhlet apparatus with acetone (Sigma-Aldrich ®) for 8 h. The efficiency extraction of phenanthrene from soil was 85 ± 3.7 %.

Samples were analysed by gas chromatography/mass spectrometry (GC/MS), using either GCQ Finnigan Mat or Top Fisons with a AS800 autosampler (see section 3.5.3).

### 3.5.1.13 Total bacteria

The Most Probable Number (MPN) technique is based on a determination of the presence or absence of microorganisms in several individual portions of each of several consecutive dilutions of soil (Alexander, 1982). To prepare soil culture tubes 2 g of soil was used. A positive reading meant that at least one microorganism was present initially in the proportion used for inoculation.

### 3.5.1.14 Hydrocarbonoclastic microorganisms

Hydrocarbonoclastic microorganisms (which have the ability to degrade and utilize hydrocarbons) were counted. The method uses a reduction assay and the number was calculated using the MPN technique (Alexander, 1982). The method uses 2 g of soil to prepare soil culture tubes. A positive reading

meant that at least one microorganism was present initially in the proportion used for inoculation.

### 3.5.1.15 Total fungi

Isolation of fungi from soil was performed by the soil dilution plate method (Parkinson, 1982), which consisted of: 1) the preparation of initial soil suspension, 2) preparation of an appropriate dilution series from this suspension and 3) plating of aliquots of appropriate dilutions on water agar. After incubation, a counting plate was used to determine the colony-forming unit (UFC) presented.

### 3.5.2 $CO_2$

The evolution of $CO_2$ was measured to quantify the heterothrophic activity (Mitchell, 1992; Dosoretz *et al.*, 1990; Saucedo *et al.*, 1994).

Headspace samples (2 ml) were taken from microcosms with a 5-ml gas-tight syringe. The $CO_2$ content of the samples was determined with a Gow Mac 550 gas chromatograph equipped with a thermal conductivity detector and an Alltech CTRI stainless steel column.

The operation conditions were, 30 °C oven temperature, 30 °C injector temperature, and 125 °C detector temperature. Helium was used as a carrier gas, and the flow rate was 45 ml/min.

Data were processed with Gow Mac software and integrated to obtain the cumulative $CO_2$ production. The $CO_2$ production per initial dry matter (IDM) was obtained using the equation (3.10).

IDM=PVW/RTG, where: (3.10)

P is pressure
V is volume of the sub sample
W is molecular weight
R is gas universal constant
T is temperature
G is the initial mass added

### 3.5.3 GC/MS

Soil samples were removed from each bottle and extracted in a Soxhlet apparatus with acetone (Sigma-Aldrich ®) for 8 h (EPA-3540). The extract was diluted 1:1 with a naphthalene standard (fisher, 98% purity). Samples were analysed by gas chromatography/mass spectrometry (GC/MS), using for Results 1 (Chapter 4) a GC/MS GCQ Finnigan Mat and for the Results 2

and 3 (Chapters 5 and 6) a GC 8000 Top Fisons with an AS800 auto-sampler.

The conditions for the GC/MS GCQ Finnigan Mat were the following. Hewlett Packard capillary column (0.32mm x 50m, 5% phenyl and 95% methyl silicon), initial column temperature 60°C for 4 min, 250°C at 5°C/m, 19 min. Helium was used as carrier gas at a flow rate of 15 ml/min. The injector temperature was 270°C, the interface temperature was 280°C and the detector was an electromultiplier. The conditions for the GC 8000 Top Fisons were the following. Fused-silica capillary column Excellence (0.25 mm x 60m, ID-BPS 0.25 mm, phase BP5 non-polar), initial column temperature 60°C for 4 min, 270°C at 5°C/m, 19.62 min. Helium was used as carrier gas at a flow rate of 13.2 ml/min. The injector temperature was 260°C and the interface temperature was 280°C.

Phenanthrene quantification was accomplished by comparing its mass spectra with the electron impact spectra of the phenanthrene standard purchased as certified. The base peak ion from the specific internal standard was used as the primary ion for quantification, which was accomplished by comparing the response of a major (quantification) ion relative to the internal standard (naphthalene) using a five-point calibration curve. The method had a detection limit of approximately 0.002 mg $Kg^{-1}$ and was in 8% reproducibility.

### 3.5.4 Microwave digestion

**5.4.1 Soil digestion.** 0.2 g of soil was digested in 9 ml of concentrated nitric acid and 3 ml hydrofluoric acid for 15 minutes using a CEM Corporation microwave, model Mars X, version 047919. The sample and acid were placed in suitably inert polymeric microwave vessels. The vessel was sealed and heated in the microwave system. The following conditions in two stages were followed (Table 3.6). After cooling, the vessel contents were filtered, diluted and analysed by GFAAS. Blank samples using acids were prepared using the same reagents and quantities used in sample preparation, placed in vessels of the same type, and processed with the samples. Each treatment was replicated 3 times.

**Table 3.6** Operation conditions for microwave digestion of soil.

| Maximum wattage | Power | Time (min) | Pressure (psi) | Hold time (min) | Temperature (°C) |
|---|---|---|---|---|---|
| 600 | 100 | 2 | 300 | 0 | 165 |
| 600 | 100 | 3 | 300 | 5 | 175 |

The multiple point calibration for a 600 W unit involved the following power settings: 100, 99, 98, 97, 95, 90, 80, 70, 60, 50, and 40% using the following procedure. One kg of reagent water is weighed (1,000.0 g + 0.1 g) into an inert polymeric beaker. The initial temperature of the water was 23 ± 2 ºC. The covered beaker was circulated continuously through the microwave field for 2 min at the desired partial power setting with the system's exhaust fan on maximum. The beaker was removed and the water vigorously stirred. A magnetic stirring bar was used inserted immediately after microwave

irradiation and record the maximum temperature within the first 30 seconds to ± 0.05 ºC.

**3.5.4.2 Plant digestion.** 0.5 g of dried plant (roots and leaves) was digested in 5 ml of concentrated $HNO_3$ and 50 ml MQ water for 15 minutes using a CEM Corporation microwave, model Mars X, version 047919. The sample and acid were placed in suitably inert polymeric microwave vessels. The vessel was sealed and heated in the microwave system. The following conditions in one stages were followed (Table 3.7). Each treatment was replicated 3 times.

**Table 3.7** Operation conditions for microwave digestion of plants.

| Maximum wattage | Power | Time (min) | Pressure (psi) | Hold time (min) | Temperature (°C) |
|---|---|---|---|---|---|
| 1200 | 100 | 20 | 170 | 10 | 200 |

After cooling, the vessel contents were filtered, diluted and analysed by GFAAS. Blank samples using acid and water were prepared using the same reagents and quantities used in sample preparation, placed in vessels and processed with the samples. A suitable multiple point calibration was used for a 600 W.

### 3.5.5 GFAAS

A 280 Perkin Elmer graphite furnace atomic absorption spectrometer was used. 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 (see Table 3.8 for each metal). When the temperature

is increased to the point where sample atomisation occurs, the atomic absorption measurement was made. The main steps will be described briefly.

1) The drying step: After the sample was placed in the furnace, it was dried at low temperature to avoid sample spattering, which would result in poor analytical precision (Beaty and Kerber, 1993). A temperature “ramp” programme was used according to the type of metal. After the temperature ramp, an appropriate drying temperature was selected until drying is completed.

2) The pyrolysis (ashing) step: This step volatilises remaining inorganic and organic matrix components selectively from the sample. The temperature was increased as high as possible to volatilise matrix components but below the temperature at which analyte loss would occur.

3) The atomisation step: To produce an atomic vapour of the metal, thereby allowing atomic absorption to be measured, temperature was increased to the point where dissociation of volatilised molecular species occurs (see Table 3.8 for each metal).

4) The clean out and cool down steps: After atomisation, the graphite furnace was heated to still higher temperatures to burn off any sample residue, which may remain in the furnace. This was performed to prevent cross contamination.

Variables under operator control include the volume of sample placed into the furnace and heating parameters for each step. Table 3.8 shows the

conditions for each metal, using the suitable lamp for each one. Since the graphite furnace signal depends on analyte mass, the control of measured absorbance was performance by controlling the sample volume. 20 μl was used as a sample volume considered a convenient volume for most analyses (Beaty and Kerber, 1993). All reagents were prepared with Milli-Q water (conductivity 18.2 $M\Omega cm^{-1}$).

**Table 3.8** Parameters used for the determination of trace metals.

| Metal | Wavelength (nm) | Dry Temp. (°C) Time (seg) | Atomisation Temp. (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|---|---|
| Cd | 228.8 | 100-130 50 | 1400 | 1 | 0 |
| Cr | 357.9 | 100-130 50 | 2300 | 1 | 0 |
| Cu | 324.7 | 100-130 50 | 1900 | 2 | 20 |
| Ni | 232.0 | 100-130 50 | 2300 | 1 | 0 |
| Pb | 217.0 | 100-130 50 | 1500 | 1 | 0 |

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. Analytical errors were determined by replication of blanks and standards and the data were interpreted by the statistical methods. The relative standard deviation was 2%.

# CHAPTER 4. RESULTS AND DISCUSSION 1

# BIOREMEDIATION PROCESS

## 4.1 Summary

This chapter reports the determination of the ability of the non-basidiomycete, filamentous fungi *Penicillium frequentans*, to remove phenanthrene in a solid-state culture. Additionally, the study investigated whether phenanthrene removal could be enhanced by manipulating the carbon-to-nitrogen-to phosphorous ratio (C:N:P) and moisture content over a relatively short-term period (29 days). To evaluate the combined effect of moisture content and the C:N ratio, a combined experimental design, composed with a $2^2$ factorial design with both central and axial points, was used (see Chapter 3, section 3.3 for the method). It was shown that the moisture content ($p<0.0003$) and the combined effect of moisture content and C:N ratio have a significant ($p<0.002$) effect on the phenanthrene removal. It was also found that heterothrophic activity was not correlated to phenanthrene removal. An optimum phenanthrene removal efficiency of 74 ± 3.8% was realised at a moisture content of 40% and a C:N ratio of 60. This suggests that *Penicillium frequentans* was able to effectively remove phenanthrene in a solid-state culture and that the combination of nutrient addition and moisture adjustment could enhance the phenanthrene removal activity.

## 4.2 Soil characterisation and effects on biodegradation

### 4.2.3 Soil texture and structure

The various chemical and physical properties of a soil determine the nature of the environment in which microorganisms are found (Parr *et al.*, 1983). A typical mineral soil is composed of approximately 45% mineral material (varying proportions of sand, silt and clay), 25% air and 25% water (*i.e.*, 50% pore space, usually half saturated with water), and 5% organic matter, although this is highly variable. Table 4.1 shows the characteristics of the sampled soil. Soil had a predominance of sand (54%), silt (28%) and clay (18%), therefore was classified as a sandy clay loam soil.

It has been shown that soil type affects phenanthrene biodegradation (Riser-Roberts, 1998). Manilal and Alexander (1991) report that mineralisation of phenanthrene occurs more slowly in soil than in liquid media. This may be caused by sorption to soil constituents, such as organic matter. After exposure to different soil types with high clay content, up to 25% of phenanthrene was not extractable (Karimi-Lotfabad *et al.*, 1996). Therefore, it is necessary to understand soil structure in order to determine the likely effectiveness of bioremediation and ways to optimise bioremoval of PAHs. The soil type used here contains clay as a minimum fraction and the concentrations of PAHs in the soil were not detectable (DL=0.002 mg $Kg^{-1}$). However, this soil is representative of the study area in the southeast part of Mexico and it was considered as a suitable soil model to carry out the present study.

Table 4.1 Major physico-chemical characteristics of experimental soil.

| Characteristic | Value |
|---|---|
| **Physical** | |
| Texture | Sandy clay loam |
| Sand | 54.00 % |
| Silt | 27.99 % |
| Clay | 17.99 % |
| Moisture | 2 % w/w |
| Water holding capacity* | 15% |
| Porosity | 54.26 |
| Real density | 2.4135 g $cm^{-3}$ |
| Apparent density | 1.104 g $cm^{-3}$ |
| **Chemical** | |
| pH | 5.7 |
| CEC | 5.1 meq 100 $g^{-1}$ |
| Total nitrogen | 0.269 % |
| Total phosphorous | 0.183% |
| Organic matter | 6.83% |
| Total organic carbon | 41.34 mg $Kg^{-1}$ |
| Polyaromatic hydrocarbons | ND |
| Iron | 11538 mg $Kg^{-1}$ |
| Copper | 59 mg $Kg^{-1}$ |
| Manganese | 226 mg $Kg^{-1}$ |
| Zinc | 103 mg $Kg^{-1}$ |
| Nickel | 32 mg $Kg^{-1}$ |
| Lead | 14 mg $Kg^{-1}$ |
| Cadmium | 18 mg $Kg^{-1}$ |
| Chromium | 23 mg $Kg^{-1}$ |
| Cobalt | 6 mg $Kg^{-1}$ |
| **Biological** | |
| Total bacteria | 20 x $10^5$ MPN $g^{-1}$ |
| Hydrocarbonoclastic | ND |
| Total fungus | 200 CFU $g^{-1}$ |

ND: not detect; MPN: most probable number; CFU: colony-forming unit;
*water holding capacity at field capacity.

Soil bulk density is a measure of dry soil weight per unit of volume and determines pore space through which water can move (Hornick *et al.*, 1983).

Pore size affects the rate of growth of organisms (McInerney *et al.*, 1993). Smaller pore sizes produced reduce growth due to a restriction of bacterial cell division. However, soil has a suitable density (2.4 g $cm^{-3}$) to let organisms grow (Dane and Topp, 2002), and therefore to consume an alternative carbon source, such as phenanthrene.

In addition to soil texture and structure, the most important soil factors that affect phenanthrene degradation are: nutrients, water, organic matter, soil pH and aeration or oxygen supply (Sims and Bass, 1984). Any treatments applied to the soil to enhance contaminant removal processes must not alter the physical or chemical environment to the extent of severely restricting microbial growth.

### 4.2.2 Nutrients

Microbial degradation requires the presence of nutrients such as nitrogen, phosphorous and potassium (Arora *et al.*, 1982). Nitrogen and phosphate are the nutrients most frequently present in limiting concentrations in soil (USEPA, 1994). Applying such nutrient solutions to natural soil bacteria often enhances the ability of the microorganisms to degrade organic molecules into carbon dioxide and water.

The addition of sugar cane bagasse pith increases microbial respiration and biodegradation (Al-Hadhrami *et al.*, 1996; Rodríguez-Vázquez *et al.*, 1999b).

Addition of nutrients increases total biomass available for degradation of PAHs (Riser-Roberts, 1998).

The carbon, nitrogen and phosphorous content of bacterial cells is generally the ratio of 100 parts carbon to 15 parts nitrogen to 3 parts phosphorous (Zitrides, 1983). However, there is some disagreement on the exact C:N: P ratio in soils. Sufficient nitrogen and phosphorous should be applied to ensure that these nutrients do not limit microbial activity (Alexander, 1994). In this study soil, C:N: P was considered as another variable to optimise phenanthrene removal, as C:N ratio is one of the primary factor to be optimised in soil (Zhou and Crawford, 1995). Theoretically, 150 mg of nitrogen and 30 mg of phosphorous are required to convert 1 g of hydrocarbon to cellular material (Rosenberg *et al.*, 1992). However the conversion of substrate to cellular material is less than 100% as the ratio of C to nutrients depends on the rate and extent of biodegradation of the chemicals and may vary according to the particular contaminant. A factorial experimental design with axial and central points established the maximum, minimum and the intermediate points for C/N ratio at the present study.

During optimisation of the bioremediation process, emphasis should be placed on meeting the requirements of microorganisms for growth (Atlas and Bartha, 1993). Nutrients are able to enhance biodegradation rates of the indigenous hydrocarbon degraders (Atlas and Cerniglia, 1995). In the study soil, the total concentration of bacteria and fungi were low and hydrocarbonoclastic microorganisms were not detected (Table 4.1). For this

reason, bioaugmentation (supplementation of microorganisms to a contaminated site to enhance bioremediation) was required in this study. To enhance the phenanthrene biodegradation, biostimulation (the stimulation of microbial degradation of organic contaminants by the addition of nutrients, or optimisation of environmental factors) was used.

Mineralisation of phenanthrene is enhanced by addition of phosphate (Riser-Roberts, 1998). Several studies have suggested that adjusting the C/N/P ratio in contaminated environments can stimulate the degradation of PAHs (Rogers *et al.*, 1993; Meulenberg *et al.*, 1997). In addition, fungi can degrade phenanthrene in nitrogen-rich culture media (Sutherland *et al.*, 1991). Measurement of soil carbon, nitrogen, and phosphorous allows the determination of its carbon-to-nitrogen-to-phosphorous (C:N:P) ratio and evaluation of nutrient availability (Sims and Bass, 1984). Although mineralisation of phenanthrene and other structurally related compounds is greater at low nitrogen levels (Field *et al.*, 1993), and the study soil has low content of nitrogen, but high content of P (Dane and Topp, 2002). A balance of C:N:P is required to obtain a suitable balance between these nutrients, therefore the most suitable conditions for phenanthrene removal efficiency.

### 4.2.3 Soil moisture and water holding capacity

Aerobic hydrocarbon decomposition is diminished under saturated moisture conditions because of low oxygen supply, while under very dry conditions, microbial activity is hindered due to insufficient moisture levels necessary for

microbial metabolisms (CONCAWE, 1980). A typical soil is about 50% pore space and 50% solid matter (JRB Associates, Inc., 1984). Water entering the soil fills the pores spaces until they are full. The water then continues to move down into the subsoil, displacing air as it goes. Soils with large pores, such as sands, lose water rapidly. In the soil that has been studied, the content of sands of 54% is not considered a major problem, as 60% is the maximum content recommended for suitable oxygenation and water content retention (Riser-Roberts, 1998). Large pores are less hospitable environments for microorganisms (Turco and Sadowsky, 1995), whereas the smaller pores inside soil aggregates retain water (Papendick and Campbell, 1981). Soil with a mixture of pore size, such as loamy soils, hold more water at saturation and lose water more slowly. The sandy clay loam texture of the study soil freely draining, therefore is a suitable soil for bioremediation process.

Water-holding capacity at the field capacity of this soil is 15% and moisture content is 2%. Therefore a different moisture content in soil was tested and maintained, in order to find the optimum moisture content to enhance phenanthrene bioremoval. Riser-Roberts (1998) report optimal biodegradation at a soil water-holding capacity of 30 to 90%. However, several authors have indicated ranges of moisture for optimum biodegradation (Bossert and Bartha, 1986; Ryan and Dhir, 1993), depending on the type of PAHs. Holman and Tsang (1995) determined that 50 to 70% of water content was the optimum for aromatic hydrocarbons biodegradation. For phenanthrene, the model provides a good fit at soil water content below

50%. The dependency on soil water content for phenanthrene biodegradation is compound specific and probably also soil specific (Holman and Tsang, 1995). The moisture content of soil may be controlled to immobilise constituents in a contaminated soils and to allow additional time for accomplishing biological degradation.

### 4.2.4 Organic matter

Since soil generally contains 5 to 12% organic matter (Cerniglia, 1992), the 6.8% organic matter of the soil that has been studied is within the average. Sorption of contaminants on soil particles can alter the molecular character and enzymatic attack of a given compound. In addition, organic matter usually is not an easily carbon source to assimilate (Atlas and Cerniglia, 1995), since 65 to 75% of the organic material in soil usually consists of humic substances (humic acid, fulvic acid, and hummin), which are refractory.

For the study soil, modified Wunder media (Wunder *et al.*, 1994), which contains glucose, was added to the system for improving phenanthrene removal rate, since glucose is usually an easily assimilable source of carbon compared to soil organic matter and phenanthrene (Bossert and Bartha, 1986). Soil organic material generally consists of humic substances with high density of reactive functional groups, which help to bind both organic and inorganic compounds that may be added to the soil. These properties also help to retain the soil bacteria, which can attack the bound compounds

(Godbout *et al.*, 1995). Manilal and Alexander (1991) also suggest that biodegradation of phenanthrene may be slowed by their sorption to soil organic matter.

### 4.2.5 pH

In general, the soil pH is between 5 and 9 (Atlas and Bartha, 1981; Sommers *et al.*, 1981). The pH of the study soil was slightly acidic (pH=5.7) and fungi predominate under acidic conditions. Biological activity in the soil is greatly affected by pH, through the availability of nutrients and toxicants and the tolerance of organisms to absolute variations in pH. Bacteria and fungi have a pH optimum near 7.0 for biological activity and growth, although fungi in general tolerate lower pH more easily.

### 4.2.6 Metals

The total concentrations of metals of the study soil (Table 4.1) are within the recent maximum metal concentrations in agricultural soils of European Union, Mexico and US (Table 4.2), except in the case of cadmium.

**Table 4.2** Maximum metal concentrations ($\mu g\ g^{-1}$) in agricultural soils of various countries and years comparing with the concentrations in the study soil.

| Country | Cd | Cr | Cu | Ni | Pb |
|---|---|---|---|---|---|
| European Union, 2001-2006 [a] | 1 | 100 | 100 | 50 | 100 |
| Mexico, 2002[b] | 3 | 75 | 100 | 75 | 100 |
| US, 2003[c] | 1.5 | 140 | 75 | 50 | 140 |
| Study soil | **18** | 23 | 59 | 32 | 14 |

[a]Commission Decision, 2001; [b]PROFEPA, 2002; [c]USEPA, 2002.

Cadmium concentration in the study soil is higher than the criteria for agricultural soil (Table 4.2, in bold), and its concentration corresponds to a soil with industrial contamination.

## 4.3 Fungal bioremediation

### 4.3.1 Choice of the microorganism

Fungi play an important role in the hydrocarbon-oxidising activities of the soil, and they seem to be at least as versatile as bacteria in metabolising aromatics (Riser-Roberts, 1998). Several fungi can cope better with the various bonding mechanisms involved with contaminants adsorbed to soil particles (Sutherland, 1991). Therefore, fungi exhibit greater hydrocarbon biodegradation than bacteria (Cerniglia, 1992). Soil fungi are generally believed to play a more important role in the formation, metabolisms, and interactions of soil-organic matter complexes than do bacteria. Most of the fungal transformation products are less mutagenic that the parent compound (Riser-Roberts, 1998).

A wide range of fungi have the enzymatic capacity to oxidise PAHs when grown on an alternative carbon source (Cerniglia, 1992). However the ability to utilise hydrocarbons occurs mainly in two orders, the Mucorales and the Moniliales. Fungi, besides bacteria, can also metabolise phenanthrene (see

Figure 2.3) and the conjugates formed may be considered detoxification products of phenanthrene (Ouyang, 2002).

*Aspergillus* and *Penicillium* are rich in hydrocarbon-assimilating strains (Sutherland, 1991). Hofrichter and Scheibner (1993) discussed the utilisation of aromatic compounds by *Penicillium* strain BI7/2. Hofrichter *et al.* (1995) described the co-metabolic degradation of o-cresol and 2,6-dimethylphenol (2,6-xylenol) by *P. frequentans* BI7/2. Wunderwald (1997) found *P. frequentans*, using phenol as a sole source of carbon and energy, transformed difluorinated phenol into difluorocatechol. Recently, Pérez-Armendariz *et al.* (2003) isolated *P. frequentans* from bagasse and demonstrated the ability of this fungus to grow in PAHs contaminated soil. The genera most frequently isolated from soils are those producing abundant small conidia, such as *Penicillium* spp (Müncnerova, and Augustin, 1994). However, the potential of the species *P. frequentans* to degrade phenanthrene, has received limited attention in the literature (Laborda *et al.*, 1999). For these reasons *P. frequentans* was chosen for this study, as a potential species capable of removing phenanthrene. In addition, there is a lack of information about the relationship between soil native microflora and *Penicillium* sp. (Chávez-Gómez *et al.*, 2003), so it is unknown how *P. frequentans* will perform in real soils.

Although, antagonism between certain species has been observed using an inoculum of a white rot fungus (Fernández-Sánchez *et al.*, 2001), the relationship between *Penicillium frequentans* to soil microflora is still unknown. Therefore, this study will provide the basis of the knowledge of the

microbial interactions between soil native microflora and *P. frequentans*, in addition to the study of phenanthrene removal kinetics.

### 4.3.2 $CO_2$ production

Cumulative $CO_2$ evolution increased as a function of time (Figure 4.1). Except for controls, which were composed of sterile soil and sterile bagasse, all treatments showed an increase in $CO_2$ production (150 μg $CO_2$ $g^{-1}$ IDM) after 15 days (Fig. 4.1). This increase might be explained by the high carbohydrate content of bagasse (Rodríguez-Vázquez, 1993, 1999b; Padley *et al.*, 2000), used by microorganisms as one of the main carbon and energy source. Since fungal growth in soil can be difficult, the fungus was grown into lignocellulosic substrates (Mitchell, 1992) to propagate and give the fungal strain a period of maturation to allow it to survive in soil.

In order to evaluate the fraction of $CO_2$ production due to soil organic matter utilisation, a control with only sterilised soil and fungi was conducted. As shown by Figure 4.1, the use of soil organic matter as an auxiliary carbon source is negligible compared to treatments with soil and phenanthrene. The $CO_2$ production is negligible when organic matter is used as a carbon source, which is not surprising since only 6-7% of soil is organic matter and this may be refractory (Cerniglia, 1992). Therefore, the use of biostimulation and bioaugmentation is essential.

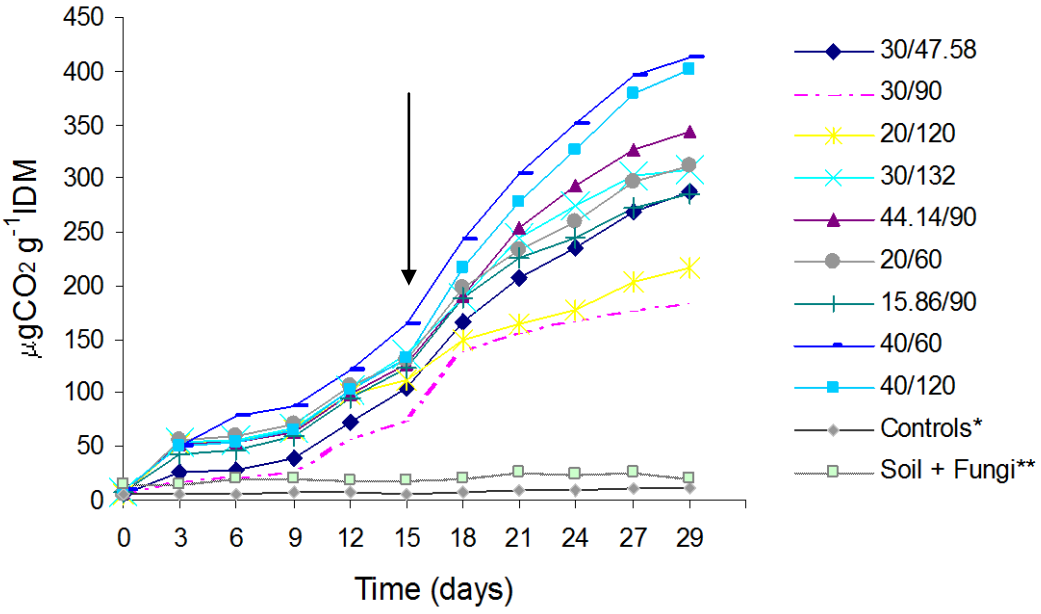



**Figure 4.1** Heterothrophic microbial activity during soil treatment by solid state culture, as a function of moisture content and C:N ratio. Arrow indicates the time when phenanthrene polluted soil and nutrients were added. IDM= Initial dry matter. *Controls included sterilised soil and sterilised bagasse.**Treatment without biostimulation. In the box legend, the first number corresponds to C:N ratio, and the second corresponds to the moisture content. Arrow indicates the time when phenanthrene polluted soil was added. Error bars are too small to show with clarity.

### 4.3.3 Phenanthrene removal

The fungus *Penicillium frequentans* was able to remove 27% of phenanthrene without any nutrient addition. Phenanthrene removal efficiency

was enhanced from 27 to 74% (Table 4.3), and there was a significant difference ($p<0.005$) between each treatment (Table 4.4), with a coefficient variation of 7.2%.

After day 15, heterothrophic activity of soil did not correlate ($r^2=0.005$) to phenanthrene removal (*i.e.* 40 μg $CO_2$ $g^{-1}$ IDM corresponds to 36% removal and 41 μg $CO_2$ $g^{-1}$ IDM to 74% removal), which is attributed to the capacity of fungus to remove more available carbon sources, contained in the Wunder media (such as glucose). It is also likely that phenanthrene was only partially degraded and not mineralised to $CO_2$. This finding is in agreement with previous reports, which describe that the presence of co-substrates, *e.g.* glucose, enhances PAH degradation (Bossert and Bartha, 1986). The variations in the phenanthrene removal using different co-substrates has been demonstrated by Yuan *et al.* (2000), who reported that phenanthrene removal was enhanced by the addition of various organic sources. Phenanthrene removal was enhanced in the following order: yeast extract> acetate> glucose> pyruvate (Yuan *et al.*, 2000).

Table 4.3 Factorial experimental design with axial and central points, including independent variables (moisture content and C:N ratio), observed and predicted phenanthrene removal.

| **Independent variables** | **Dependent variable phenanthrene** |
|---|---|

| Moisture (%) | C:N ratio | Removal observed (%) | Removal predicted (%) |
|---|---|---|---|
| 30 | 47.58 | 65 | 73 |
| 30 | 90 | 61 | 61 |
| 30 | 90 | 61 | 61 |
| 30 | 90 | 60 | 61 |
| 30 | 90 | 59 | 61 |
| 30 | 90 | 63 | 61 |
| 20 | 120 | 62 | 65 |
| 30 | 132 | 68 | 64 |
| 44.1 | 90 | 33 | 36 |
| 20 | 60 | 53 | 47 |
| 15.9 | 90 | 41 | 42 |
| 40 | 60 | 74 | 67 |
| 40 | 120 | 36 | 37 |

According to the results from the statistical analysis (Table 4.4), the factors that were significant are: moisture content ($p<0.0003$) and the interaction between moisture and C:N ratio ($p<0.003$).

Table 4.4 Estimated parameters during the analysis of variance, which include interaction between variables: $X_1$ (moisture content) and $X_2$ (C:N ratio).

| Variable | Coefficient | P value > \|F\| |
|---|---|---|

| | | |
|---|---|---|
| Intercept | 90.86 | 0.0371 |
| $X_1$(moisture) | 9.79 | 0.0003 |
| $X_2$ (C:N) | 0.27 | 0.583 |
| $X_1 X_2$ | -0.039 | 0.0029 |
| $X_1 X_1$ | -0.108 | 0.0010 |
| $X_2 X_2$ | 0.004 | 0.0856 |

$R^2$ =0.89995287, C.V.= 9.29%

Figure 4.2 presents data of the surface response of phenanthrene removal as a function of moisture and C:N ratio, obtained from the regression analysis (equation 4.1), where z represents the phenanthrene removal, x the moisture content and y the C:N ratio. Results showed that, after 29 days, the highest phenanthrene removal efficiency of 74% was obtained with the combination of a moisture content of 40% and a C:N ratio of 60:1. The moisture content had a statistically significant effect ($p<0.0003$) on the phenanthrene removal. At the highest moisture content (44.14%) and C:N ratio (90:1), the phenanthrene removal was 33%.

$$z=-90.9+9.8x+0.27y-0.11x^2-0.04xy+0.004y^2 \qquad (4.1)$$

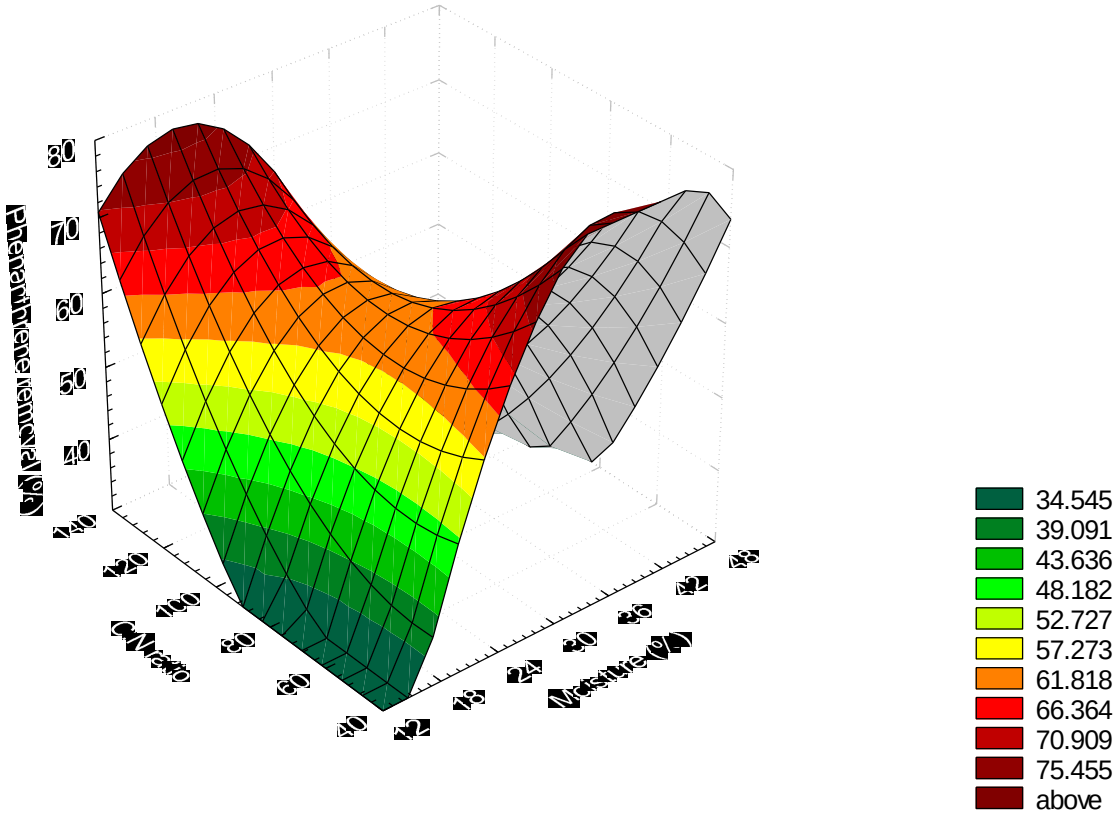



**Figure 4.2** Surface response of phenanthrene removal as a function of moisture content and C:N ratio. The value of the independent variables were calculated according to the combined factorial experimental design, composed by $2^2$ factorial with both central and axial points (Table 4.3).

The optimal C:N ratio and moisture content in a soil both depend upon the rate and extent of biochemical degradation and may vary according to the particular type of contaminant and soil. For this reason, equation 4.1 is only useful for this particular soil and contaminant. Therefore, an optimal ratio and moisture would need to be assessed for any soil type and contaminant undergoing further investigation.

Zhou and Crawford (1995) reported that the optimal C:N ratio from a clay/loam soil was 50:1. In the results presented here, the greatest phenanthrene removal was found under C:N ratio of 60:1, which corresponds to 8 mM N in this soil. This is consistent with, but slightly higher than, the levels reported by Kirk (1980), who mentioned that a nitrogen-deficient culture (2-3 mM N) is the optimum concentration for contaminant metabolism, and higher concentrations severely suppressed this metabolism, favouring non-ligninolytic fungi. Additionally, excessive nitrogen (>20 mM) can impair biodegradation, possibly due to ammonia toxicity (Zhou and Crawford, 1995), which is consistent with our data.

The surface response of phenanthrene removal as a function of moisture and C:N ratio (Figure 4.2) shows two optimal conditions on the phenanthrene removal, which may imply two types of processes. In the first case, where C:N ratio was low and the moisture content high, the system may be saturated, and therefore oxygen deficient (Arora *et al.*, 1982). Addition of the bulking agent (bagasse) forms empty spaces allowing oxygen transfer into the system (LaGrega *et al.*, 1996), favouring fungal aerobic metabolism. In the second case, where the C:N ratio was high and the moisture content low, the increased efficiency of phenanthrene removal could be due to a low nitrogen content, avoiding a possible toxic effect by ammonium (Zhou and Crawford, 1995).

As observed, C:N ratio significantly interacts with moisture content. Moisture content has a significant effect during this experiment (Figure 4.2). Previously moisture was shown to be a critical parameter for the degradation of two-, three-and four-ring polycyclic aromatic hydrocarbons (Loehr, 1992). The density and texture of soil determine the water-holding capacity and moisture content, both of them affect oxygen availability, redox potential and biological activity, so potentially affecting degradation. In this study, the optimum moisture content of 40% agrees well with the value of below 50% previously reported (Holman and Tsang, 1995).

## 4.4 Conclusion

It was found that *Penicillium frequentans* was able to remove up to 74% of phenanthrene, under optimal culture conditions (nutrient ratio and moisture content). In this laboratory-based study, the use of *P. frequentans* has been shown to be a potentially important method for degradation of phenanthrene, and, by implication, for the degradation of other PAHs and for bioremediation of contaminated soils. Further work is required to optimise the experimental conditions for different soil types and different pollutants and to elucidate the enzymatic pathways used in the biodegradation process.

# CHAPTER 5. RESULTS AND DISCUSSION 2
# IMPACT OF MICROBIAL ACTIVITY ON METAL BEHAVIOUR DURING PHENANTHRENE BIOREMOVAL

## 5.1 Summary

Assessment of potential metal biouptake from soils before and after bioremediation was studied considering the solution speciation and the kinetics of exchange between solution and solid phase. These parameters were measured in phenanthrene-contaminated soils by the techniques of Diffusive Gradient in Thin-films (DGT) and filtration. A number of different treatments were performed on both previously sterilised (prior to inoculation with *Penicillium frequentans*) and non-sterilised soils (which include soil microflora and *P. frequentans* inoculum). Treatment A contained soil, fungus and phenanthrene (S + F + P), Treatment B contained soil and fungus (S + F), Treatment C contained soil and phenanthrene (S + P), and Treatment D contained soil only (S). Treatments C and D contained no fungus and were controls to allow calculation of the net impact of the fungus only. Heterothrophic activity was measured indirectly by $CO_2$ evolution. Phenanthrene was measured by GC/MS. All measurements (metals by DGT, metals in soil solution, $CO_2$ and phenanthrene) were performed in triplicate every three days for 30 days before and after inoculation of fungi (see Chapter 3, section 3.4 for the method). It was found that $CO_2$ production and phenanthrene removal increases in the presence of both soil microflora and fungi, since microorganisms appear to act in a synergistic manner. In addition, removal of phenanthrene by either fungal or mixed fungal and native microflora resulted in an increased pool of potentially bioavailable and

toxic metal species and increased fluxes from solid to solution. These results highlight the impact of bioremediation process on metal behaviour.

## 5.2 Microbial activity

### 5.2.1 $CO_2$ production

Figure 5.1 shows the $CO_2$ production before and after mixing of soil and fungus. The increase observed is due to microbial action by *P. frequentans* in sterilised soil (Fig. 5.1a) and by *P. frequentans* inoculum and soil microflora in non-sterilised soil (Fig. 5.1b). Higher activities are seen in the non-sterilised soil and with the addition of phenanthrene. This was due to the synergistic action of native microflora and fungi and to the use of phenanthrene as a carbon and energy source. The type of treatment (Treatment A-D), time of treatment and soil type (sterilised and non-sterilised soil) were found to significantly ($p< 0.0001$) affect the $CO_2$ production.

The maximal heterothrophic activity (180 $\mu$g $CO_2$ $g^{-1}$ IDM) was found in non-sterilised soil spiked with phenanthrene (Treatment A). In the case of sterilised soil, the maximal activity (90 $\mu$g $CO_2$ $g^{-1}$ IDM) was found due to the microbial activity in the soil without phenanthrene (Treatment B). This result indicates that the added fungus and native microflora acted synergistically and were able to minimise potential deleterious effects of the phenanthrene and maximise the use of carbon and energy sources. In contrast, growth and activity of the *P. frequentans* alone was reduced due to the harmful effects of

the phenanthrene, despite the ability of the fungus to degrade phenanthrene (see section 5.2.2).

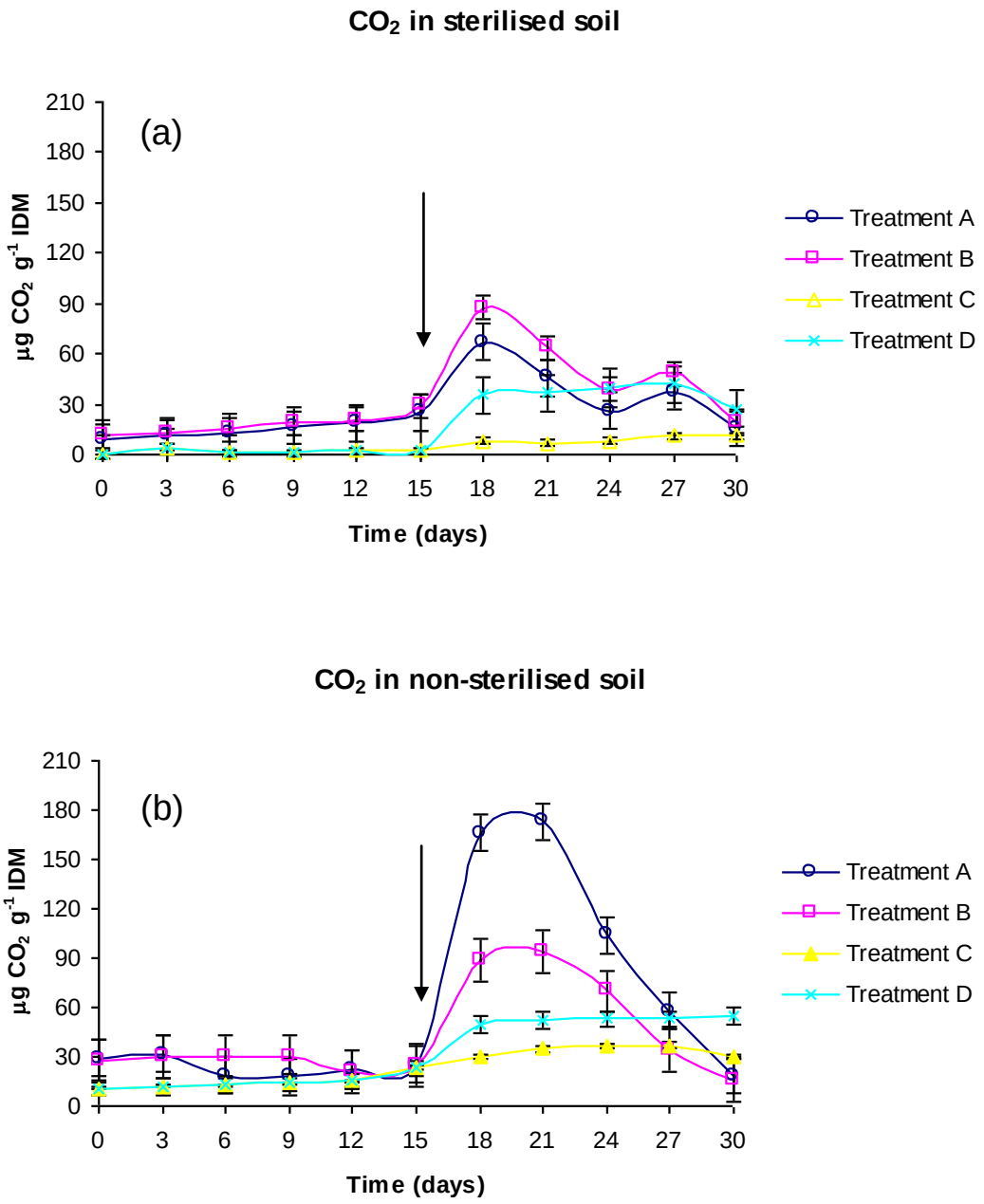



**Figure 5.1** $CO_2$ evolution during the phenanthrene bioremoval process: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi, Treatment C= Soil + Phenanthrene, Treatment D= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. IDM= Initial dry matter.

### 5.2.2 Phenanthrene removal

The highest phenanthrene removal was observed in non-sterilised soil (with *P. frequentans* inoculum and soil microflora) at day 21 (Figure 5.2), with a total degradation of 73% ± 2.3 in 30 days. With sterilised soil, maximum phenanthrene removal was observed at day 24 and total phenanthrene removal was lower (60% ± 1.8). The type of treatment ($p < 0.0005$) and time of treatment ($p < 0.0001$) were found to significantly affect the phenanthrene removal.

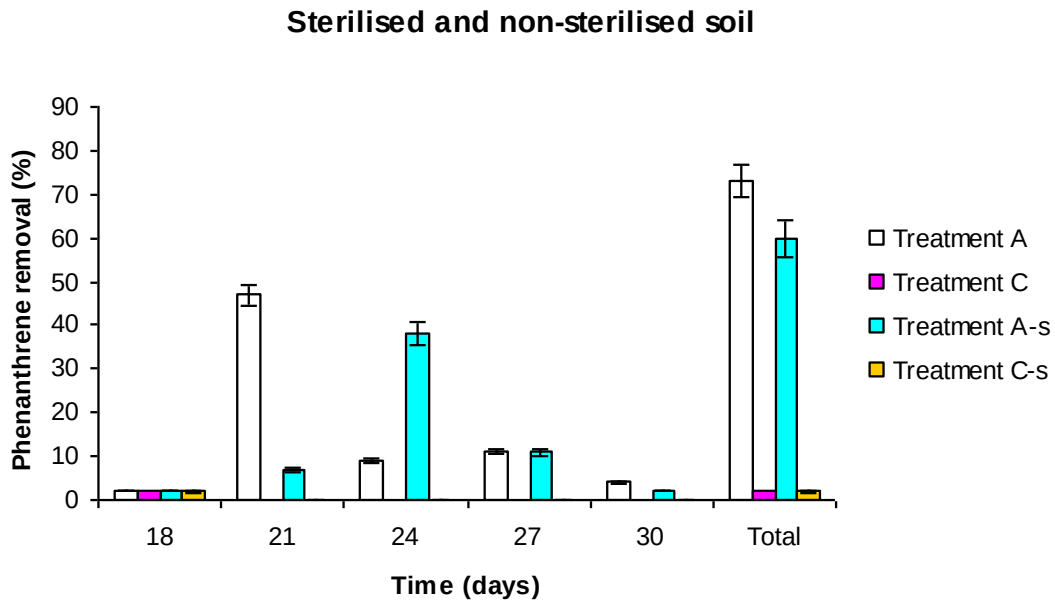



**Figure 5.2** Phenanthrene removal in sterilised (s) and non-sterilised soil as a function of time. Treatment A= Soil + Fungi + Phenanthrene, Treatment C= Soil + Phenanthrene. T= 28°C, Moisture= 44.5%, C:N= 40.

As with the $CO_2$ production, both soil microflora and *P. frequentans* appear to act in a synergistic manner, that is, phenanthrene removal increases in the

presence of both soil microflora and fungi. Such behaviour in the filamentous fungi group (Deuteromycetes), which includes *P. frequentans*, has largely been neglected (Hofrichter *et al.*, 1994), in bioremediation studies. However, antagonism between certain species has been observed using an inoculum of a white rot fungus (Fernandez-Sánchez *et al.*, 2001). In the case of *P. frequentans*, the synergistic behaviour during bioaugmentation should prove useful in improving phenanthrene removal efficiencies (Figure 5.2).

This result agrees with the study of Mahmood and Rao (1993), in which a mixed culture was more successful in degrading PAHs. However, the individual isolates could efficiently remove the more water-soluble PAHs, such as phenanthrene, but not less soluble compounds, like anthracene. Anthracene degradation was dominated by *Agrobacterium* spp., *Pseudomonas* spp., *Aspergillus terreus*, *A. flavus*, and *Penicillium tordum*. *Sclerotium rolfii* and *Trichoderma harzianum* were associated with the degradation of phenanthrene. In this study, higher phenanthrene removal was obtained with the action of both *P. frequentans* inoculum and soil native microflora and agrees with the results of Trzesicka-Mlynarz and Ward (1995), who used a mixed culture to PAHs degradation.

## 5.3 Metal behaviour results

A number of general trends in the behaviour of metals in soils have been found. DGT fluxes, metal concentration measured by DGT and metal concentration in soil solution by filtration all increased after fungal treatment. Treatment A showed the highest values in non-sterilised soil, while in sterilised soil, Treatment B showed the highest values. Sterilised soils have significantly ($p<0.0003$) lower fluxes and concentrations, presumably due to the absence of native soil microflora. The presence of phenanthrene has a significant effect on metal fluxes ($p<0.0003$), metal DGT-concentration ($p<0.0003$) and metal soil solution by filtration ($p<0.0005$). However, comparison of data between metals reveals differences in magnitude. Results will be described for each metal, considering fluxes, DGT-concentration and concentration by filtration. There was a change in pH from 5.7 before treatment to 5.4 after treatment. Although pH is one of the main parameters that impact the metal solubility, in this study this pH change is considered negligible on metal behaviour. At this pH range, a much smaller variation of dissolved metal concentration by natural particles is found (Lead *et al.*, 1999) than the one observed here. In addition, the same change in pH values was found in blanks, which therefore accounts for the pH changes. In fact, all figures presented in this section are the net value obtained after the subtraction of blanks.

### 5.3.1 Metal fluxes measured by DGT

#### 5.3.1.1 Cadmium

The fluxes derived from measurements using DGT are presented in Figure 5.3, showing the effect of fungal activity on Cd behaviour. Prior to fungal addition, Cd fluxes are low (< 0.1 pg $cm^{-2}$ $s^{-1}$) and increase to 0.3-0.4 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in the sterilised soil and by similar, but slightly elevated (0.4-0.9 pg $cm^{-2}$ $s^{-1}$), amounts in non-sterilised soil. The DGT fluxes of Cd in sterilised soil were lower (by 2 fold) from treatments in non-sterilised soil. Note the difference in scales to clarify the trends of sterilised and non-sterilised soils. There was no significant difference ($p<0.005$) between treatments A and B in sterilised soil, but there was a significant difference ($p<0.005$) between treatments A and B in non-sterilised soil.

#### 5.3.1.2 Chromium

Figure 5.4 shows fluxes of Cr in both sterilised and non-sterilised soil. Cr fluxes increased after fungal inoculation, but proportionally to a greater degree compared with Cd. Prior to fungal addition, Cr fluxes are low (< 0.04 pg $cm^{-2}$ $s^{-1}$) and increase to 0.08-0.14 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in sterilised soil and by similar, but slightly elevated (0.3-0.4 pg $cm^{-2}$ $s^{-1}$), amounts in non-sterilised soil. Note the difference in scales. There was no significant difference ($p<0.05$) between treatments A and B in non-sterilised soil, but significant differences ($p<0.005$) in sterilised soil.

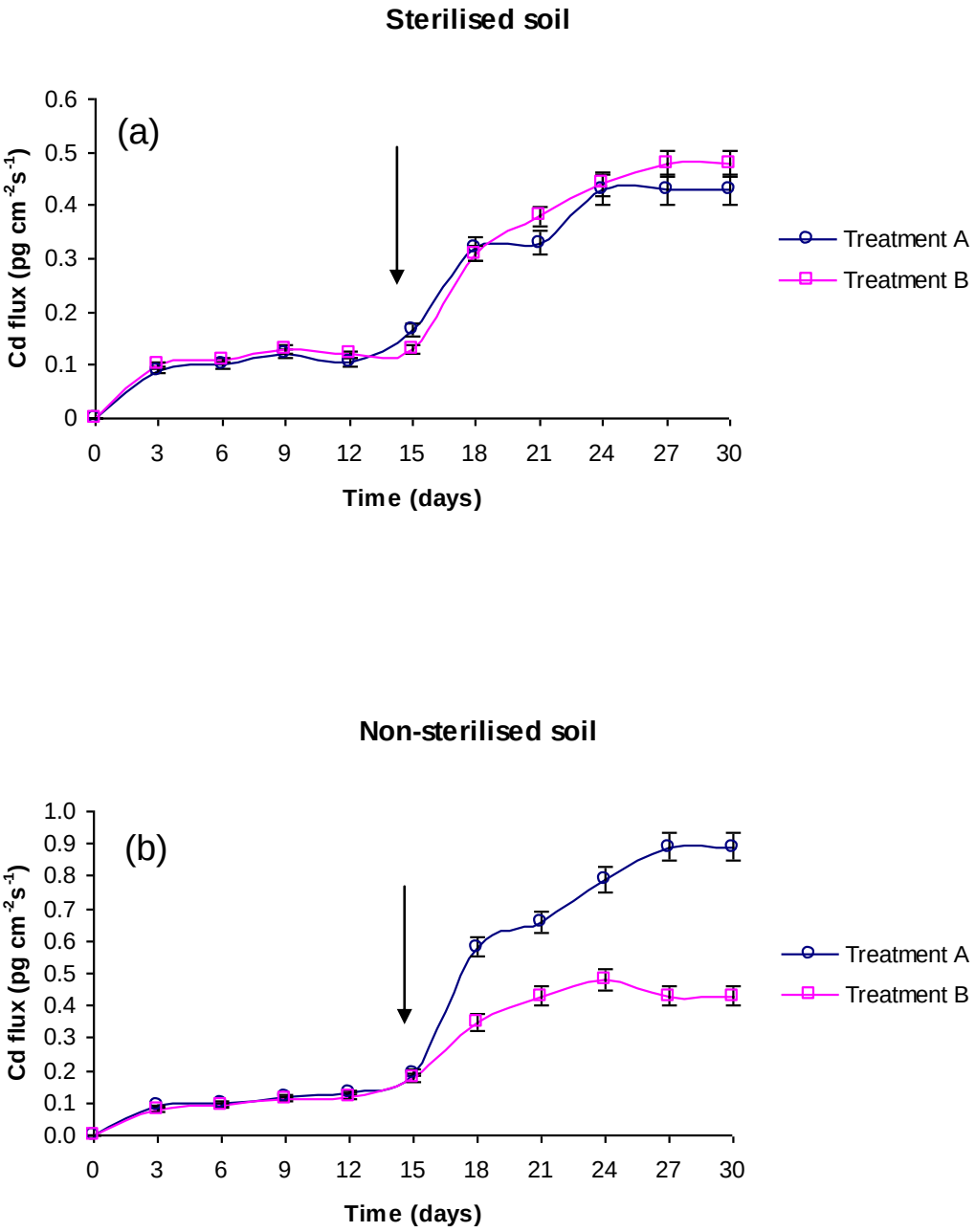



**Figure 5.3** Cadmium fluxes measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

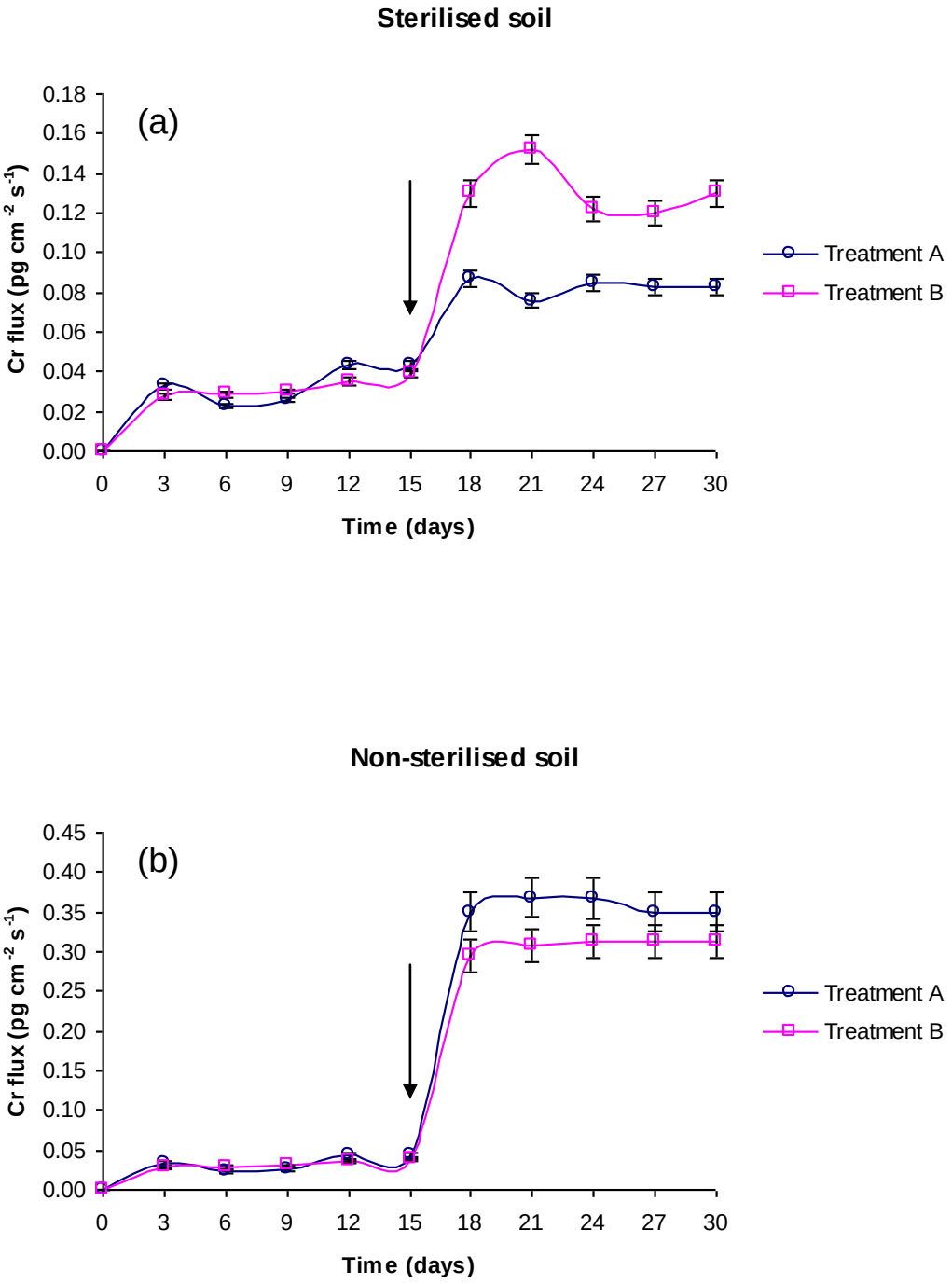



**Figure 5.4** Chromium fluxes measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

#### 5.3.1.3 Copper

DGT fluxes of Cu are shown in Figure 5.5. Metal fluxes significantly ($p < 0.05$) increase after mixing of soil and fungus. Before fungal addition, Cu fluxes are low (< 0.1 pg $cm^{-2}$ $s^{-1}$) and increase to 0.25-0.35 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in the sterilised soil and by similar, but slightly elevated (0.3-0.5 pg $cm^{-2}$ $s^{-1}$), amounts in non-sterilised soil.

Fluxes were just slightly elevated in non-sterilised soil compared with sterilised soil (0.50 pg $cm^{-2}$ $s^{-1}$ and 0.40 pg $cm^{-2}$ $s^{-1}$, respectively) after phenanthrene bioremoval process. Significant differences ($p<0.005$) were found between treatments A and B in both sterilised and non-sterilised soil.

#### 5.3.1.4 Nickel

DGT fluxes of Ni are shown in Figure 5.6. Metal fluxes significantly ($p < 0.03$) increase after mixing of soil and fungus. Before fungal inoculation, Ni fluxes are low (< 0.05 pg $cm^{-2}$ $s^{-1}$) and increase to 0.25-0.35 pg $cm^{-2}$ $s^{-1}$ in the sterilised soil and by similar, but highly elevated (1.3 pg $cm^{-2}$ $s^{-1}$), amounts in non-sterilised soil. Significant differences ($p<0.003$) were found between Treatments A and B in both sterilised and non-sterilised soil. Maximal fluxes of Ni of 0.35 pg $cm^{-2}$ $s^{-1}$ in sterilised soils, compared with 1.4 pg $cm^{-2}$ $s^{-1}$ in non-sterilised soil were observed.

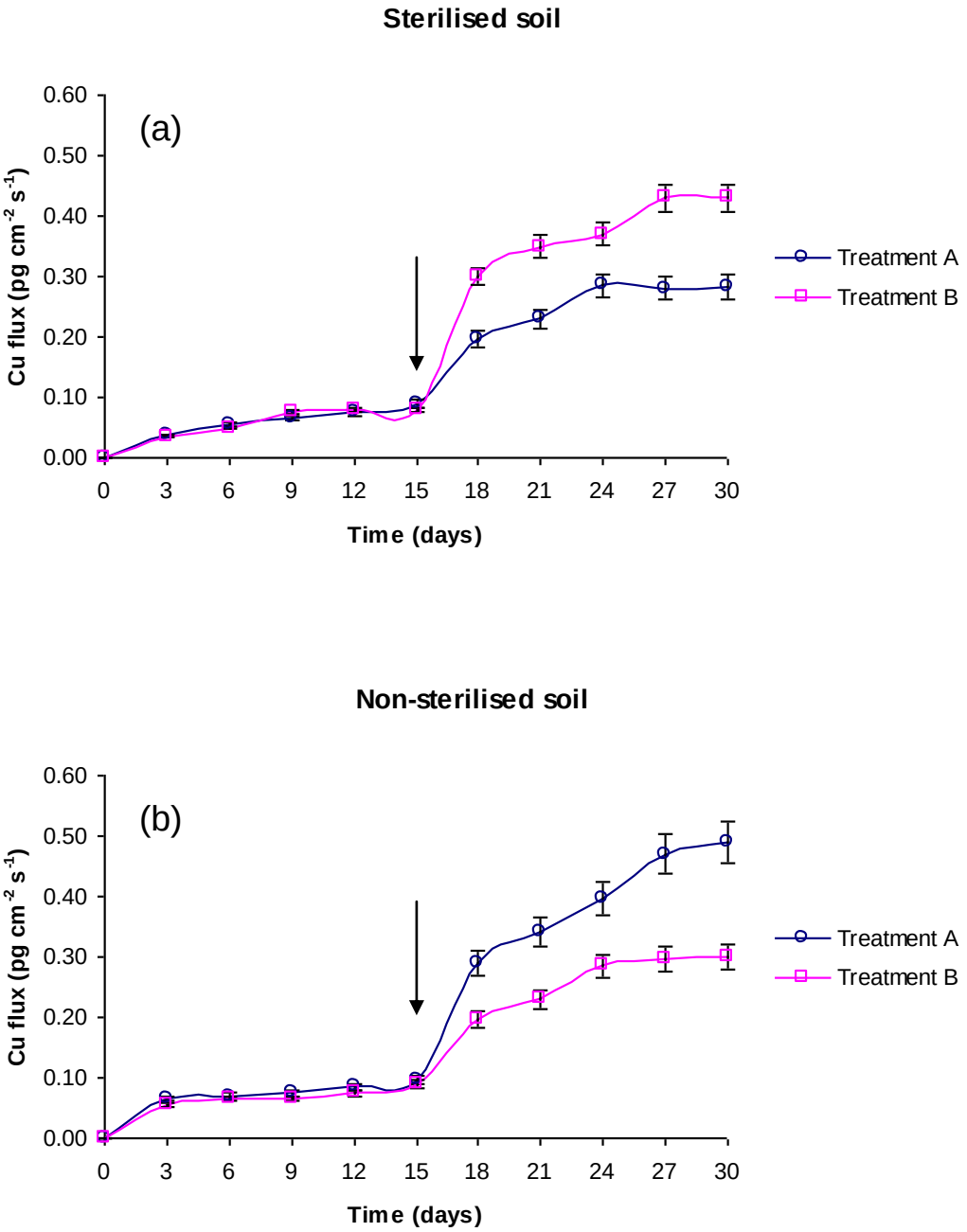



**Figure 5.5** Copper fluxes measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

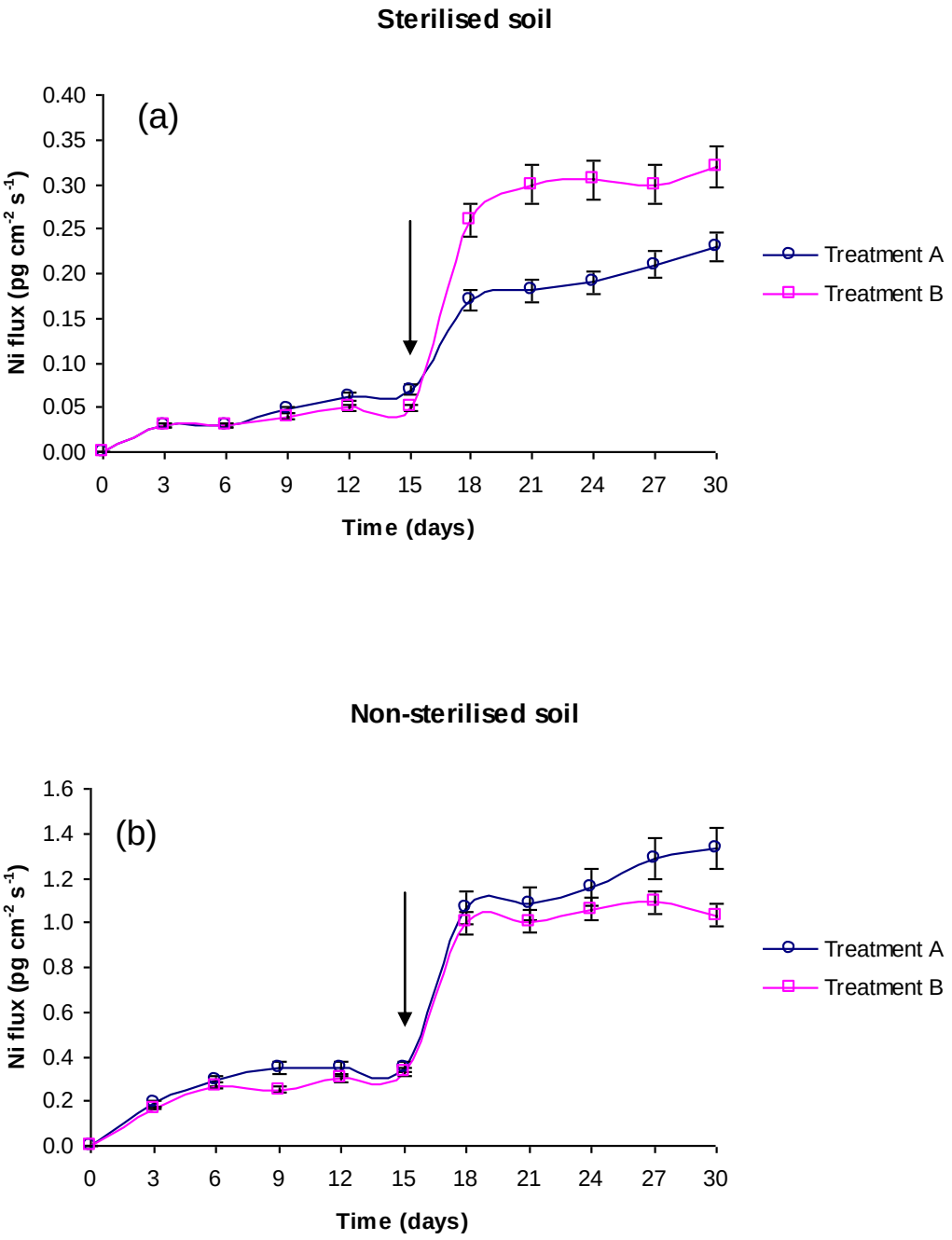



**Figure 5.6** Nickel fluxes measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

#### 5.3.1.5 Lead

The Pb fluxes measurements using DGT are presented in Figure 5.7, showing the effect of fungal activity on Pb behaviour. Prior to fungal addition, Pb fluxes are low (< 0.1 pg $cm^{-2}$ $s^{-1}$) and slightly increase to 0.15-0.3 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in the sterilised soil (Figure 5.7a) and by similar, but higher (0.45 pg $cm^{-2}$ $s^{-1}$) in non-sterilised soil (Figure 5.7b). Significant differences ($p<0.003$) were found between treatments A and B in both sterilised and non-sterilised soil.

### 5.3.2 Metal concentration measured by DGT

#### 5.3.2.1 Cadmium

Cadmium concentrations (in pore waters) measured by DGT are shown in Figure 5.8. DGT-Cd concentrations were 1.5 μg $l^{-1}$ and 4 μg $l^{-1}$ in sterilised and non-sterilised soil respectively, prior to fungal addition. This increased by 3-4 fold to in sterilised (Figure 5.8a) and by 5-8 fold to in non-sterilised soil after addition of fungi (Figure 5.8b), in line with DGT fluxes as expected. Significant differences were found ($p<0.005$) between treatments A and B in both sterilised and non-sterilised soil.

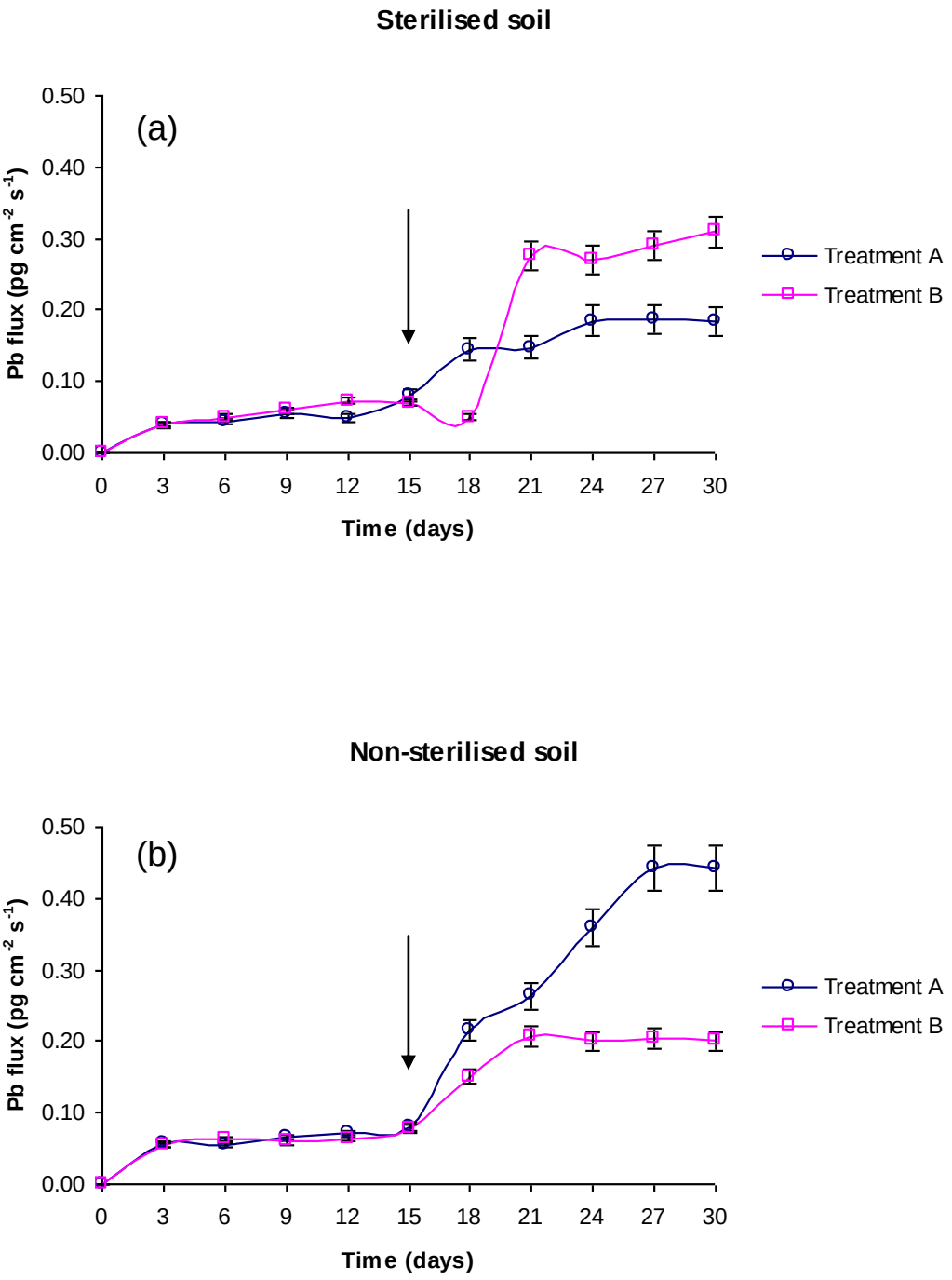



**Figure 5.7** Lead fluxes measured by DGT. (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

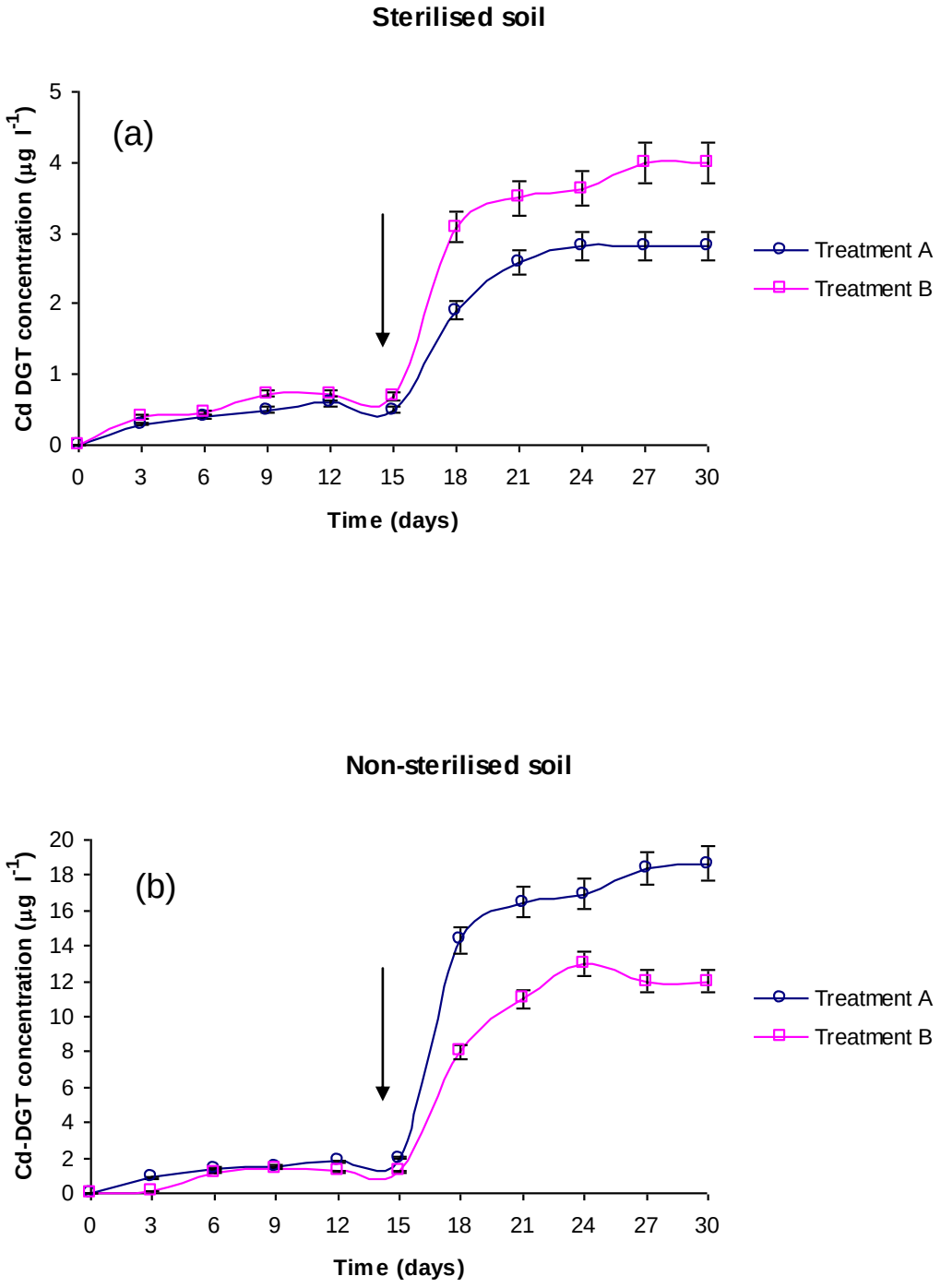



**Figure 5.8** Cadmium concentration measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

### 5.3.2.2 Chromium

Metal concentration of Cr in pore waters (Figure 5.9) significantly ($p < 0.05$) increase after adding soil with or without microflora and *P. frequentans* inoculum. Prior to fungal addition, the concentration of Cr is <0.2 $\mu g\ l^{-1}$ in both type of soils and increase to 0.5 $\mu g\ l^{-1}$ in sterilised soil and to 10 $\mu g\ l^{-1}$in non-sterilised soil after addition of fungus. This represents an increase of 50 fold in Cr fluxes. The highest rate of increase in DGT concentration between sterilised and non-sterilised soil was found in Cr. In sterilised soil, treatment B was higher by 1 $\mu g\ l^{-1}$ than treatment A. However, in non-sterilised soil, concentration increased by 4 fold in Treatment A.

### 5.3.2.3 Copper

The concentration of Cu measured by DGT (Figure 5.10) increased after fungal treatment. Before fungal addition, Cu concentrations are low (<1 $\mu g\ l^{-1}$) and increase to 4-6 $\mu g\ l^{-1}$ after addition of fungus in the sterilised soil (Figure 5.10a) and by similar, but slightly elevated (5-7 $\mu g\ l^{-1}$), amounts in non-sterilised soil (Figure 5.10b). There was a statistically significant difference ($p<0.05$) in Cu DGT concentration before and after fungal treatment. Significant differences ($p<0.003$) were found between treatments A and B in both sterilised and non-sterilised soil.

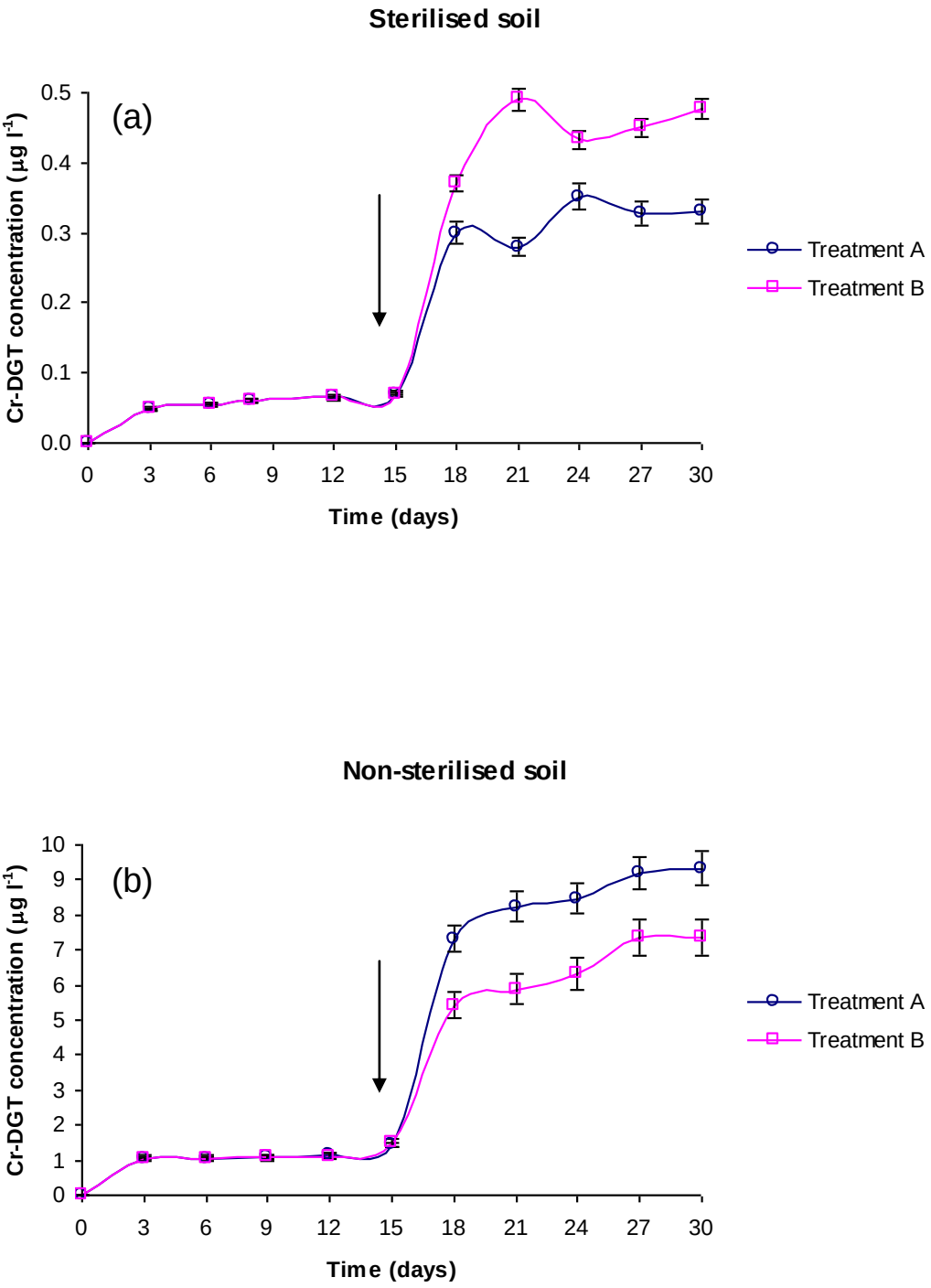



**Figure 5.9** Chromium concentration measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

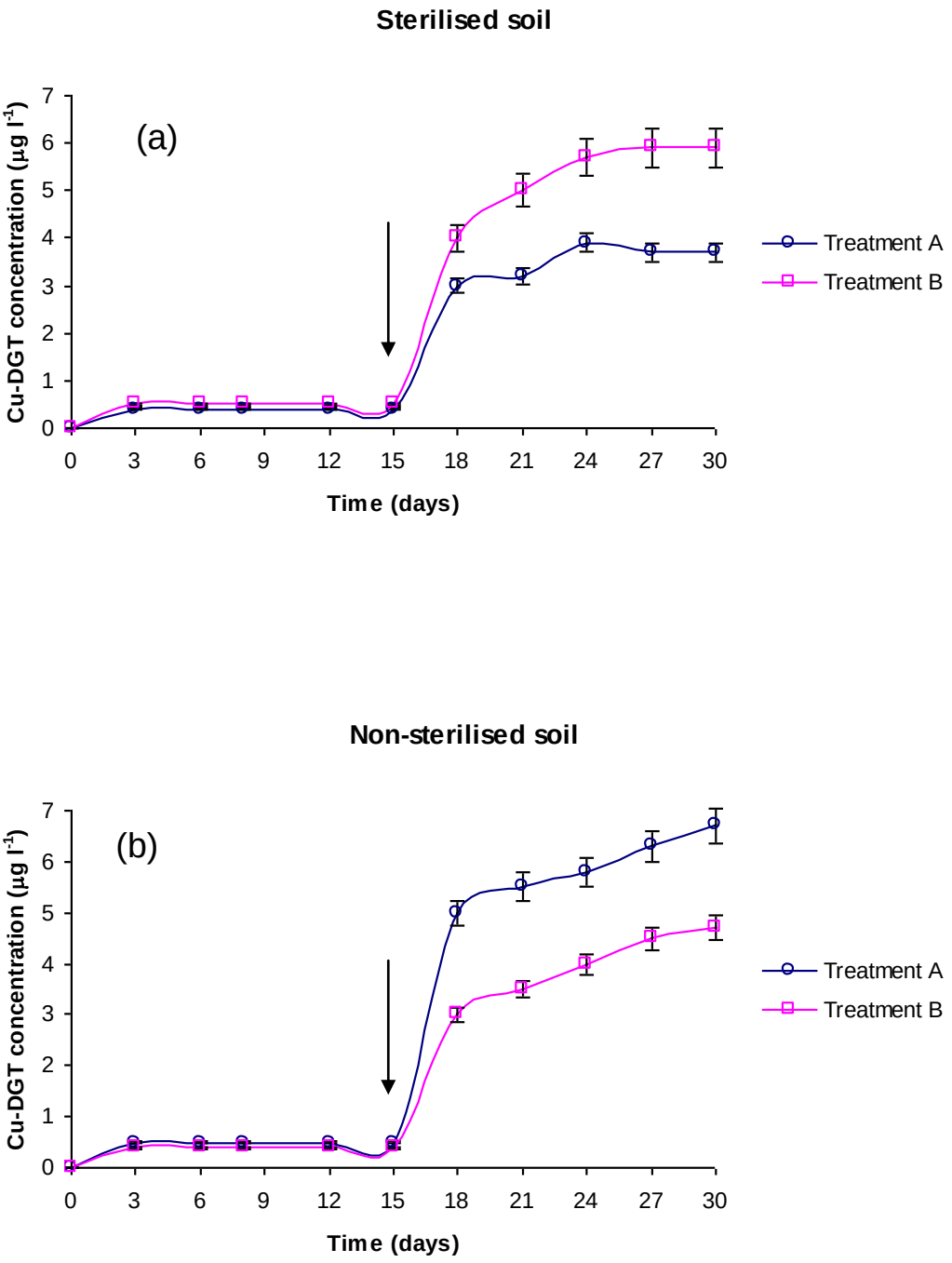



**Figure 5.10** Copper concentration measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 5.3.2.4 Nickel

The concentration of Ni in soil solution increased after fungal treatment (Figure 5.11). There was a statistically significant difference ($p<0.03$) in Ni concentration before and after fungal treatment (especially in Treatment A). Both type of treatment ($p<0.021$) and time of treatment ($p<0.001$) were found to significantly affect the metal in pore waters. Treatment A was significantly different from Treatment B in sterilised ($p<0.022$) and non-sterilised ($p<0.05$) soil.

### 5.3.2.5 Lead

Lead concentrations in pore waters are 0.2 $\mu g\ l^{-1}$ in both sterilised and non-sterilised soil, before fungal addition. These increased by 5 fold in sterilised soil and by 9 fold in non-sterilised soil after addition of fungi (Figure 5.12).

Significant differences ($p<0.003$) were found between treatments A and B in both sterilised and non-sterilised soil. The highest Pb concentrations measured by DGT in sterilised soil was on Treatment B (without phenanthrene), and in non-sterilised soil, in Treatment A (with phenanthrene).

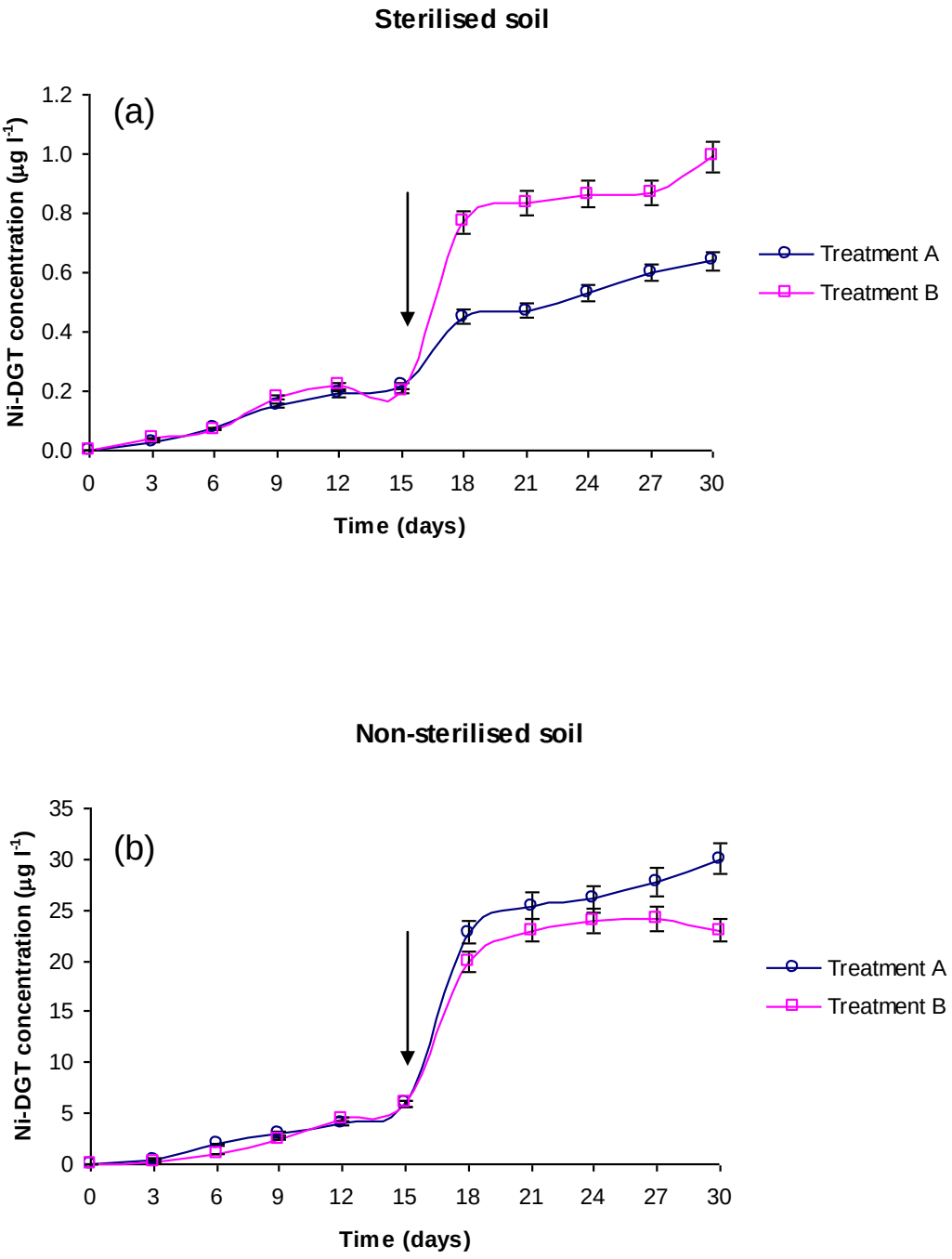



**Figure 5.11** Nickel concentration measured by DGT: a Sterilised soil; b Non-sterilised soil. S=soil, P= phenanthrene, F= fungi T= 28°C, Moisture= 44%, C:N= 40. Arrow indicates when soil was added. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

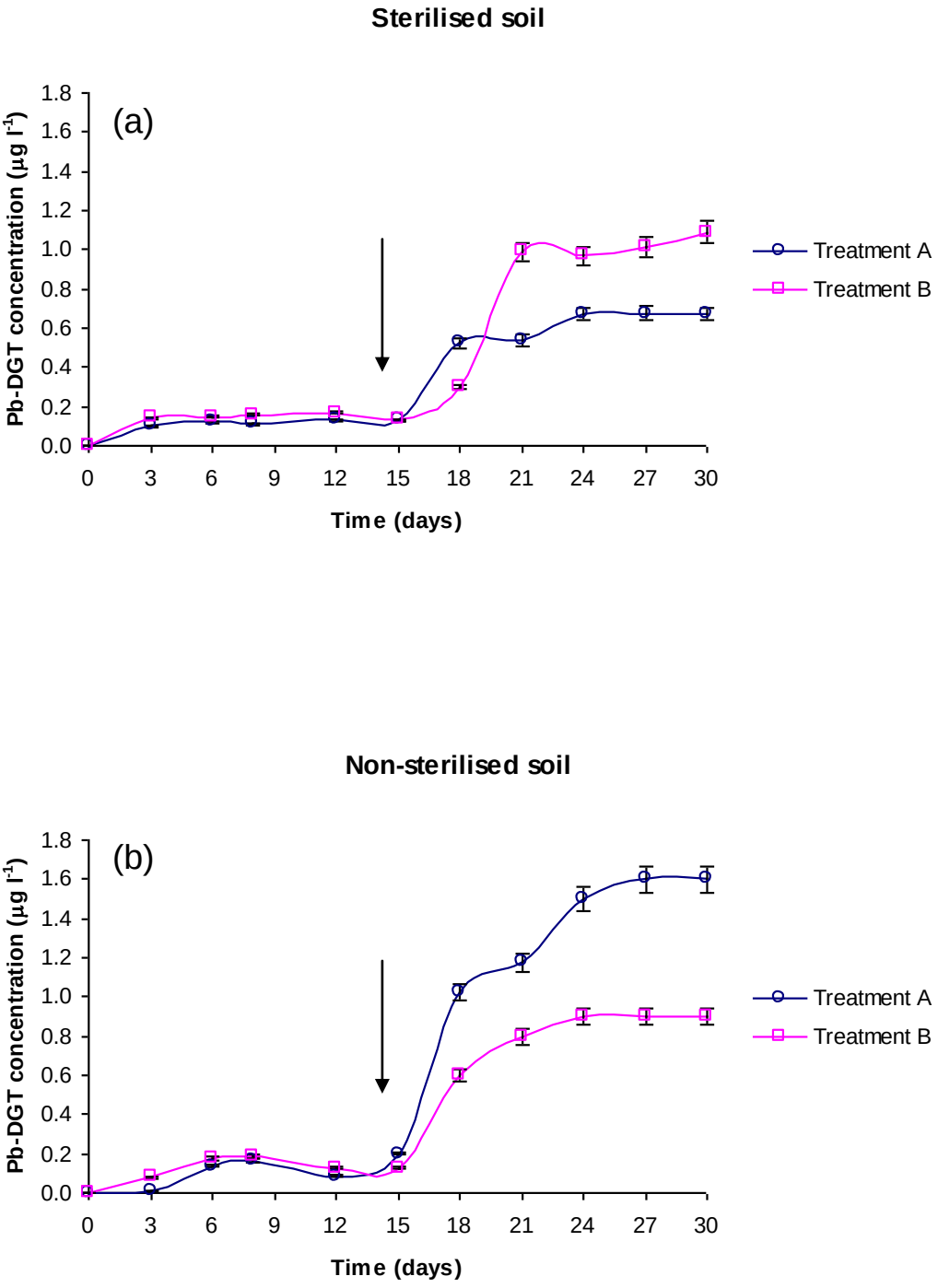



**Figure 5.12** Lead concentration measured by DGT: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 5.3.3 Metal concentration in soil solution by filtration

#### 5.3.3.1 Cadmium

Figure 5.13 shows the soil solution concentration (defined as Cd filtrate through a 0.45 μm membrane). Unlike DGT devices, which show a continuous increase in flux and concentration as a function of time, the solution concentration from filtration shows a clear bimodal distribution. Peaks in concentration occur at 18 and 24 days in all cases (except in sterilised soil, Treatment A, with a peak at day 21). The two peaks were 18 and 15 $\mu g\ l^{-1}$ for sterilised soil and 50 and 40 $\mu g\ l^{-1}$ for non-sterilised soil.

Significant ($p<0.005$) differences were found between Treatments A and B. Treatment B showed the highest Cd concentrations in soil solution in sterilised soil, while Treatment A showed the highest values in non-sterilised soil.

#### 5.3.3.2 Chromium

Two peaks were found at day 18 and 24 days in all cases (Figure 5.14) as found with cadmium. In sterilised soil, the highest peaks (Treatment B) were found at 3.5 and 3 $\mu g\ l^{-1}$, respectively. In non-sterilised soil, the highest peaks were found at 25 and 23 μg l-1, respectively. A significant ($p<0.005$) difference was found between Treatment A and B in sterilised soil, and a

significant ($p<0.05$) difference was found between Treatments A and B in non-sterilised soil.

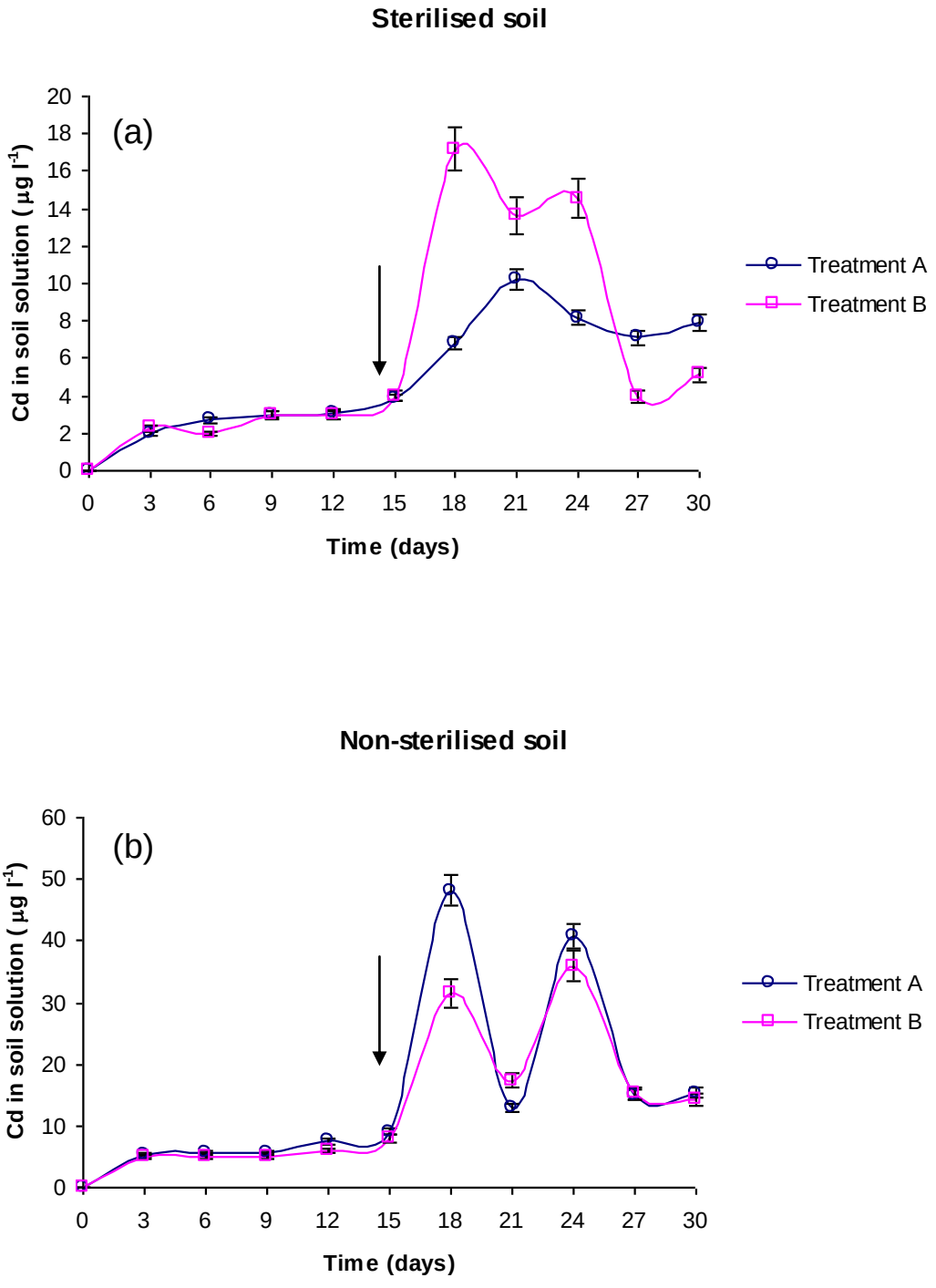



**Figure 5.13** Cadmium in soil solution measured by filtration: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

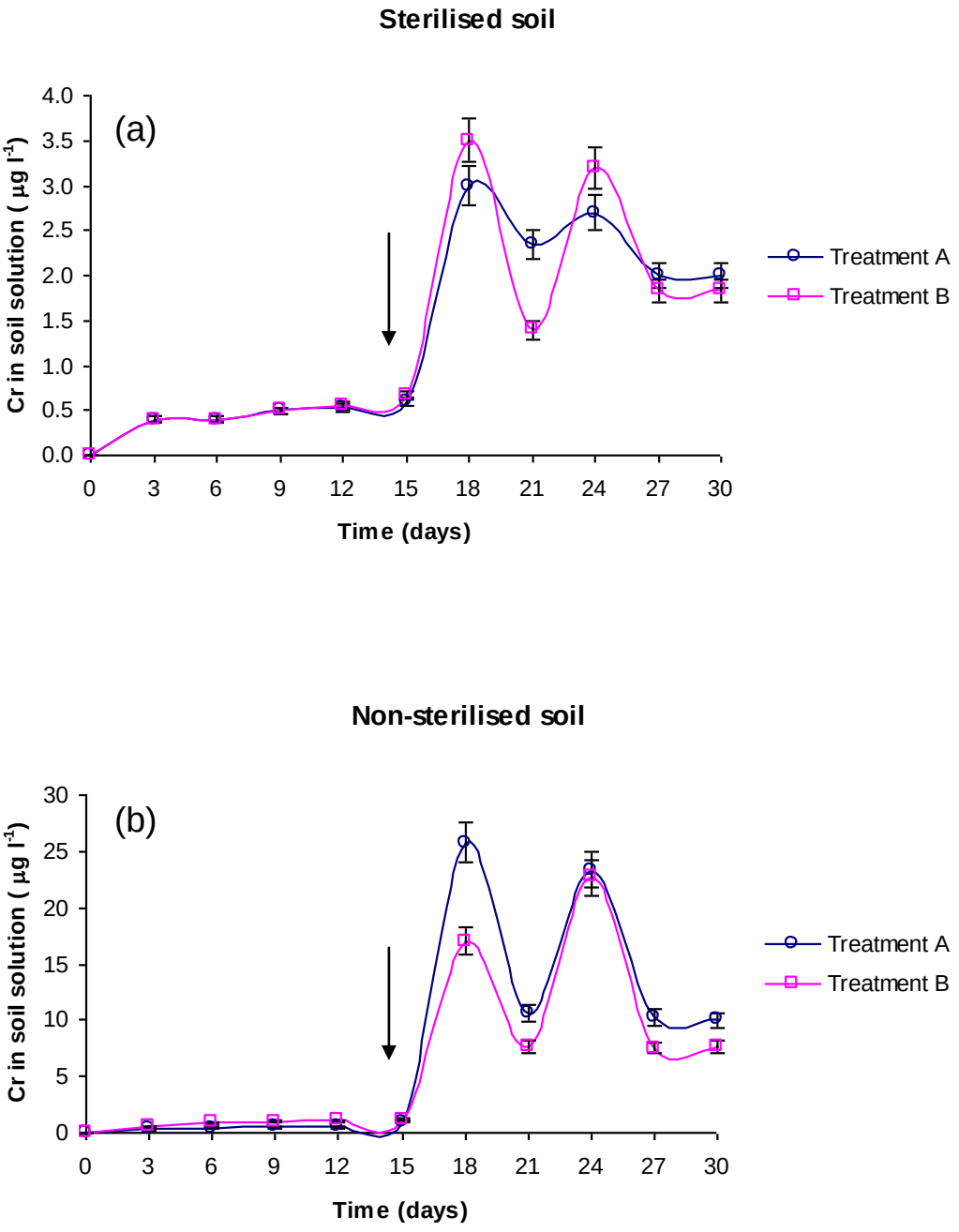



**Figure 5.14** Chromium in soil solution measured by filtration: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

#### 5.3.3.3 Copper

The Cu concentrations in soil solution are shown in Figure 5.15. Prior to fungal inoculation, concentrations were around 2 $\mu g\ l^{-1}$, and increased by 4 fold after fungal inoculation in non-sterilised soil (8 $\mu g\ l^{-1}$) and by 4-5 fold (8-10 $\mu g\ l^{-1}$) in sterilised soil. Unlike Cd concentration (which shows a clear bimodal distribution), Cu shows a continuous increase in soil solution as a function of time, finding the highest concentration at the end of the experiment (day 30). Treatments A and B were significantly ($p<0.005$) different in both sterilised and non-sterilised soil.

#### 5.3.3.4 Nickel

Figure 5.16 shows the concentration of Ni in the soil solution. Like DGT devices, Cu and Ni show a continuous increase in soil solution as a function of time, showing the same trend as in DGT fluxes. Metal in soil significantly ($p <0.03$) increases after mixing of soil with or without microflora and *P. frequentans* inoculum. Before fungal addition, the concentration of Ni in soil solution was <4 $\mu g\ l^{-1}$ in sterilised soil and increase to 10-18 in sterilised soil and to 120-140 $\mu g\ l^{-1}$ in non-sterilised soil after addition of fungus. Treatments A and B are significantly ($p<0.003$) different in both sterilised and non-sterilised soil.

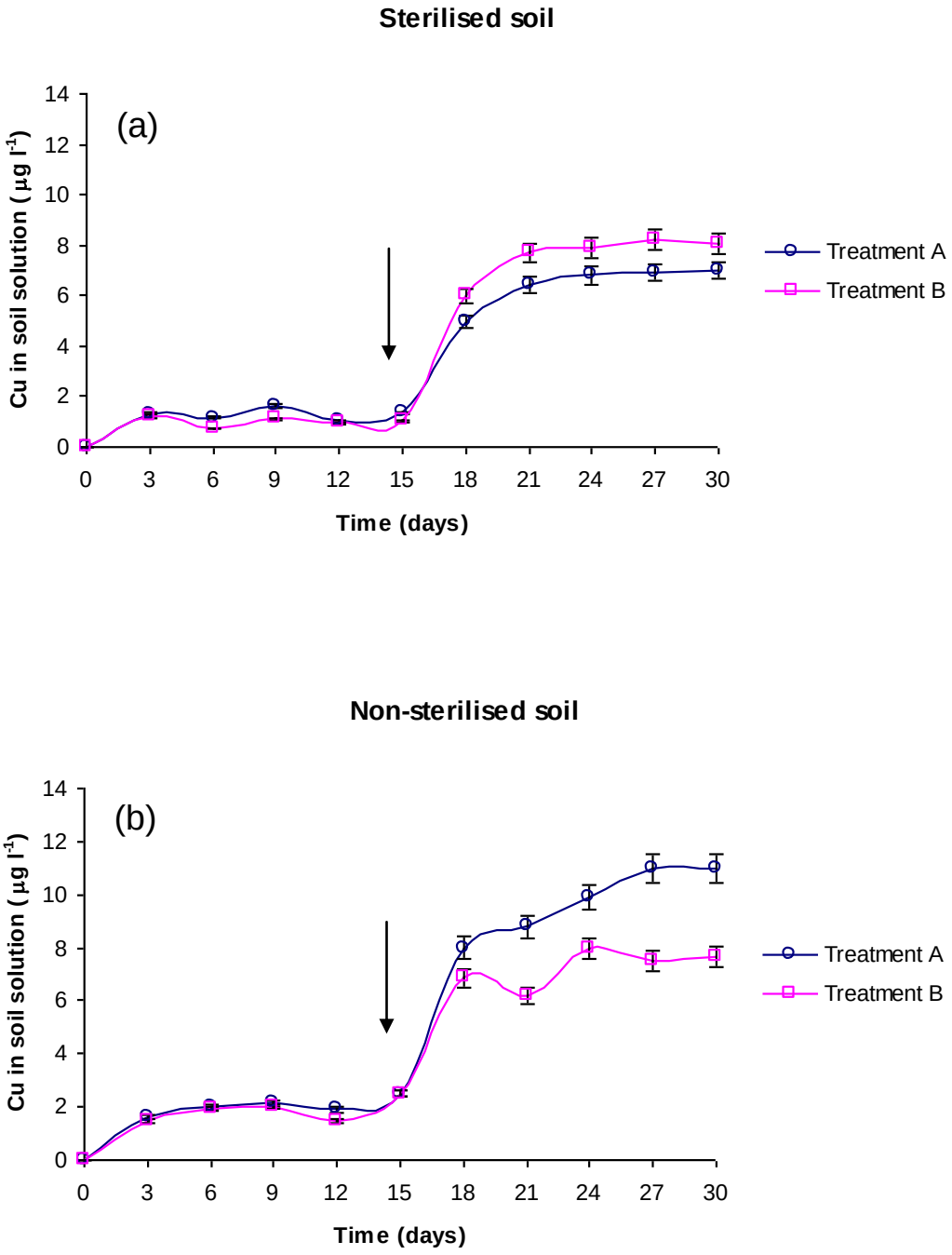



**Figure 5.15** Copper in soil solution measured by filtration: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

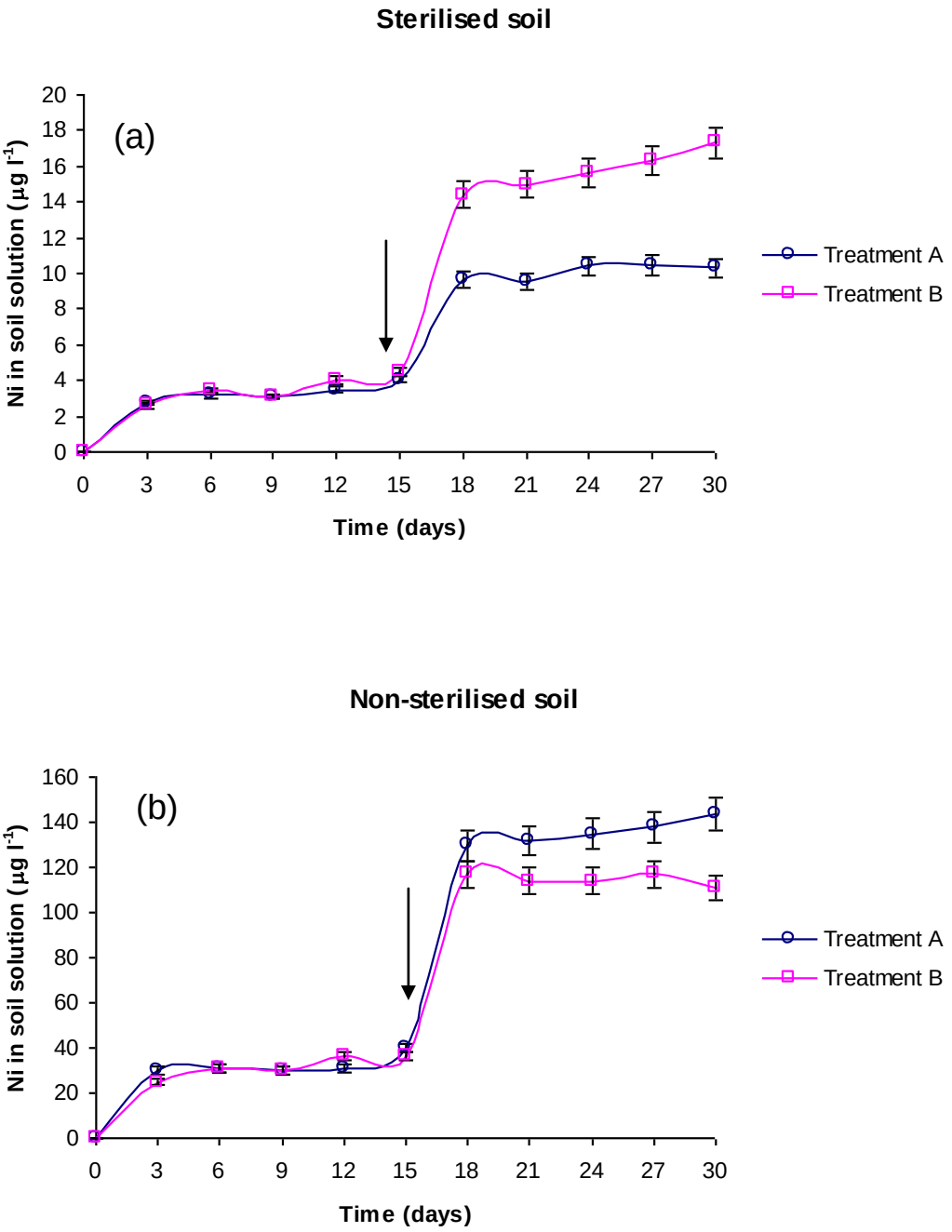



**Figure 5.16** Nickel in soil solution measured by filtration: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together. Two different scales are presented to clarify the trends of sterilised and non-sterilised soils.

### 5.3.3.5 Lead

Figure 5.17 shows the Pb soil solution concentration by filtration. Unlike the other metals studied, lead shows a decrease in soil solution at the end of the experiment. Unlike DGT devices, which show a continuous increase in flux and concentration as a function of time, the solution concentration by filtration shows an increase just after fungal inoculation (from day 15 to 21), but after day 21, a continuous decrease in concentration was found (Figure 5.17a).

Two different behaviours in a non-sterilised soil (Figure 5.17b) were found. Without phenanthrene, a bimodal distribution was found from day 18 to 24 (3 $\mu g\ l^{-1}$). In the presence of phenanthrene (Treatment A), a unimodal peak at day 21 (5 $\mu g\ l^{-1}$), which decreases similarity to the sterilised soil.

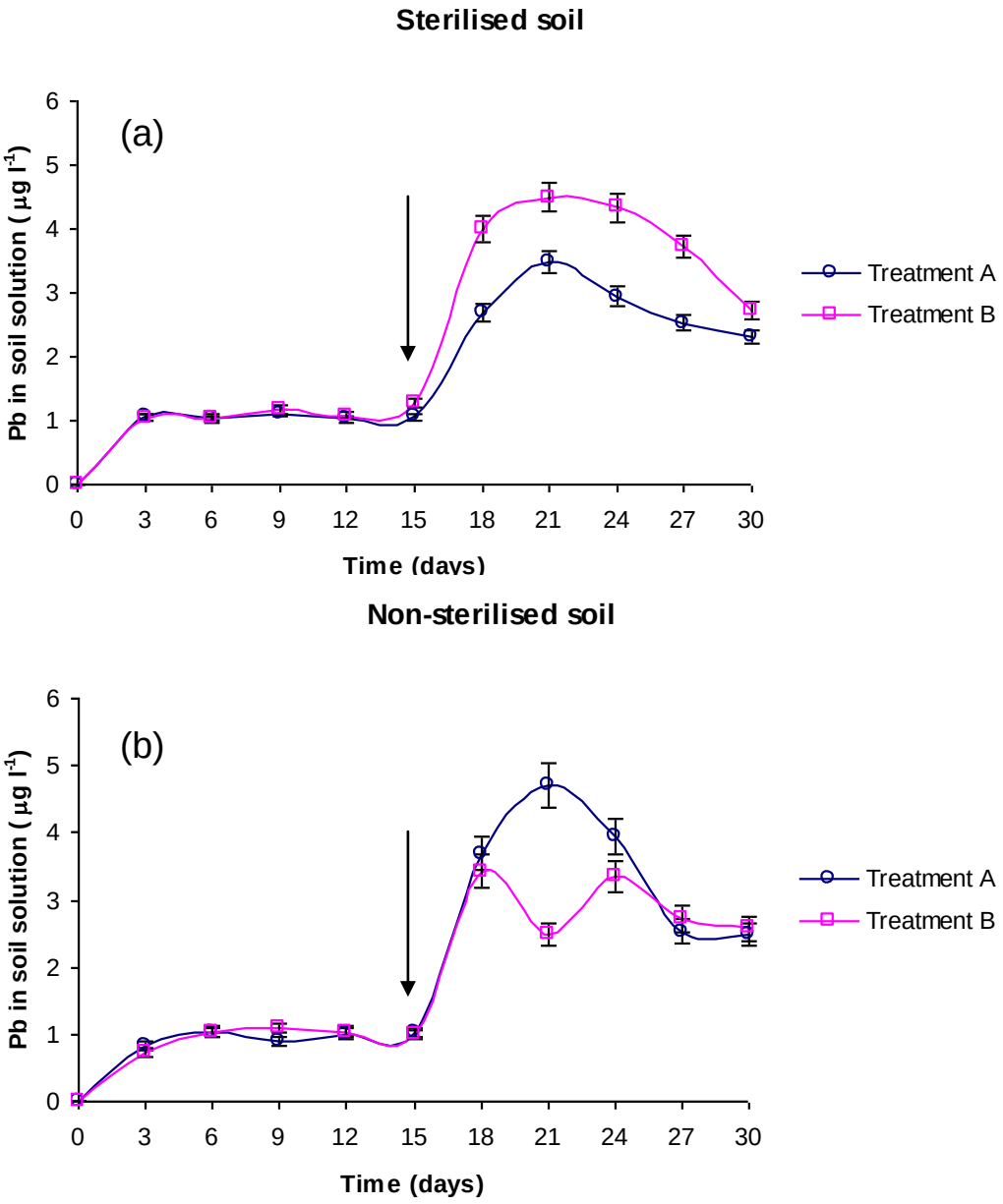



**Figure 5.17** Lead in soil solution measured by filtration: (a) Sterilised soil; (b) Non-sterilised soil. Treatment A= Soil + Fungi + Phenanthrene, Treatment B= Soil + Fungi. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

## 5.4 Metal behaviour- discussion

### 5.4.1 Differences between techniques

DGT theory has been discussed in detail in Chapter 2 (section 2.4.3.4). DGT provides an accurate measure of fluxes from the solid phase to solution in the presence of the perturbation caused by DGT. DGT removes labile metal in soil solution causing metal depletion. The solid-solution equilibrium adjusts and the solution is resupplied from the solid phase. It is this process (resupply from solid phase), which is measured over the period of deployment.

DGT concentrations are therefore not absolute measures of solution metal concentration. As resupply may be insufficient, DGT concentrations are not absolute. Cd and Zn have shown good resupply, while Cu and others metals have shown that resupply from solid phase to solution is poor (Harper *et al.*, 1998). However, the trends in DGT concentrations are comparable within this study, even though it cannot be assumed the metal concentration to be accurate. In addition, DGT concentrations can be interpreted as a speciation measurement, based on diffusive mobility, size and chemical lability of the metal-ligand complex (Lead *et al.*, 1997; van Leeuwen, 1999; Davison *et al.*, 2000a). Although the speciation measurement made is not exactly known, it does measure the most potentially bioavailable fraction, *e.g.* most mobile and labile metal species.

Although the experiments are all of a relatively short timespan (30 days), the period of time was fixed according to the extent of microbial activity. In addition, an indication of DGT saturation was inferred towards the end of the experiment. This inference was made due to the DGT fluxes becoming more constant over time. This may also have been due to the reduction in heterothrophic activity. In order to avoid DGT saturation and to study metal behaviour before $CO_2$ production (and therefore heterothrophic activity) levelled off, metal behaviour was studied for 30 days.

#### 5.4.1.1 DGT-bioavailability

The bioavailability and mobility of potentially toxic trace metals largely determine the environmental impact of metal in contaminated soils, rather than total metal concentration. Measurement of labile metal species in soil solutions has been suggested as a more sensible and sensitive approach for evaluating metal bioavailability than chemical extraction based procedures (McGrath *et al.*, 1999). However, such measurements do not provide any estimate of those labile components associated with the solid phase that, in response to local removal by the plant, may contribute to the local solution pool and consequently plant uptake (Davison *et al.*, 2000b).

In saturated soils, measured DGT fluxes are proportional to the initial solute concentrations in the soil solution, in agreement with theoretical predictions (Zhang *et al.*, 1998). However, it was not possible to resolve whether DGT fluxes or solute concentrations mimic plant uptake more effectively. A test

with DGT and metal uptake was performed to determine which of these measurements was a better surrogate for plant uptake (Davison *et al.*, 2000b). It was found that metal uptake does not depend simply of free ion activities in soil solution, but rather is governed by diffusional transport of labile solution species augmented by the solid phase. Since, in practice, mechanisms other than resupply due to local depletion may prevail, further more comprehensive studies are required. If membrane transport is sufficiently slow, local concentrations will remain intact and plant uptake can be expected to be related to the concentration of particular solutes species (the free ion model). In some cases, convective transport to the root may dominate or the effects of root microenvironment or exudates may be important in active uptake. Moreover, as roots grow, their surface area continually increased and they contact fresh soil. None of these effects can be expected be accurately mimicked by DGT. DGT can only be expected to be a good surrogate in some circumstances metal uptake by plants. Further work is required to establish the range of conditions over which DGT measurements can serve as a good surrogate for plant uptake. However, DGT may be a useful tool for assessing the hazards posed by metals in soils (Davison *et al.*, 2000b), and will be useful for this reason in this study.

#### 5.4.1.2 Filtration

Filtration has been discussed in Chapter 2 (section 2.4.3.2.1). Problems of filtration, such as unsatisfactory size resolution and only small quantities can be collected (Lead *et al.*, 1997) were described. Despite these problems, or

because they are known and be potentially accounted for, filtration is a useful tool for gaining more information on soil solution behaviour. Filtration gives an indication of the solution phase behaviour of metals and performs a different speciation measurement than DGT. Assuming the filtration works ideally, all material <0.45 μm will be measured in the solution phase, *e.g.* metals measured will be bound to material <0.45 μm in size. However, DGT only measures the most diffusive and most labile species. In consequence, we would expect DGT concentration to be lower than filtration concentration, which agrees with the results found by Odzak *et al.* (2002). In addition, as indicated in section 5.4.1, DGT concentration may be low because of the limited resupply from the solid phase (Harper *et al.*, 1998).

In this study, Cd concentrations by DGT were comparable to concentrations measured by filtration. However, Cd is generally weak binder (Lead *et al.*, 1999) and Cd is well re-supplied by the solid phase, while Ni is partial resupply from solid phase (Zhang *et al.*, 1995). Cu concentrations measured by DGT were slightly lower than Cu measured by filtration. Cr, Ni and Pb concentrations were much larger in soil solution measured by filtration than by DGT. This is relevant to metal bioavailability since biological responses of organisms often have been shown to be related to the concentration of labile metal species in solution (Hudson and Morel, 1990; Morel and Hering, 1993; Hooda *et al.*, 1999). The two techniques perform different measurements. Combination of the methods allows the consideration of a number of aspects of metal behaviour relevant to biological uptake. DGT provides a good way of measuring concentrations of labile species over time, while soil solution by

filtration is able to measure metals in material <0.45 μm at the time of sampling.

### 5.4.2 Impact of bioremediation on metal mobility and potential biouptake

Biological activity is usually low in soils contaminated with PAHs and heavy metals. In these situations, bioaugmentation (the supplementation of microorganisms) and biostimulation (the stimulation of microbial degradation by the addition of nutrients) are ideal methods to apply (Riha *et al.*, 1993). In addition, it is well known that bioremediation of organic pollutants has advantages over other techniques in terms of cost and because the soil as a living systems suitable for plant growth is not destroyed (Riser-Roberts, 1998). Furthermore, the contamination is not transferred elsewhere, as with land filling (Wilson and Jones, 1993). However, if after other technical and economic feasibility studies, bioremediation by bioaugmentation and biostimulation is the best option to apply, this study indicates that subsequent metal behaviour would need to be considered as a substantial extra risk.

It is clear from data in section 5.3 that all measures of potential metal availability increased after phenanthrene bioremoval. These results highlight the impact of bioremediation of organic contaminants on trace metal behaviour. Although a number of metals have nutrient properties, especially Cu, they are all potentially toxic. The bioremediation process potentially therefore makes these toxic metals more available to plants. In addition, it is

likely that more metal will be incorporated into the human food chain, if crops grown on bioremediated soil are used for human/animal consumption.

Cd is one of the most ecotoxic metals that exhibit highly adverse effects on soil biological activity, plant metabolism, and the health of humans and the animal kingdom (Kabata-Pendias, 2001). Increased lability of Cd due to bioremediation process is therefore highly undesirable.

The increase in Cr flux/concentration is likely to be because of the mobile Cr (VI) ion as soils were under aerobic conditions. Readily soluble Cr (VI) in soils is toxic to plants and animals (Kabata-Pendias and Pendias, 1999) and is much more toxic than Cr (III).

Cu occurs in enzymes having vital functions in plant metabolism. Moreover, Cu is involved in some mechanisms of disease resistance. This resistance of plants to fungal diseases is likely to be related to an adequate Cu supply (Kabata-Pendias, 2001). Prediction of the Cu content of soil that results in toxic effects on plants is extremely complex (McGrath, 1998). The increase supply of Cu may have beneficial results in some cases. However, Cu is unlikely to be limiting in soil requiring bioremediation. Therefore, even here Cu mobilization is likely to be at best neutral in its consequences. At high levels Cu is also toxic and therefore bioremediation may also be concern when applied to Cu.

Ni inhibits soil nitrification, carbon mineralisation, and the activities of acid and alkaline phosphatase and arylsulphatase (JRB Associates, Inc., 1984).

Orientation of ligands at the cell surface must be important, since only surface-active substances, could compete effectively for Ni binding (Galun *et al.*, 1983). Ni is very toxic to microfungi of soils and its increase in the growth medium (up to 1 mg $L^{-1}$) decreases ciliate, such as Protozoa and *Copoda* sp. (Pankhurst *et al.*, 1998). Therefore, bioremediation increase the risks of Ni toxicity.

Inorganic lead is toxic to a broad range of microorganisms, including cyanobacteria, fungi and protozoa (Roane and Kellogg, 1996). Lead and its compounds also affect microbial activities in soil, including inhibition of nitrogen mineralisation, stimulation of nitrification and the synthesis of soil enzymes. Species diversity is lower in Pb-contaminated soils (Pattee and Pain, 2003). The mobilisation of Pb by bioremediation could therefore have severe consequences for soil fertility and potentially plant uptake.

#### 5.4.2.1 Metal biouptake

Since metal fluxes and concentration of metal labile fractions increased, and this fraction is potentially bioavailable, metal uptake potentially could increase. This has been discussed, from a quantitative point of view, through the free-ion activity model (see Chapter 2, section 2.4.2.1). In has been showed that total concentration of a metal is not a good predictor of its 'bioavailability', *i.e.* the metal's speciation will greatly affects its availability to organisms (Tessier *et al.*, 1994), and metal biouptake is likely to be based only on labile and mobile metal species (van Leeuwen, 1999).

It has been shown in this study that bioremediation increase fluxes and concentrations of labile metal species. Bioavailability is therefore likely to be enhanced, and this is discussed in the next chapter.

#### 5.4.2.3 Possible mechanism

Although it is not possible to decide which mechanisms are taking place during the bioremediation process, it has been demonstrated that bioremediation increased the mobility and lability of metals.

Filtration results for Cd, Cr and Pb indicate peaks and troughs in metal concentration, suggesting that the microbial breakdown of solid soil brings metal into solution. The metal concentration peaks and then falls as soil solution matter is used as an available carbon and energy source until depleted. Then microorganisms further break down the solid phase. However, trend similar to the DGT trends were observed in Cu and Ni. These possibly relate to differences in biological uptake or subsequent re-sorption on solid phases, which alter metal solubilisation. Cu in soils exhibits a great ability to chemically interact with soil mineral and organic components (Kabata-Pendias, 2001) because Cu binds more strongly than Cd (Lead *et al.*, 1999). However, Ni is highly toxic to microfungi in soils (Pankhurst *et al.*, 1998), therefore the fraction potentially bioavailable Ni increases as a function of time, since the chance for Ni uptake by *P. frequentans* and soil microflora is reduced, due to its toxicity. However, further work is necessary to fully establish the mechanisms by which metal is moved from the solid to solution phase.

### 5.4.3 Differences between sterilised and non-sterilised soil, in presence or absence of phenanthrene

Although all data show essentially the same general trends, comparison of data between sterilised and non-sterilised soils and soils where phenanthrene has and has not been added, reveals differences in magnitude of metal flux and concentration.

#### 5.4.3.1 Sterilised soil in presence and absence of phenanthrene

Sterilised soil in the presence of phenanthrene shows lower DGT fluxes and metal concentrations in soil solution, compared with sterilised soil in the absence of phenanthrene. This result suggests that metal mobility is higher when *P. frequentans* is taking glucose from Wunder media as carbon and energy source than when *P. frequentans* is breaking phenanthrene down. Possibly, phenanthrene is toxic for the fungi alone, reducing its metabolism, with a consequent impact on metal mobility.

#### 5.4.3.2 Non-sterilised soil in presence and absence of phenanthrene

Non-sterilised soil in the presence of phenanthrene results in higher DGT fluxes and metal concentrations in soil solution, compared with non-sterilised soil in the absence of phenanthrene. This result suggests that metal behaviour is more impacted when both *P. frequentans* and soil microflora are metabolising phenanthrene, using glucose as a co-substrate (Bossert and

Bartha, 1986) than when *P. frequentans* and soil microflora have glucose as a sole source of carbon. The native microflora and *P. frequentans* therefore act synergistically, utilising phenanthrene as a carbon source while minimising its toxicity.

#### 5.4.3.3 Comparison between sterilised and non-sterilised soil in presence of phenanthrene

Metal fluxes and metal concentrations in soil solution in all cases were higher in non-sterilised soil than in sterilised soil in the presence of phenanthrene. This was most evident in the case of Ni, which showed an increase of 7 fold, and Cr of 5 fold. Cd, Cu and Pb showed an increase of about two fold. In non-sterilised soil, the synergistic behaviour of soil microflora and *P. frequentans,* allows phenanthrene to be used as a carbon and energy source giving higher fluxes and concentrations in all metals.

#### 5.4.3.4 Comparison between sterilised and non-sterilised soil in absence of phenanthrene

Comparison between sterilised and non-sterilised soil in the absence of phenanthrene showed different trends. Cr fluxes and concentrations were 2 fold higher in sterilised soil than in non-sterilised soil. Fluxes and concentrations of Cd, Cu and Pb were also slightly higher in sterilised soil than in non-sterilised soil. However, fluxes and concentrations of Ni were by 3 fold higher in non-sterilised soil than in sterilised soil. Possibly this behaviour could be explained due to the Ni toxicity to soil microflora which

has been reported in the literature (Pankhurst *et al.*, 1998; Riser-Roberts, 1998).

## 5.5 Conclusions

The highest phenanthrene removal was found in the presence of both *Penicillium frequentans* and native microflora, which seems to act synergistically and were able to minimise potential deleterious effects of the phenanthrene. In contrast, phenanthrene removal in the presence of *P. frequentans* only was reduced due to the harmful effects of the phenanthrene, despite the ability of the fungus to remove phenanthrene.

It was found that the removal of phenanthrene either by fungus or mixed fungus and native microflora resulted in an increased pool of potentially bioavailable and toxic metal species. These results highlight the behaviour of metals as a side effect of the bioremediation process.

# Chapter 6. Results and discussion 3
# Impact of microbial activity on metal behaviour and metal uptake during phenanthrene bioremoval

## 6.1 Summary

To assess Cd, Cr, Cu, Ni and Pb uptake by plants before and after phenanthrene bioremoval, DGT was used as a surrogate for plant uptake. Changes on metal fluxes, metal DGT concentrations and soil solution concentration by filtration were performed. In addition, metal uptake by plants was studied using two different types of plants: *Echinochloa polystachia* (alemangrass) and *Triticum aestivum* L. (wheat). Metal concentration was measured in both roots and leaves. All treatments were performed in triplicate and were carried out on non-sterilised soils only (including soil microflora) every three days for 30 days. Treatments were designed through a factorial experimental design ($2^3$), including plant (Pl), fungi (F), phenanthrene (P) and time (Pl*F*P) as independent variables, and metal uptake by plants as dependent variable. 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 (see Chapter 3, section 3.4 for the method). It was found that the removal of phenanthrene by mixed fungal and native microflora results in an increased pool of potentially bioavailable and toxic metal species, which were up taken by both types of plants, even in roots and leaves. These results highlight the increase in trace metal bioavailability after phenanthrene bioremoval and confirm DGT as a good surrogate for plant uptake.

## 6.2 Phenanthrene removal

Results of phenanthrene removal are shown in Figure 6.1. The highest phenanthrene removal (77% ± 1.3) in presence of *E. polystachia* (Figure 6.1a) was obtained in Treatment 1 (S + F + P + Pl). This value was higher than the one obtained in absence of *E. polystachia* (73% ± 2.3). According to this, possibly this 4% more could be attributed to the action of *P. frequentans* by itself or to the action of both fungi and microorganisms associated to the rhizosphere of *E. polystachia*. There was no significant ($p<0.02$) uptake of phenanthrene by *E. polystachia* and the phenanthrene was not detected in plant tissue (DL=0.002 $\mu g\ g^{-1}$). Escudero (2003) found that in a PAH polluted soil (fluorene, 2385 $\mu g\ g^{-1}$ phenanthrene, anthracene, fluoranthrene, pyrene and chrysene), phenanthrene was not detected in *E. polystachia* tissue, as were fluoranthrene and pyrene. However, in this study, the presence of *E. polystachia* enhanced phenanthrene removal. This is possibly because of the microorganisms associated to the plant rhizosphere. The rhizosphere flora plays a special role in the bioactivity of soils (Riser-Roberts, 1998) and rhizhospheric microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Anderson, 1997; Wenzel *et al.*, 2001). Another reason could be that the plant exudates increased native soil microflora (Larsen *et al.*, 1998), and perhaps those microorganisms were able to remove phenanthrene.

Treatment 3 (S + P + Pl) showed 67% (± 1.2) phenanthrene removal (Figure 6.1a). This bioremoval was attributed to both soil microflora and microorganisms associated to the rhizosphere of *E. polystachia*.

Figure 6.1b showed the results of phenanthrene removal in presence of *T. aestivum*. The highest phenanthrene removal obtained in Treatment 1 (75%± 1.8) was slightly lower than in the presence of *E. polystachia* (77% ± 1.3). However, there was no significant ($p<0.05$) difference on phenanthrene removal between treatments in presence of *E. polystachia* or *T. aestivum*. Moreover, Treatment 3 (S+ P+ Pl) showed 67% of phenanthrene removal in the presence of either *E. polystachia* or *T. aestivum*. This bioremoval could be attributed to both soil microflora and microorganisms associated to the rhizosphere of plants.

Comparison between Treatments 1 and 3 showed that the presence of *P. frequentans* with either plant (Treatment 1) increased the phenanthrene removal (10% in presence of *E. polystachia* and 8% in presence of *T. aestivum*). It seems that there is a slight synergism between *P. frequentans* and microorganisms associated with the rhizosphere. Moreover, plant exudates could help to increase the number of microorganisms in soil (Wenzel *et al.*, 2001). However, further work is required to know the type of microorganisms associated with plant rhizosphere and soil native microflora, and to elucidate what interactions occur during phenanthrene removal.

On day 21, the highest phenanthrene removal was found in Treatment 1 and the lowest in Treatment 3. However, the opposite result was found at day 24, when Treatment 3 showed the highest phenanthrene removal and Treatment 1 showed the lowest phenanthrene removal. Although overall Treatments 1 and 3 differ (by *ca* 10%) in their total phenanthrene removal, plant and fungi were more efficiently (*i.e.* more quickly) to remove the phenanthrene, while the plant alone requires an extra 3 days to achieve maximal degradation.

In order to compare the phenanthrene removal obtained in the presence or absence of plant (Treatments 1 and 3) and without any plant, but in the presence or absence or *P. frequentans* (Treatments A and C), all treatments are shown in Figure 6.1. Results showed that the total phenanthrene removal was higher in Treatment 1 (S + F + P+ Pl), followed by Treatment A (S + F + P) and Treatment 3 (S + P + Pl). Treatment C (S + P) showed the lowest phenanthrene removal, due to the presence of only soil native microflora.

It was not possible to measure heterothrophic activity because of the presence of *P. frequentans*, plants, plant microorganisms and soil native microflora. However, heterothrophic activity in non-sterilised soil was measured and described in Chapter 5 (section 5.2.1).

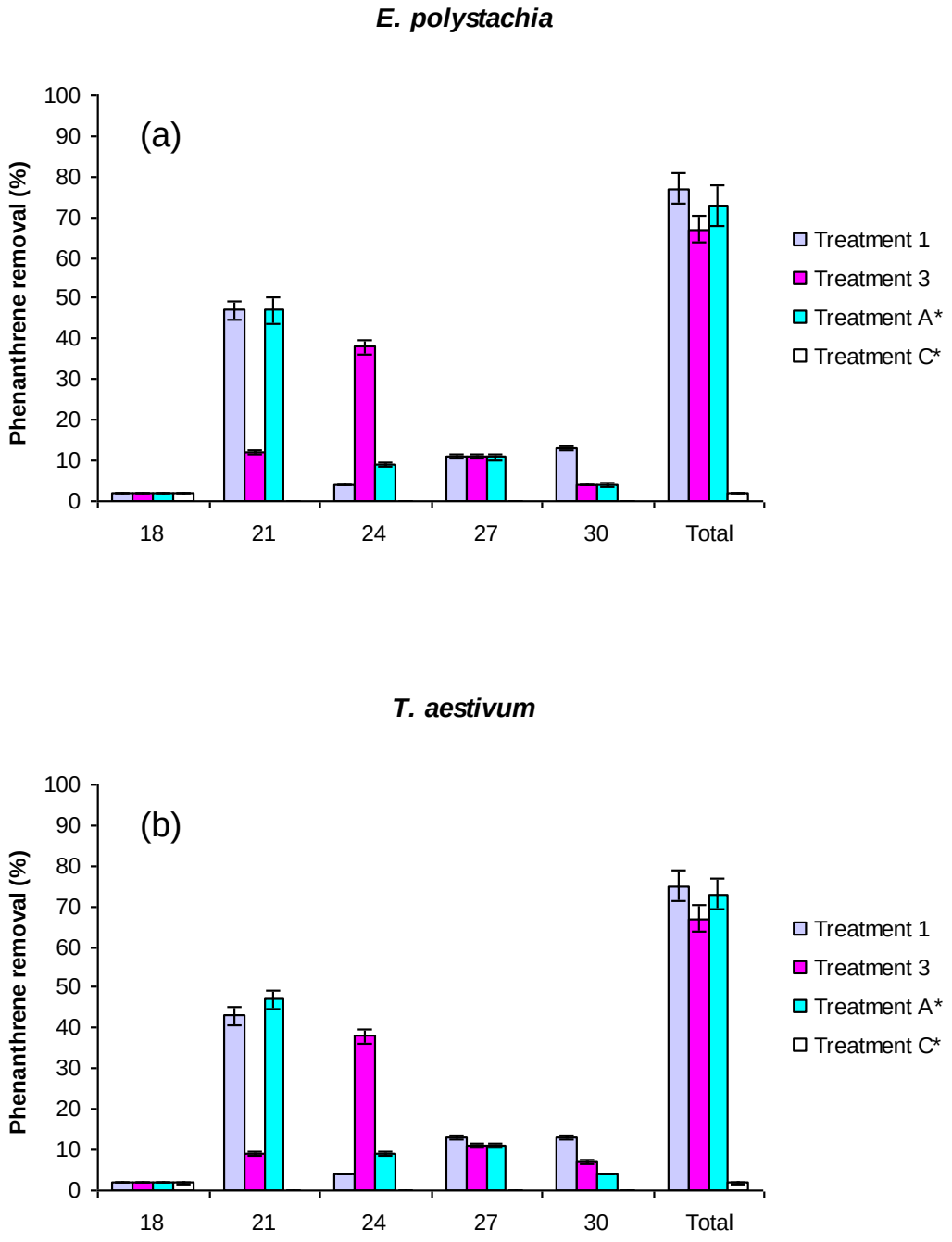



**Figure 6.1** Phenanthrene removal as a function of time: (a) In presence of *E. polystachia*; (b) In presence of *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 3= Soil + Phenanthrene + Plant. *Treatments A and C are in absence of plant. Treatment A= Soil + Fungi + Phenanthrene, Treatment C= Soil + Phenanthrene.

## 6.3 Metal behaviour

A number of general trends in the behaviour of metals in soils have been found. DGT fluxes, metal concentration measured by DGT and metal concentration in soil solution by filtration all increased after fungal treatment, as what previously found, but now in the presence of both fungi and plants the increase is higher. Treatments 1 (S + F + P + Pl) showed the highest values followed by Treatments 2, 3, 4 and 5. The data obtained in Treatment 5 is comparable with the values obtained before (Chapter 5) with non-sterilised soil. The presence of either type of plants and fungi has a significant effect on metal fluxes ($p<0.0002$), metal DGT-concentration ($p<0.0003$) and metal soil solution by filtration ($p<0.0003$). However, comparison of data between metals reveals differences in magnitude. Moreover, these differences are even large in presence of *E. polystachia*, except in the case of nickel. Results will be described for each metal, considering fluxes, DGT-concentration and concentration by filtration. All figures presented in this section are the net value obtained after the subtraction of blanks (absence of fungi or plant).

### 6.3.1 Metal fluxes measured by DGT

#### 6.3.1.1 Cadmium

The Cd fluxes measured by DGT are presented in Figure 6.2, showing the effect of fungal activity and the presence of plants on Cd behaviour. There was a significant difference ($p<0.003$) between fluxes before and after fungal inoculation, as well as in presence or absence of plants.

Prior to fungal addition, Cd fluxes are low (0.1 pg $cm^{-2}$ $s^{-1}$) and increase to 1 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in the presence of *E. polystachia* (Treatment 1), 0.5 pg $cm^{-2}$ $s^{-1}$ (Treatment 2) and are slightly elevated (0.2 pg $cm^{-2}$ $s^{-1}$) in Treatments 3, 4 and 5 (Figure 6.2a). Fluxes in Treatment 5 correspond to the fluxes measured in non-sterilised soil (Chapter 5, section 5.3.1). There was no significant difference ($p<0.05$) between Treatments 3 and 4 in presence of *E. polystachia*, but there was a significant difference ($p<0.003$) with Treatment 5.

In presence of *T. aestivum* (Figure 6.2b), fluxes are slightly lower than in the presence of *E. polystachia*, but followed the same trend as with non-sterilised soil. In descending order, fluxes were higher in Treatment 1, 2, 3, 4 and 5. Prior to fungal addition, Cd fluxes were low (0.1 pg $cm^{-2}$ $s^{-1}$) and increased to 0.8 pg $cm^{-2}$ $s^{-1}$ after addition of fungus in the presence of *T. aestivum* (Treatment 1), 0.45 pg $cm^{-2}$ $s^{-1}$ (Treatment 2) and similar (0.2 pg $cm^{-2}$ $s^{-1}$) in Treatments 3 and 4 (Figure 6.2b). There was no significant difference

($p<0.005$) between Treatments 3 and 4 in presence of *T. aestivum*, but there was a significant difference ($p<0.003$) with Treatment 5, which confirms the influence of plant on metal mobilisation.

The trends observed in both types of plants are in accordance with assumed number and type of microbial activity, as in Chapter 5 (section 5.2.1). For instance, Treatment 1 has more microorganisms and types of microorganisms, due to the presence of native soil microflora, *P. frequentans* inoculum and microorganisms associated with the rhizosphere. In addition to microbial activity to remove phenanthrene, plants acted as a metal sink by taking up the metal into the soil system. Cadmium is mobilised lower in Treatment 2 than in Treatment 1, due to the lack of microbial activity to remove phenanthrene. Treatment 3, 4 and 5 (in absence of *P. frequentans* inoculum) showed cadmium mobilisation, but lower than in the presence of the fungus. As with previous results in Chapter 5 in the absence of plants, DGT was able to differentiate between the treatments.

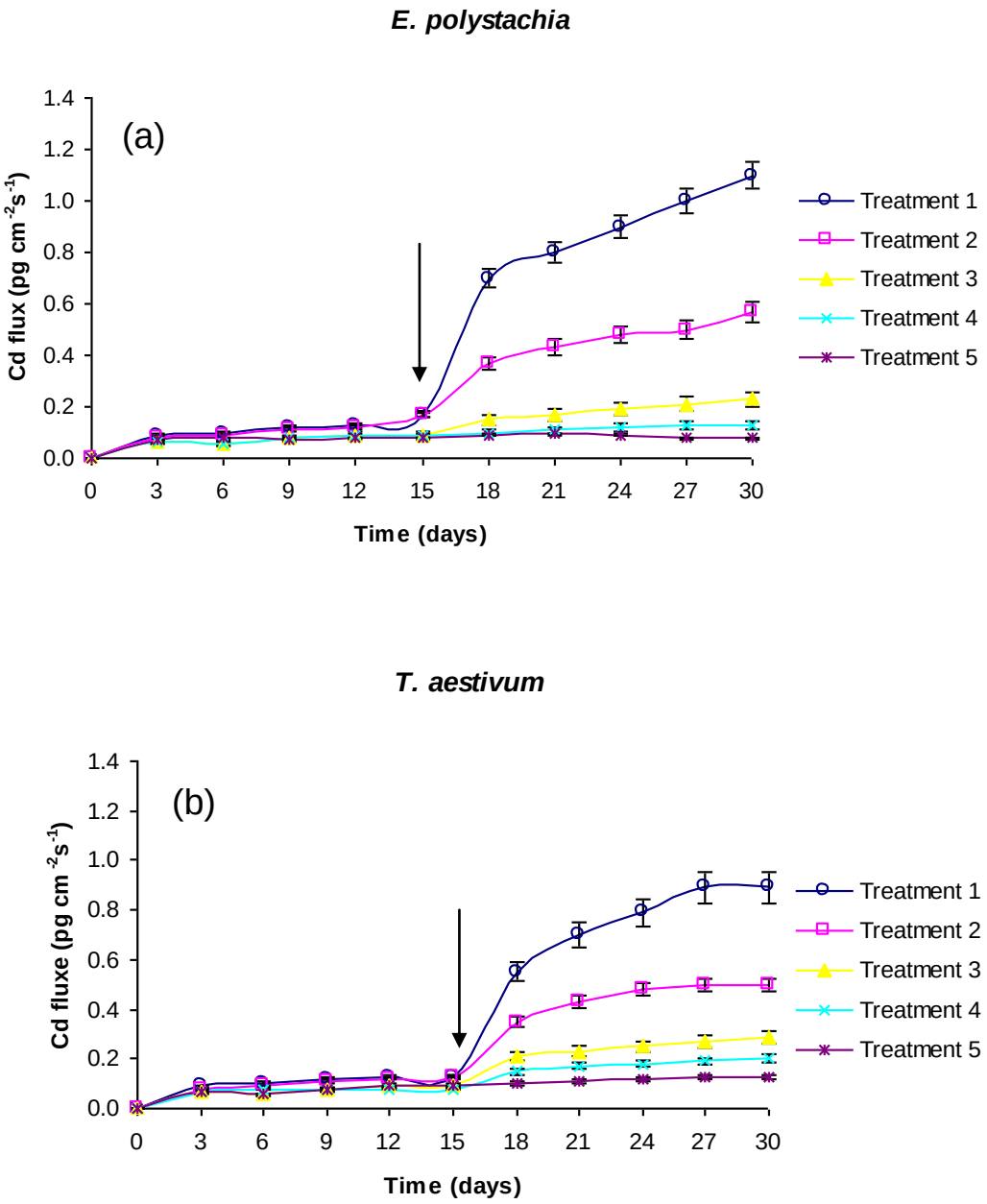



**Figure 6.2** Cadmium fluxes measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

#### 6.3.1.2 Chromium

The Cr fluxes measured by DGT are presented in Figure 6.3, showing the effect of fungal activity and the presence of plants on Cr mobilisation. Prior to fungal addition, Cr fluxes were low (<0.04 pg $cm^{-2}$ $s^{-1}$) and increased to 0.3-0.4 pg $cm^{-2}$ after the addition of fungus in the presence of *E. polystachia* and by similar amounts in the presence of *T. aestivum*. There was a significant difference ($p<0.005$) between fluxes before and after fungal inoculation in all cases. Fluxes were slightly higher in the absence of plants (Chapter 5, Figure 5.9), but this difference was not significant ($p<0.05$).

Both type of plants affect the metal behaviour in the sense that they lower the concentration in the immediate vicinity of the root. However, comparison between all treatments showed that *P. frequentans* inoculum has the highest impact on metal mobilisation (the highest metal fluxes), then soil microflora (comparison with previous results of both sterilise and non-sterilised soil) and at last plants. All this mechanism let metal uptake by plants, however, with the phenanthrene bioremoval process, metal mobilisation is increased. Further information of metal uptake by plants will be discussed later in section 6.3.4.

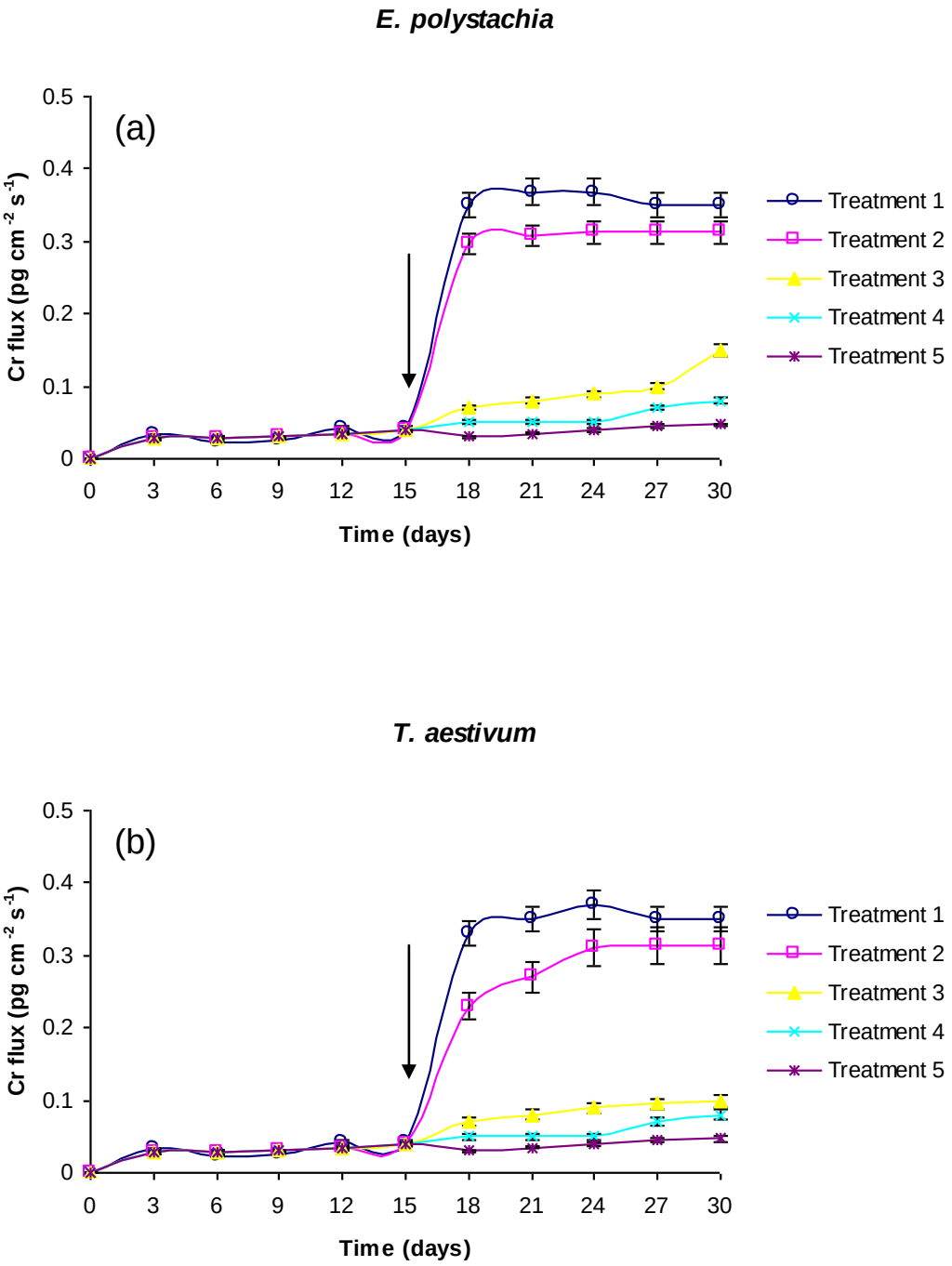



**Figure 6.3** Chromium fluxes measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.1.3 Copper

Figure 6.4 shows the effect of fungal activity and the presence of plants on Cu mobilisation. Prior to fungal addition, Cu fluxes were low (<0.1 pg $cm^{-2}$ $s^{-1}$) and increased to 0.45 pg $cm^{-2}$ after the addition of fungus in presence of *E. polystachia* and by slightly lower amounts in the presence of *T. aestivum*. Treatment 1 showed the highest fluxes (0.5 pg $cm^{-2}$ $s^{-1}$), followed by Treatment 2 (0.35 pg $cm^{-2}$ $s^{-1}$), Treatment 3 (0.15 pg $cm^{-2}$ $s^{-1}$), Treatment 4 (0.07 pg $cm^{-2}$ $s^{-1}$) and Treatment 5 (0.06 pg $cm^{-2}$ $s^{-1}$). Fluxes were in similar amounts in the previous experiment (Chapter 5). There was a significant difference ($p<0.005$) between fluxes before and after fungal inoculation.

Comparison between Treatments 1 and 2 showed different trends according to the type of plant. There was a statistically significant difference between Treatments 1 and 2 ($p<0.005$) in presence of *E. polystachia*, but there was no significant difference ($p<0.003$) between these treatments on metal fluxes in the presence of *T. aestivum*. These results could suggest two main aspects that have an influence on metal fluxes: 1) Microorganisms associated to the rhizosphere of *E. polystachia* are able to metabolise phenanthrene or at least make it less recalcitrant to the plant. This ability has been observed with another PAH using *E. polystachia* for soil bioremediation (Escudero, 2003), 2) Microorganisms associated to the rhizosphere of wheat have less influence to phenanthrene removal, *i.e.* use it as an energy source. This behaviour agrees with the previous results obtained in section 6.2 (phenanthrene removal).

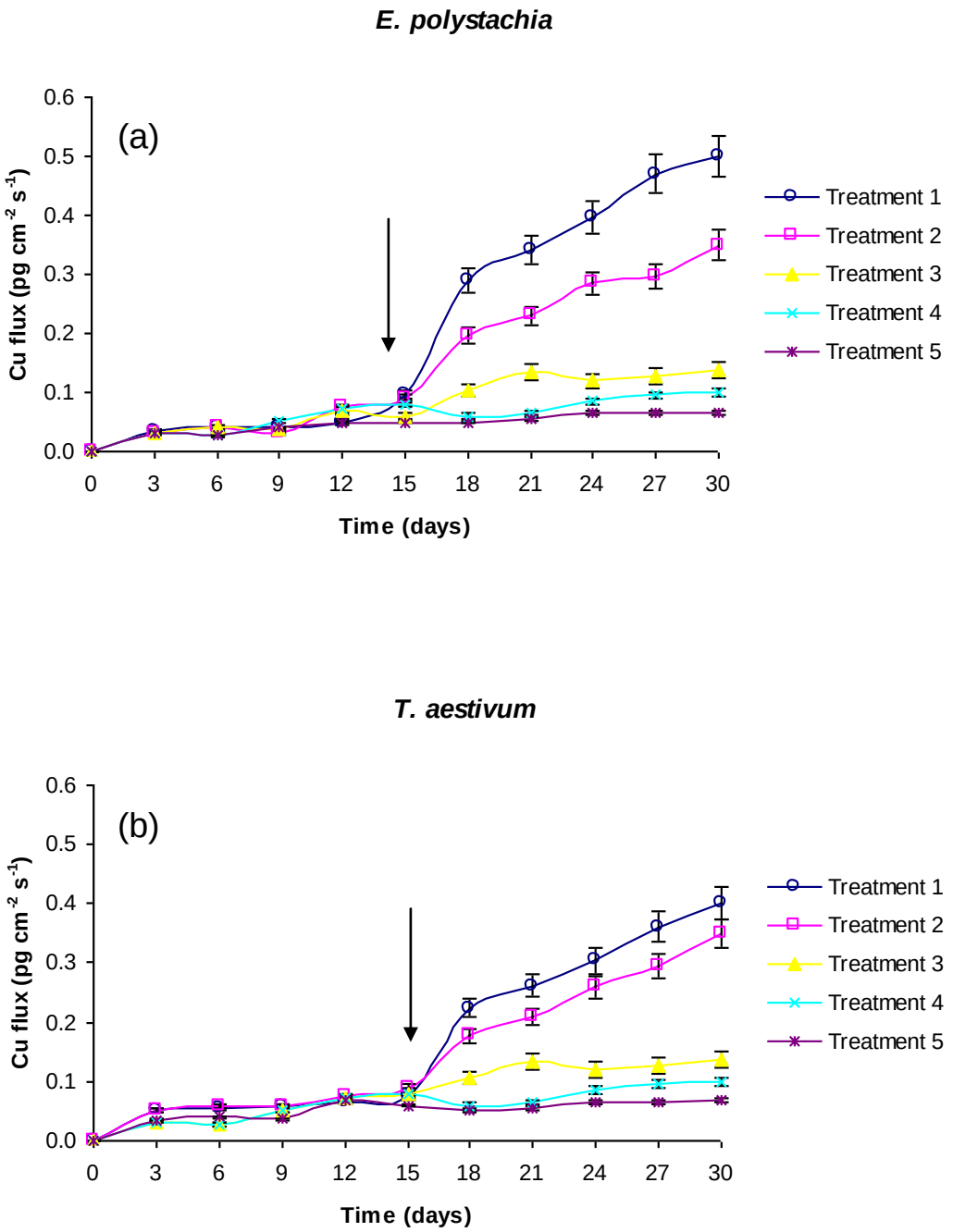



**Figure 6.4** Copper fluxes measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.1.4 Nickel

The results of fluxes of nickel obtained by DGT technique are presented in Figure 6.5. Nickel fluxes significantly ($p<0.0002$) increased after fungal inoculation.

Before fungal inoculation and plant transplant, Ni fluxes were low (<0.07 pg $cm^{-2}$ $s^{-1}$) (concentration are slightly higher, but no significant at $p<0.05$, than those obtained in Chapter 5, *i.e.* <0.05 pg $cm^{-2}$ $s^{-1}$) and significantly ($p<0.05$) increased to 1.4 pg $cm^{-2}$ $s^{-1}$ in the presence of *E. polystachia* (Figure 6.5 a) and 1.8 pg $cm^{-2}$ $s^{-1}$ in the presence of *T. aestivum* (Figure 6.5 b).

However, Treatments 3, 4 and 5 (in the absence of *P. frequentans* inoculum) showed almost the same fluxes (there was no significant difference at $p<0.005$, but there was a significant difference at $p<0.05$) in the presence of both type of plants: 0.7 pg $cm^{-2}$ $s^{-1}$, 0.4 pg $cm^{-2}$ $s^{-1}$, 0.2 pg $cm^{-2}$ $s^{-1}$, respectively.

Nickel is the only metal that showed higher fluxes in the presence of *T. aestivum* than in the presence of *E. polystachia*, but both of them in the presence of *P. frequentans* inoculum. Thus, in the presence of fungus, the differences in Ni fluxes are based on the type of plant, possibly due to the interaction between rhizosphere microorganisms and *P. frequentans*.

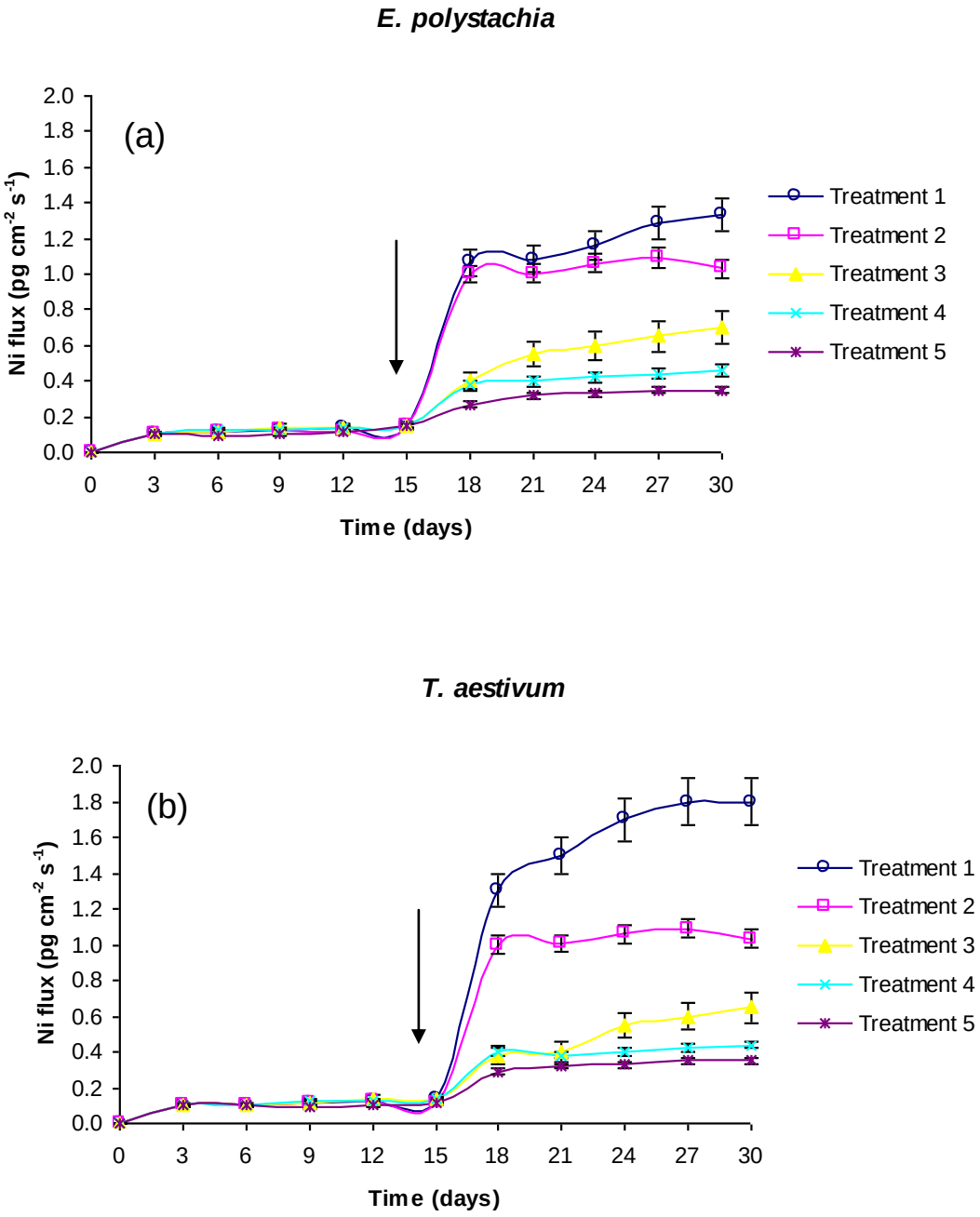



**Figure 6.5** Nickel fluxes measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.1.5 Lead

Figure 6.6 showed the results of lead fluxes obtained by DGT. Prior to fungal addition, Pb fluxes were low (<0.1 pg $cm^{-2}$ $s^{-1}$). This concentration corresponded to the previous one obtained with non-sterilised soil in Chapter 5.

After fungal inoculation, fluxes increased to 0.45 pg $cm^{-2}$ $s^{-1}$ in Treatment 1 and 0.30 pg $cm^{-2}$ $s^{-1}$ in Treatment 2 in the presence of both type of plants. A slightly increase (from 0 to 0.15 pg $cm^{-2}$ $s^{-1}$) was found in Treatment 3 and (from 0 to 0.07 pg $cm^{-2}$ $s^{-1}$) in Treatment 4. Treatment 5 showed the lowest fluxes (0.05 pg $cm^{-2}$ $s^{-1}$) and results are comparable with the measurements in Chapter 5.

The type of treatment ($p<0.0003$), the presence of plant ($p<0.005$) and the presence of phenanthrene have a significant ($p<0.002$) positive effect on lead fluxes. However, there was no any significant difference between the plant species ($p<0.005$) (Figure 6.6 a for *E. polystachia*, Figure 6.6 b for *T. aestivum*).

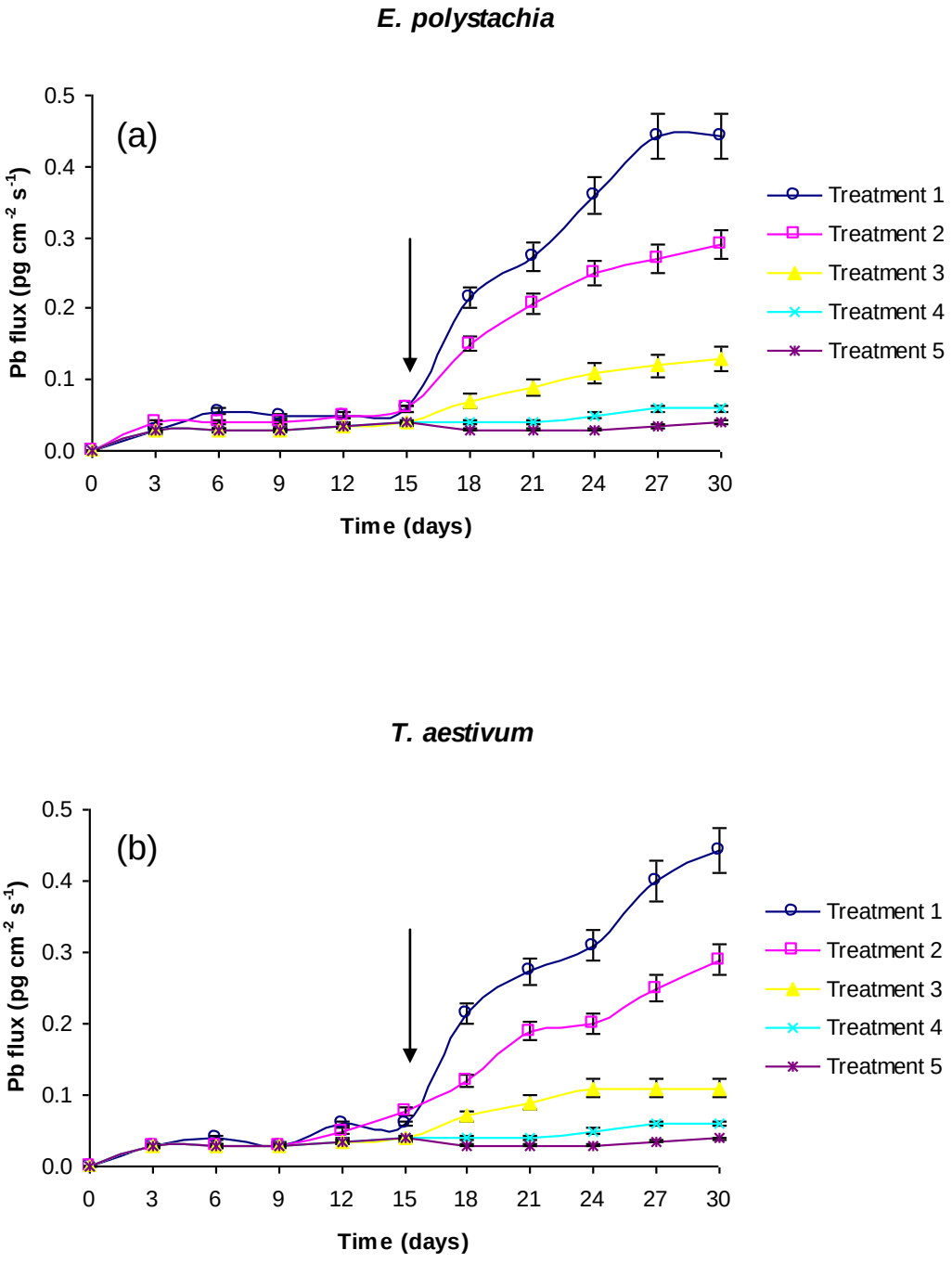



**Figure 6.6** Lead fluxes measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.2 Metal concentration measured by DGT

#### 6.3.2.1 Cadmium

The Cd concentration in pore waters measured by DGT is presented in Figure 6.7, showing the effect of fungal activity on Cd behaviour. Concentrations followed the same trends as metal fluxes. Prior to bioremediation concentrations were 2 $\mu g\ l^{-1}$. After bioremediation, the highest Cd-DGT concentration was found in Treatment 1 (18 $\mu g\ l^{-1}$), followed by Treatment 2 (11 $\mu g\ l^{-1}$), Treatment 3 (3.2 $\mu g\ l^{-1}$), Treatment 4 (2.8 $\mu g\ l^{-1}$) and Treatment 5 (1.1 $\mu g\ l^{-1}$).

There was a significant difference ($p<0.005$) between metal concentration in pore waters before and after fungal inoculation, as well as in the presence or absence of plants ($p<0.003$). Cadmium concentrations were 4 $\mu g\ l^{-1}$ in non-sterilised soil, prior to fungal addition. This increased by more than 4 fold (Figure 6.7) after addition of fungi was similar to the one obtained previously in Chapter 5.

In the case of Treatments 1 and 2, metal in pore waters increased to 18 $\mu g\ l^{-1}$ in the presence of *E. polystachia* (Figure 6.7 a) and 9 $\mu g\ l^{-1}$ in the presence of *T. aestivum* (Fig. 6.7 b). The presence of fungi + phenanthrene + plant and fungi + plant, has a positive significant ($p<0.005$) influence on metal concentration measured by DGT.

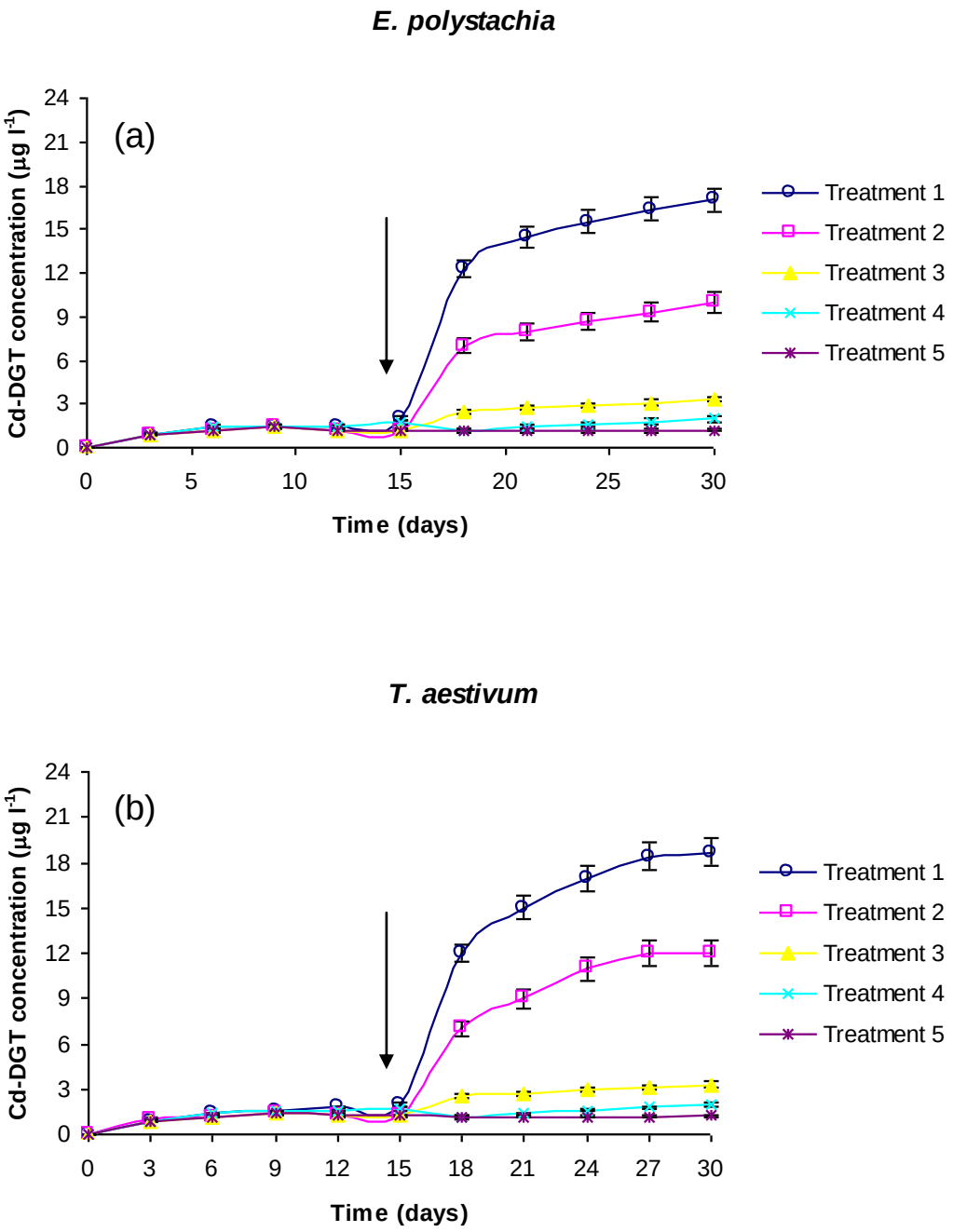



**Figure 6.7** Cadmium concentration measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.2.2 Chromium

The concentration of Cr in pore waters measured by DGT is shown in Figure 6.8. As with fluxes, DGT concentrations increased after the fungal inoculation, and follow the same trends as DGT fluxes, as expected.

Prior to fungal addition, the concentration of Cr in pore waters was 0.37 $\mu g\ l^{-1}$ in non-sterilised soil and increased by 25 fold times after addition of fungus. The highest DGT concentration was found in Treatment 1(8.5 $\mu g\ l^{-1}$), followed by Treatment 2 (7.5 $\mu g\ l^{-1}$). There was significant ($p<0.005$) difference between all treatments. The influence of microbial activity on metal behaviour due to the phenanthrene removal is even higher in presence of plants.

Treatment 3 (3.5 $\mu g\ l^{-1}$), 4 (2 $\mu g\ l^{-1}$) and 5 (3.5 $\mu g\ l^{-1}$) were significantly ($p<0.005$) different between each other and lower than Treatment 1 and 2. The lowest concentrations were found in soil only (as the previous results in Chapter 5 with non-sterilised soil). Treatment 3 showed the highest concentrations in the absence of fungi, due to the ability of both soil microflora and plants to mobilise chromium in the absence of *P. frequentans*.

The influence of microbial activity on metal behaviour is high in the presence of plants, but this impact is not the highest one. The highest impact on metal behaviour is in the presence of both fungi and plants. This will be discussed further in section 6.4.

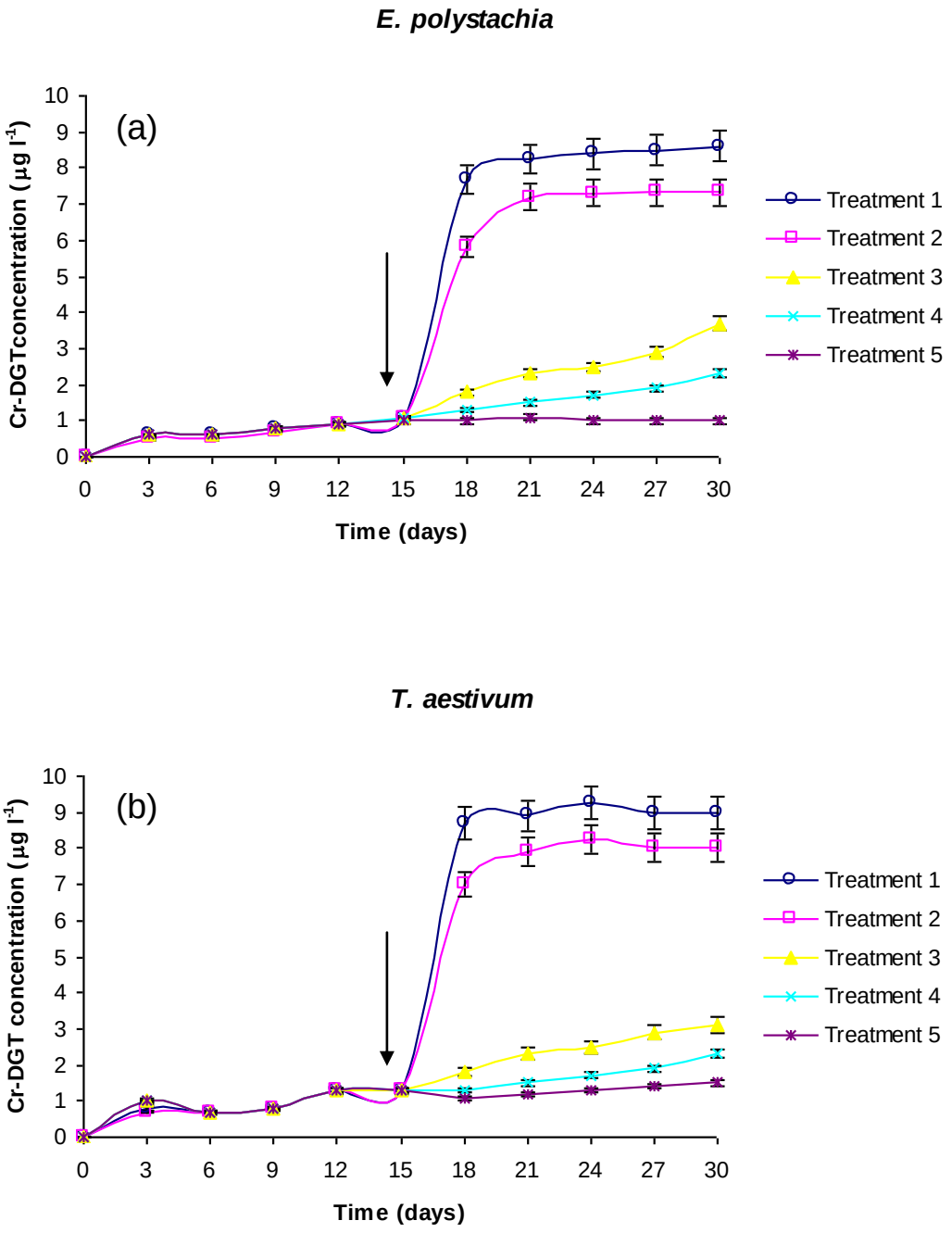



**Figure 6.8** Chromium concentration measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.2.3 Copper

Figure 6.9 shows the effect of fungal activity and the presence of plants on Cu concentration in pore waters. Before fungal addition, Cu concentrations were low (<1 $\mu g\ l^{-1}$) and increased to 6.5 $\mu g\ l^{-1}$ after addition of fungus in the presence of *E. polystachia* (Figure 6.9 a) and to 5.6 $\mu g\ l^{-1}$ in the presence of *T. aestivum* (Figure 6.9b). The concentrations in Treatments 5 were similar to the copper concentrations obtained in the last chapter with non-sterilised soil.

As with fluxes, comparison between copper concentration in pore waters in Treatments 1 and 2 showed different trends according to the type of plant. There was a statistically significant difference between Treatments 1 and 2 ($p<0.03$) in the presence of *E. polystachia*, but there was no significant difference ($p<0.03$) between these treatments on metal fluxes in the presence of *T. aestivum*.

As it was observed previously with fluxes and now with metal concentrations in pore waters measured by DGT, the highest impact on metal behaviour is in the presence of both fungi and plant.

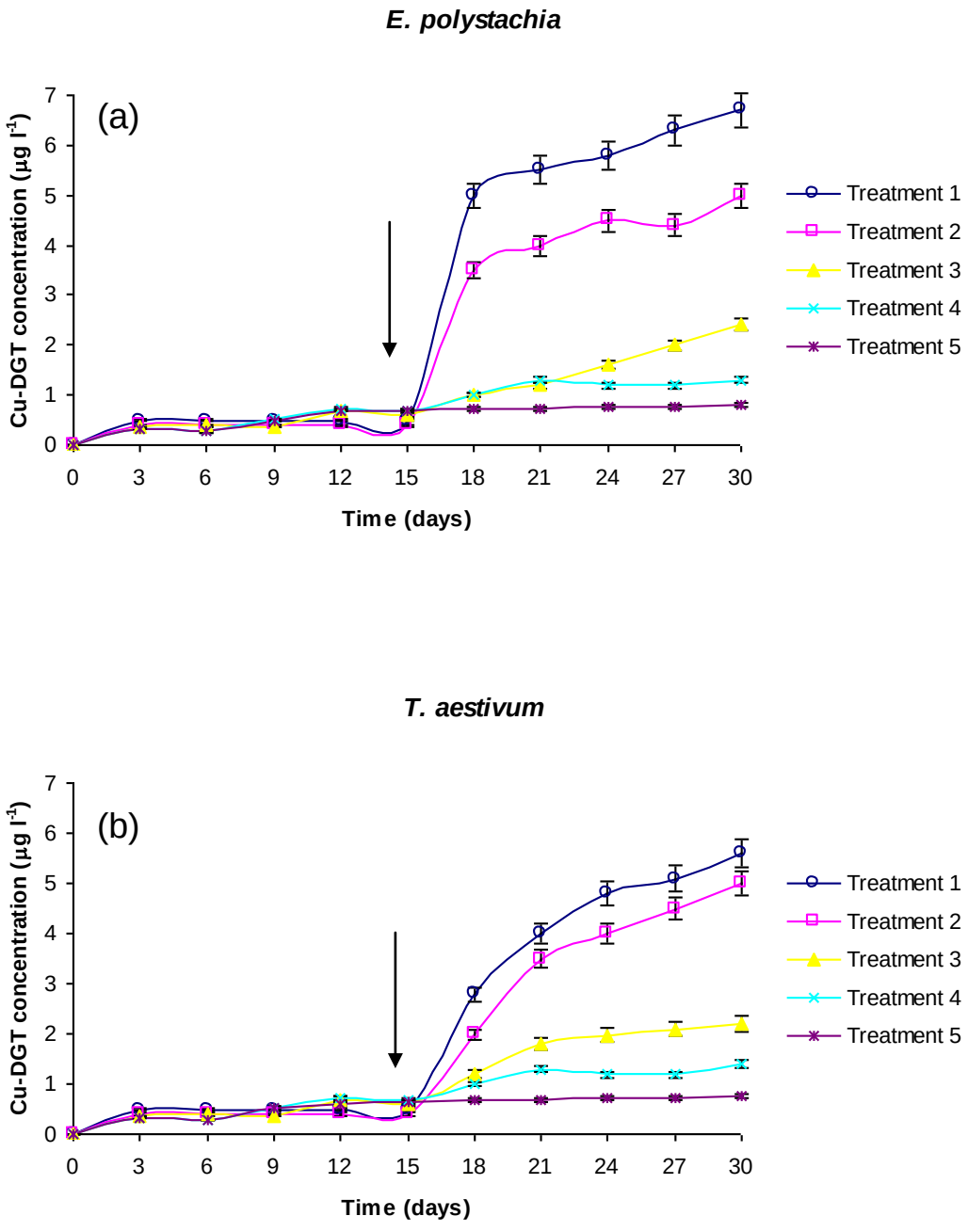



**Figure 6.9** Copper concentration measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

#### 6.3.2.4 Nickel

The concentration of nickel in pore waters measured by DGT is shown in Figure 6.10. There was a statistically significant difference ($p<0.005$) in Ni DGT concentration before and after fungal treatment in all treatments, except in Treatment 5. As observed before, DGT concentrations followed the same trend as fluxes. There was a statistical significant difference between Treatments 1 and 2 ($p<0.003$) in the presence of *E. polystachia* ($p<0.05$) (Figure 10a) and in the presence of *T. aestivum* (Figure 6.10b).

Treatments 3, 4 and 5 followed the same trend as previously. Treatment 3 (S + P + Pl) showed the highest concentrations, due to the influence of plant root microbial activity on nickel mobilisation, in addition to the presence of plants. Treatment 4 (S + Pl) also mobilised metal, but at lower rate than in the presence of microbial activity to remove phenanthrene. The last treatment (S) showed no change in nickel DGT concentrations as a function of time.

The type of treatment ($p<0.021$), time of treatment ($p<0.001$) and type of plant ($p<0.001$), were found to significantly affect the nickel concentration in pore waters measured by DGT.

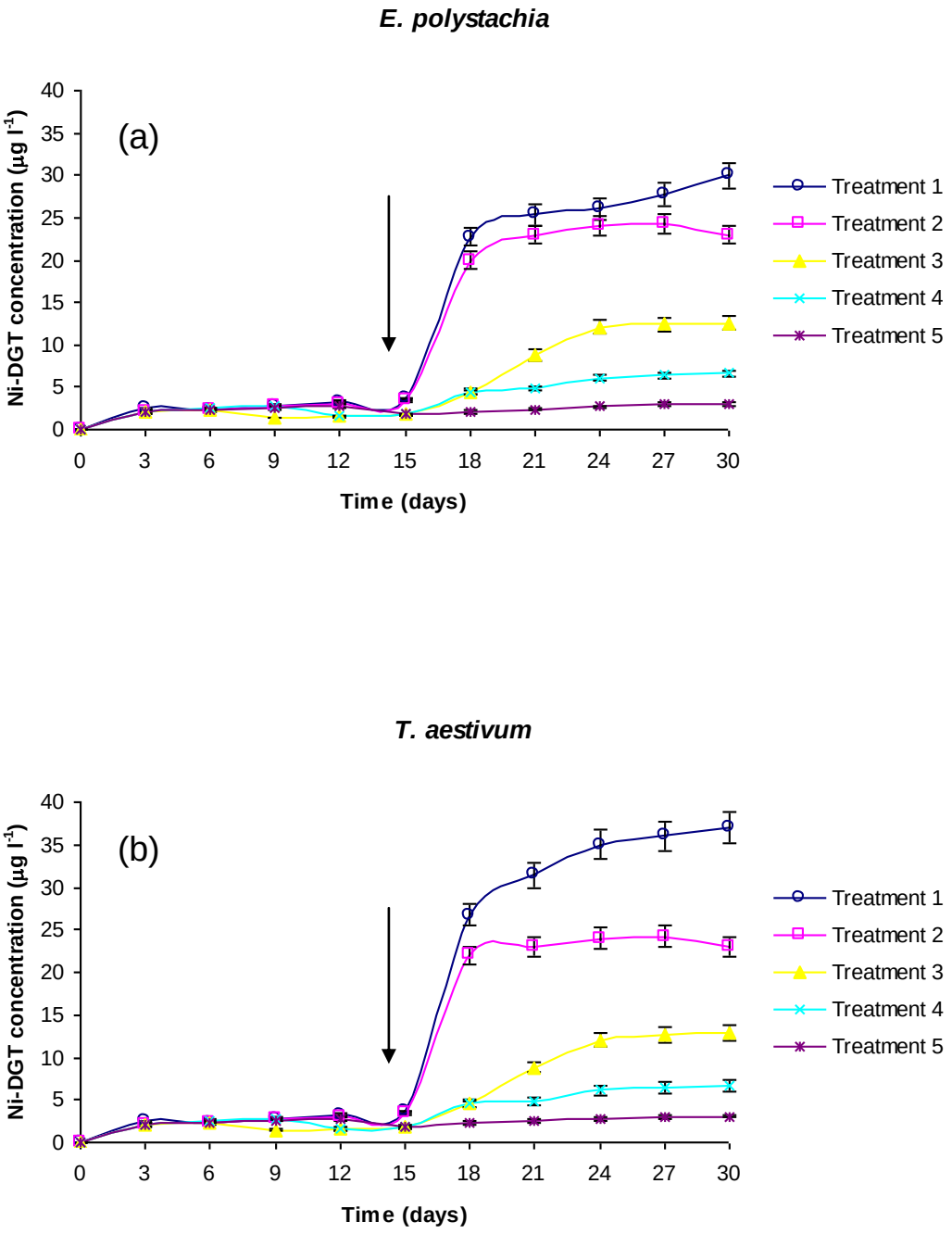



**Figure 6.10** Nickel concentration measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.2.5 Lead

Figure 6.11 showed the lead DGT concentration as a function of time. DGT concentrations were <0.2 μg $l^{-1}$ prior to bioremediation, as observed in non-sterilised soil (Chapter 5, Figure 5.12). After fungal inoculation DGT concentrations increased to 1.4-1.7 μg $l^{-1}$, in the same manner as fluxes. There was a statistical significant difference between all treatments ($p<0.003$) in the presence of either plant.

In the case of lead, comparison of Treatments 3, 4 and 5 (all in the absence of *P. frequentans*), showed that the lowest concentrations were found in Treatment 5 due to the minimal microbial activity, since only native soil microflora is present, with a lack of activity to remove phenanthrene. However, the presence of plants in both treatment 3 and 4 showed an increase in DGT metal concentration, especially in the case when presumably native soil microflora is removing phenanthrene (Treatment 3).

The type of treatment ($p<0.005$), time of treatment ($p<0.001$) and type of plant ($p<0.05$), were found to significantly affect the lead concentration in pore waters measured by DGT.

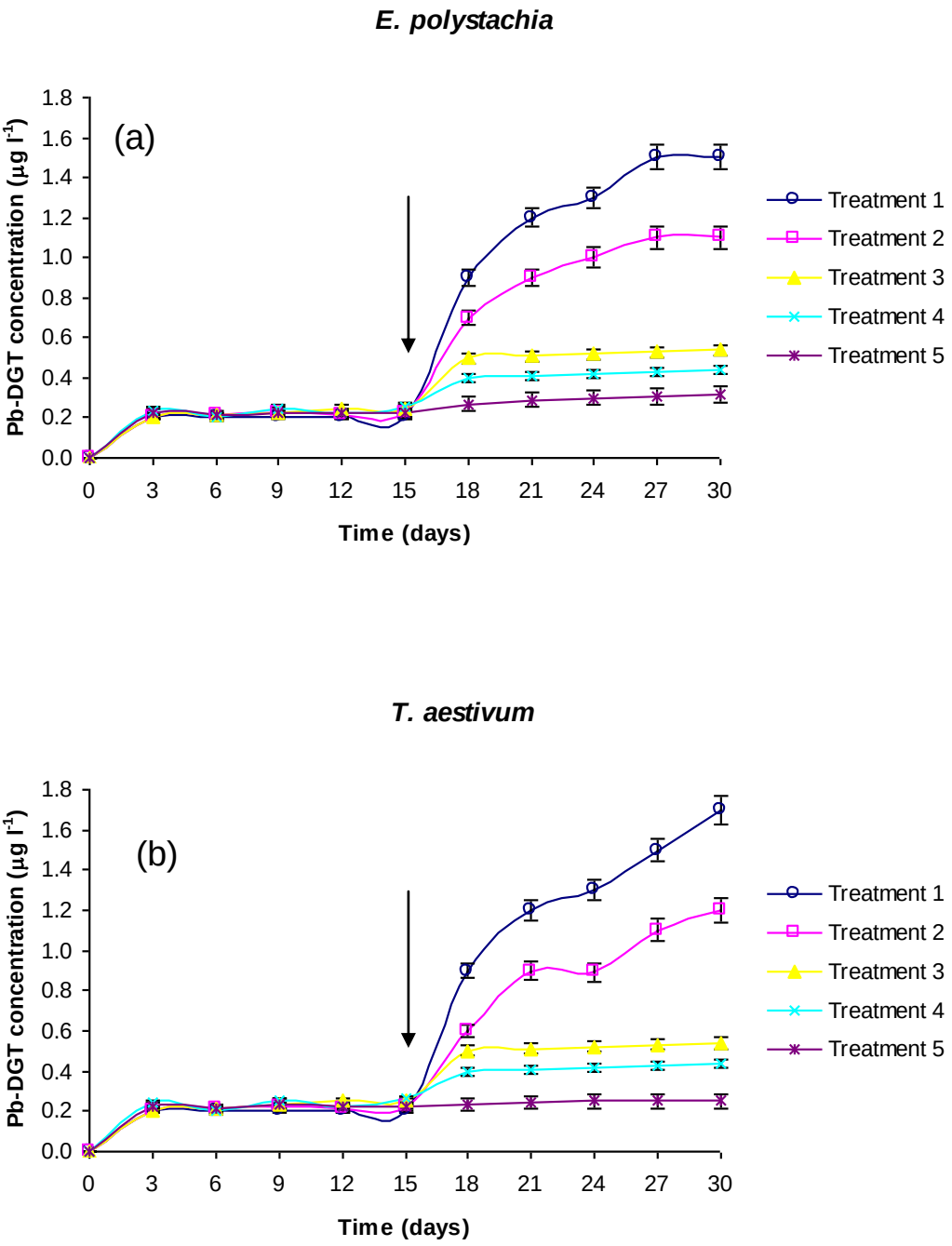



**Figure 6.11** Lead concentration measured by DGT in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.3 Metal concentration in soil solution measured by filtration

#### 6.3.3.1 Cadmium

Concentration in soil solution by filtration is shown in Figure 6.12. Cadmium concentrations by filtration were <5 $\mu g\ l^{-1}$ prior to bioremediation, as observed in non-sterilised soil (Chapter 5, Figure 5.13). Trends observed in the previous experiment with fungal inoculation in non-sterilised soil are almost identical to those observed in the absence of plants. The lowest concentration in soil solution measured by filtration was observed in soil (5 $\mu g\ l^{-1}$), where the microbial activity and phenanthrene removal was the lowest, while the highest concentration was found in Treatment 1 (45 $\mu g\ l^{-1}$).

DGT devices show a continuous increase in soil solution as a function of time because of the time averaged measurement after constant deployment. Solution concentration measured by filtration shows a clear bimodal distribution with both type of plants (Figure 6.12) in Treatments 1 and 2. In the presence of *E. polystachia* (Figure 6.12a), peaks in concentration occur at 18 (45 $\mu g\ l^{-1}$) and 24 days (40 $\mu g\ l^{-1}$) in Treatments 1, and on the same day at 30 $\mu g\ l^{-1}$ and 37$\mu g\ l^{-1}$ in Treatment 2, respectively. In presence of *T. aestivum* (Figure 6.12b), peaks in concentration were the same at day 18 and 24 (40 $\mu g\ l^{-1}$) in Treatment 1, and 35 $\mu g\ l^{-1}$ in Treatment 2. The tendency is the same as the comparable results (Chapter 5, Figure 5.13).

Comparison of Treatments 3, 4 and 5, showed the influence of microbial activity on metal in soil solution, due to the presence of 1) native soil microflora, 2) microorganisms of plant rhizosphere. In fact, differences between soil solutions obtained from rhizosphere and non-rhizosphere soils (Lorenz *et al.*, 1994) showed that Cd was depleted from the rhizosphere soils, which confirms the influence of rhizosphere microorganisms on cadmium in solution.

#### 6.3.3.2 Chromium

Figure 6.13 shows two peaks in Cr concentration in treatments 1 and 2 at day 18 and 24 in presence of both *E. polystachia* and *T. aestivum*. The highest concentration was found in Treatment 1 and 2, with 25 $\mu g\ l^{-1}$ for *E. polystachia* and 23 $\mu g\ l^{-1}$ for *T. aestivum*. In the absence of fungi, there was no peak. There was a significant ($p<0.002$) difference between Treatments 3, 4 and 5. Treatment 3 showed the highest soil solution concentration (5 $\mu g\ l^{-1}$). Treatment 5 showed the same trend as with Cd, showing the lowest solution concentration (2 $\mu g\ l^{-1}$). Although a higher sampling frequency is required to identify the trends, tentatively it may be said that fluctuations in Treatments 1 and 2 could be due to the action of microorganisms on the solid phase. Soil colloids (<0.45 $\mu m$) were perhaps solubilised from the solid phase, and made more readily available to microorganisms as carbon and source energy. Once depleted (day 21), the fungi degraded the solid soil again, leading to a peak at day 24. A subsequent trough and start of a new peak is detected at day 27 and 30. Analysis of data for other metals will help test this tentative hypothesis.

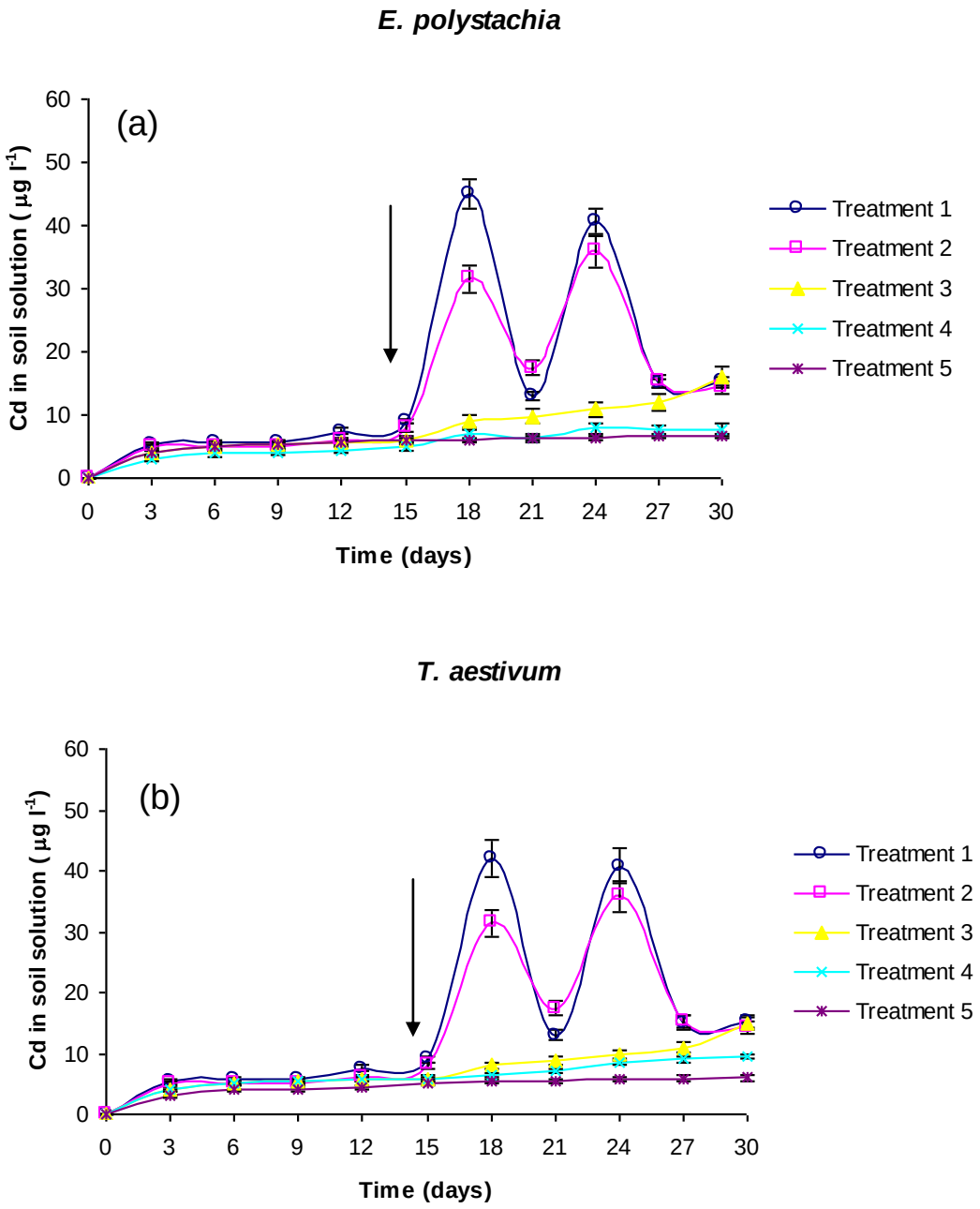



**Figure 6.12** Cadmium concentration measured by filtration in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

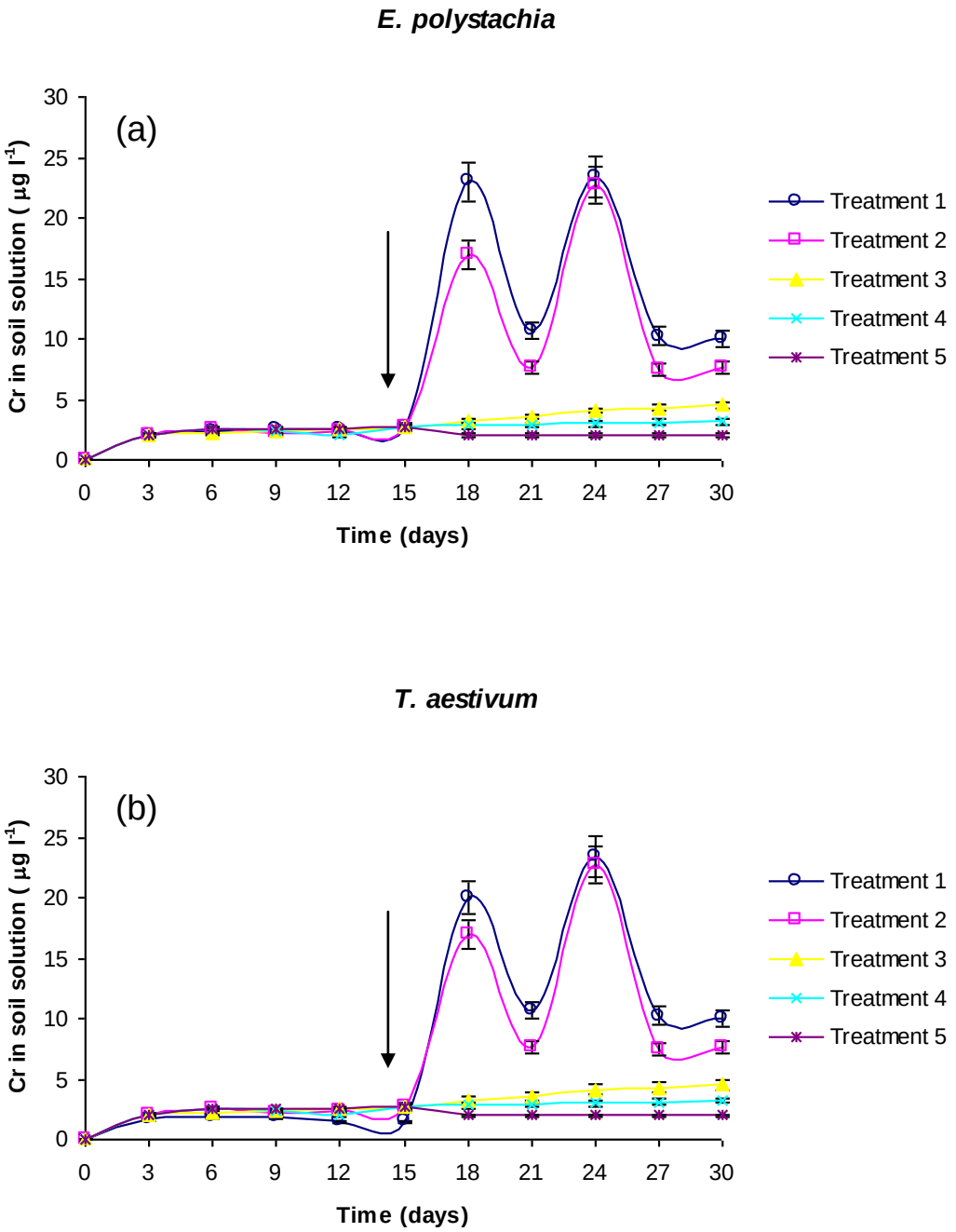



**Figure 6.13** Chromium concentration measured by filtration in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

#### 6.3.3.3 Copper

Cu concentration in soil solution is shown in Figure 6.14. Unlike Cd and Cr, Cu showed the same pattern as was previously found in DGT measurements. That is, the highest concentration was found in Treatment 1 (12 $\mu g\ l^{-1}$), followed by Treatment 2 (8 $\mu g\ l^{-1}$), 3 (5 $\mu g\ l^{-1}$), 4 (3.8 $\mu g\ l^{-1}$) and 5 (2 $\mu g\ l^{-1}$). There was no significant difference between treatments in presence of either plant ($p<0.05$).

Unlike Cd and Cr, no bimodal peaks were observed in Cu. There was a significant difference between each treatment ($p<0.03$), indicating the impact of fungi, phenanthrene and plants. However, differences can be observed between copper concentration by DGT and Cu concentration by filtration. As with DGT concentrations, there was no difference between different plants. However, the filtration results showed concentrations *ca.* twice as high as measured by DGT. This indicates the differences between DGT and filtration.

The Cu results question the hypothesis developed in the previous section. The lack of a bimodal distribution after day 15 indicates there may be a difference in microbial activity with Cu in comparison to Cr and Cd. Alternatively, Cd and Cr may readsorb to solid particles more readily than Cu. This is unlikely for Cd, which as mentioned previously, binds less strongly to solid phases than Cu. Alternatively the metals may not be evenly distributed within the soil. A definitive answer is not available based on these data.

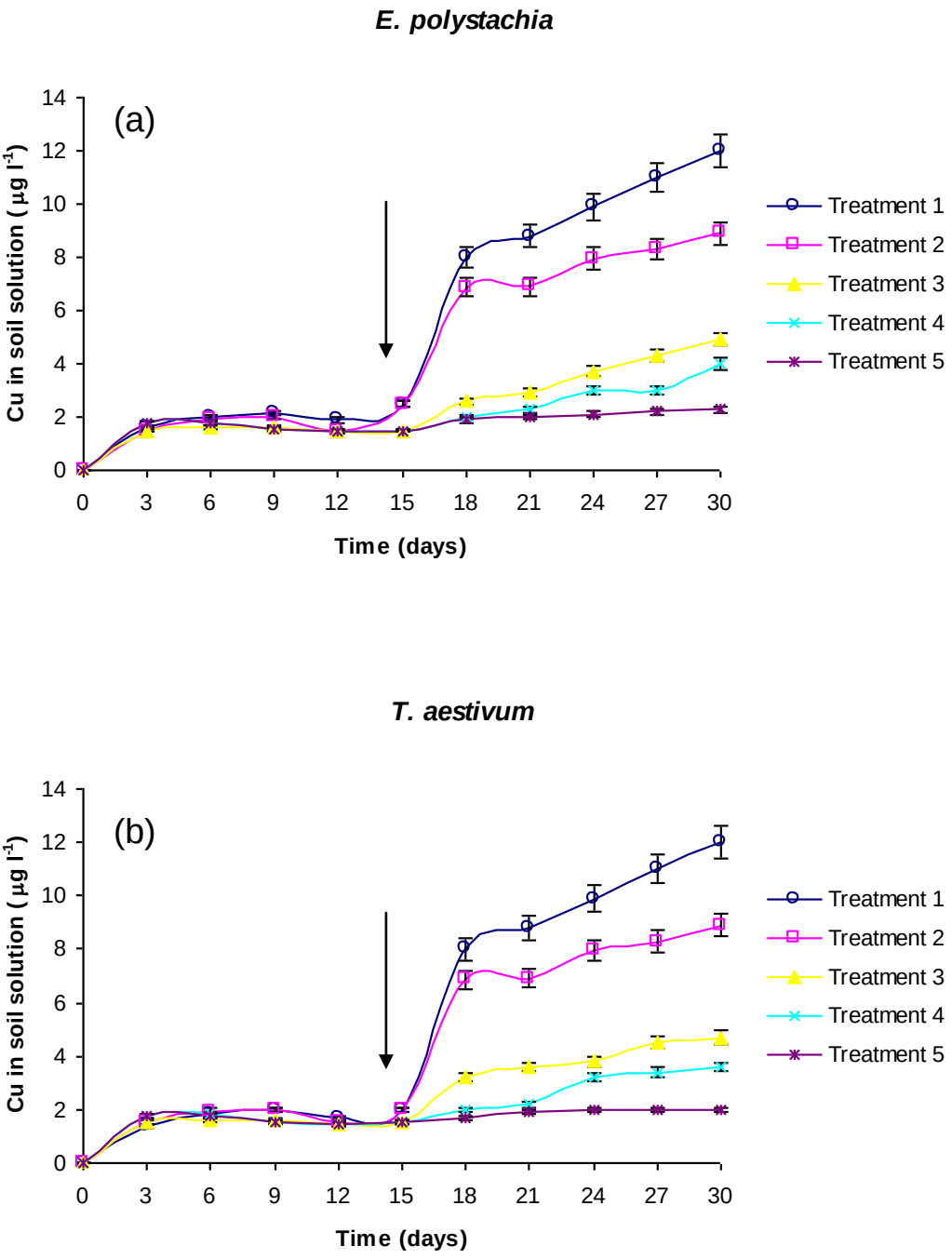



**Figure 6.14** Copper concentration measured by filtration in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.3.4 Nickel

Figure 6.15 showed the nickel concentration in soil solution measured by filtration. Nickel in soil solution significantly ($p<0.03$) increases after mixing of soil with soil microflora, and *P. frequentans* inoculum and plant. Prior to fungal addition, the concentration of Ni in soil solution is <30 $\mu g\ l^{-1}$ and increases to 140 $\mu g\ l^{-1}$ in the presence of *E. polystachia* (Figure 6.15a) and 160 $\mu g\ l^{-1}$ in the presence of *T. aestivum* (Figure 6.15b).

As with Cu, Nickel does not show a bimodal distribution. However, unlike Cu, Ni soil solution concentration was significantly ($p<0.05$) different in *E. polystachia* and *T. aestivum*. Moreover, Treatments 1 and 2 were significantly ($p<0.03$) different in presence of the same plant.

Comparison of Treatments 3, 4 and 5 (all in the absence of *P. frequentans*), showed that the lowest nickel concentrations by filtration were found in Treatment 5 due to the minimal microbial activity. However, the presence of plants in both Treatment 3 and 4 showed an increase in Ni metal concentration, especially in Treatment 3.

The type of treatment ($p<0.001$), time of treatment ($p<0.001$) and type of plant ($p<0.05$), were found to significantly affect the nickel concentration in pore waters measured by DGT.

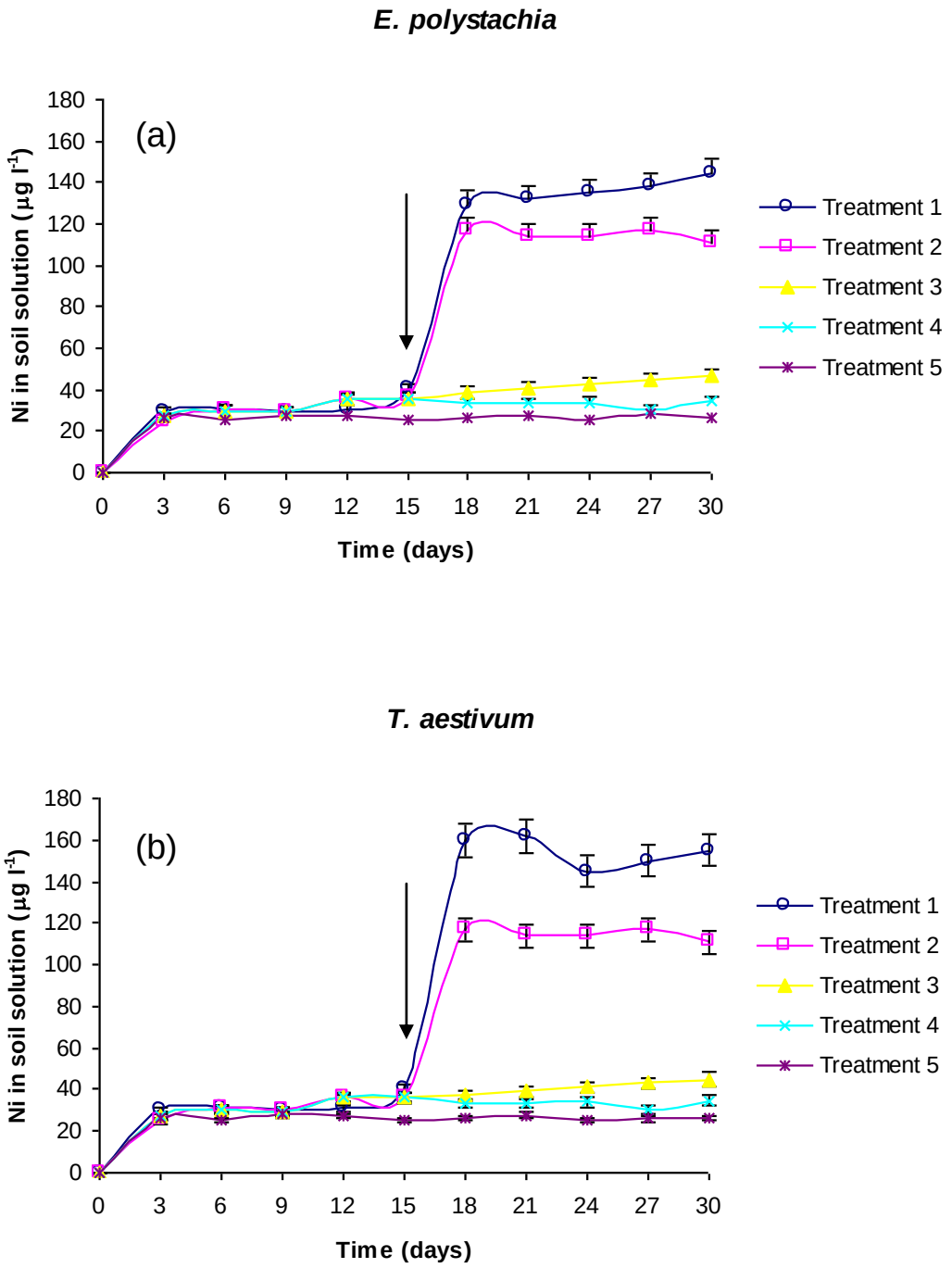



**Figure 6.15** Nickel concentration measured by filtration in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.3.5 Lead

Further information on lead concentration in soil solution is obtained from Figure 6.16. Unlike the other metals studied, lead shows a unimodal distribution in Treatment 1 with a peak of 5 $\mu g\ l^{-1}$ on day 21 (day in which phenanthrene removal was the highest, see section 6.2), in the presence of either type of plant. There was a bimodal distribution in Treatment 2 with two peaks of 3.5 $\mu g\ l^{-1}$ on day 18 and 24 and in presence of either type of plant.

The Pb results question again the hypothesis about solid soil (<0.45 $\mu m$) was perhaps solubilised and was made more readily available to microorganisms. Once depleted, the fungi degraded the solid soil again, leading to a new peak. However, a definitive answer is not available based on these data. It is clear that lead shows different behaviour to Cd and Ni and also to Cr and Cu. Possibly differences may be due to the microbial activity at different metal and/or to chemical behaviour of different metal.

Treatments 3, 4 and 5 followed the same trend as it was previously observed. That is, soil showed the lowest soil solution concentration, while Treatment 3 showed the highest concentration, which is increased as a function of time.

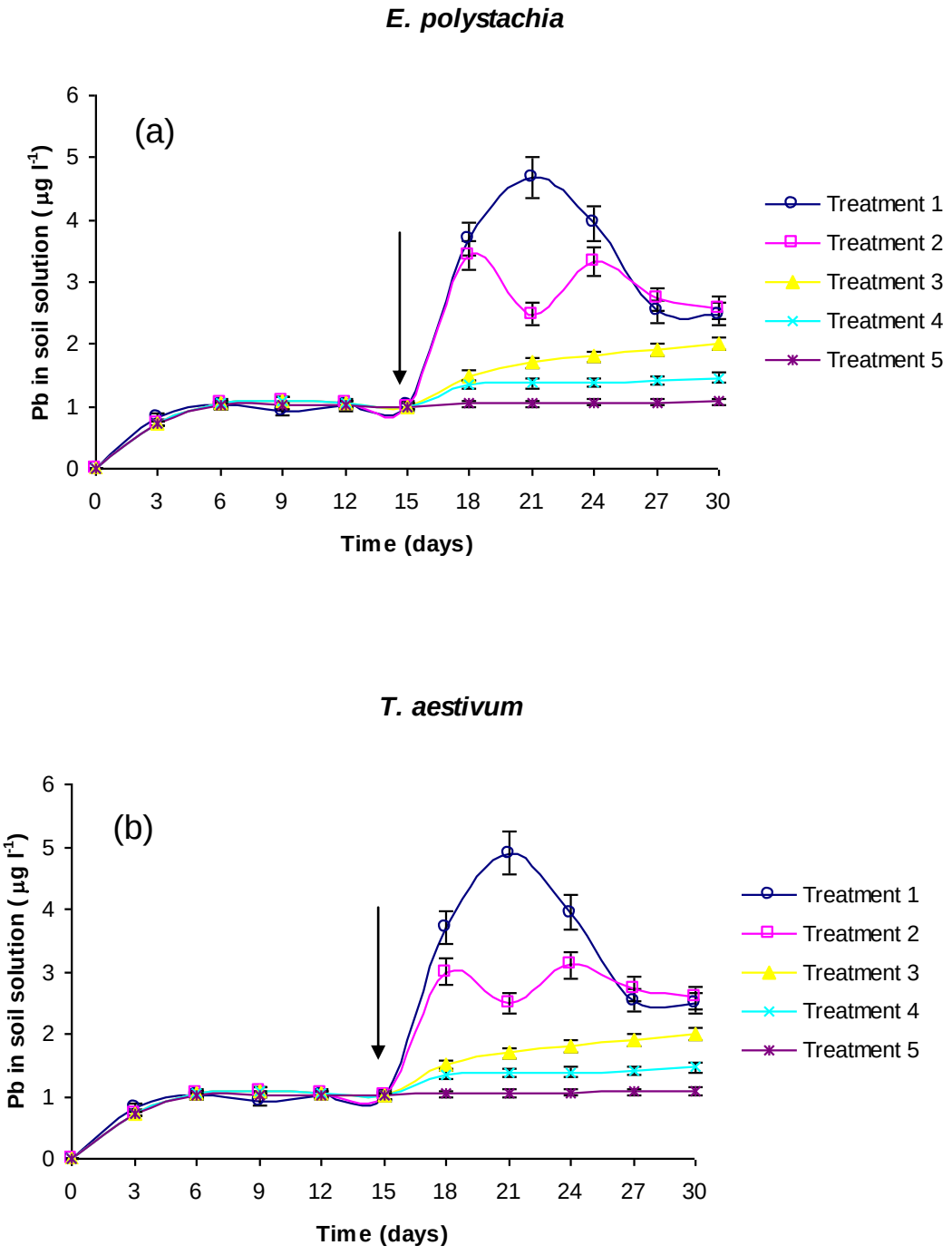



**Figure 6.16** Lead concentration measured by filtration in non-sterilised soil and in presence of: (a) *E. polystachia*; (b) *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant, Treatment 4= Soil + Plant and Treatment 5= Soil. T= 28°C, Moisture= 44.5%, C:N= 40. Arrow indicates when soil + fungi mixed together.

### 6.3.4 Metal uptake by plants

A number of general trends in the behaviour of metals in soils have been found. Metal uptake by both plants increased after fungal treatment. Treatments 1 showed the highest values followed by Treatments 2, 3 and 4. Furthermore, in some cases a metal translocation from roots to leaves was detected. Metal uptake by plants depended significantly on the type of plant ($p<0.005$) and treatment ($p<0.002$). However, comparison of data between metals reveals differences in magnitude. Results will be described for each metal, considering plant uptake in roots and translocation to leaves.

#### 6.3.4.1 Cadmium

#### 6.3.4.1 *Echinochloa polystachia*

Figure 6.17 shows a continuous increase in metal uptake by *E. polystachia* as a function of time. The highest metal uptake was found in Treatment 1 (20 $\mu g\ g^{-1}$) after 30 days, followed by Treatment 2 (17 $\mu g\ g^{-1}$), Treatment 3 (5 $\mu g\ g^{-1}$) and Treatment 4 (4 $\mu g\ g^{-1}$).

Further information on the Cd biouptake can be obtained from comparing Treatments 1 and 2 with Treatments 3 and 4, which have a significant ($p<0.005$) difference between each other. The presence of fungi + phenanthrene and fungi increases metal uptake compared to the absence of fungi.

The total uptake of *E. polystachia* was 44 μg $g^{-1}$ at day 30 in Treatment 1. In addition to total plant uptake, both roots and leaves uptake was measured, as shown in Figure 6.18.

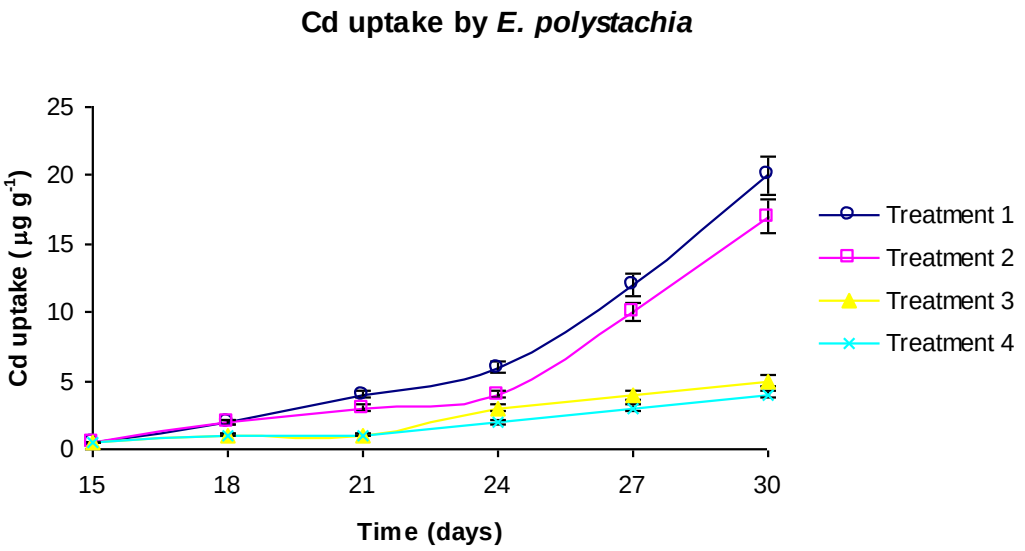



**Figure 6.17** Cadmium uptake by *E. polystachia*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

From day 15 to 30, maximum Cd concentrations in roots were 15 μg $g^{-1}$ (Figure 6.18a) and in leaves 29 μg $g^{-1}$ (Figure 6.18b) after fungal addition. The larger Cd concentration in leaves compared with roots must be due to translocation within *E. polystachia*. Although Cd is considered to be a nonessential element for metabolic processes (Kabata-Pendias, 2001), Cd in this study was effectively absorbed by roots and moved around the plant. Fungal bioremediation induces Cd uptake by plant, therefore, phenanthrene removal results in increase Cd uptake, which has been a very toxic metal on the plant growth (Athar and Ahmad, 2002).

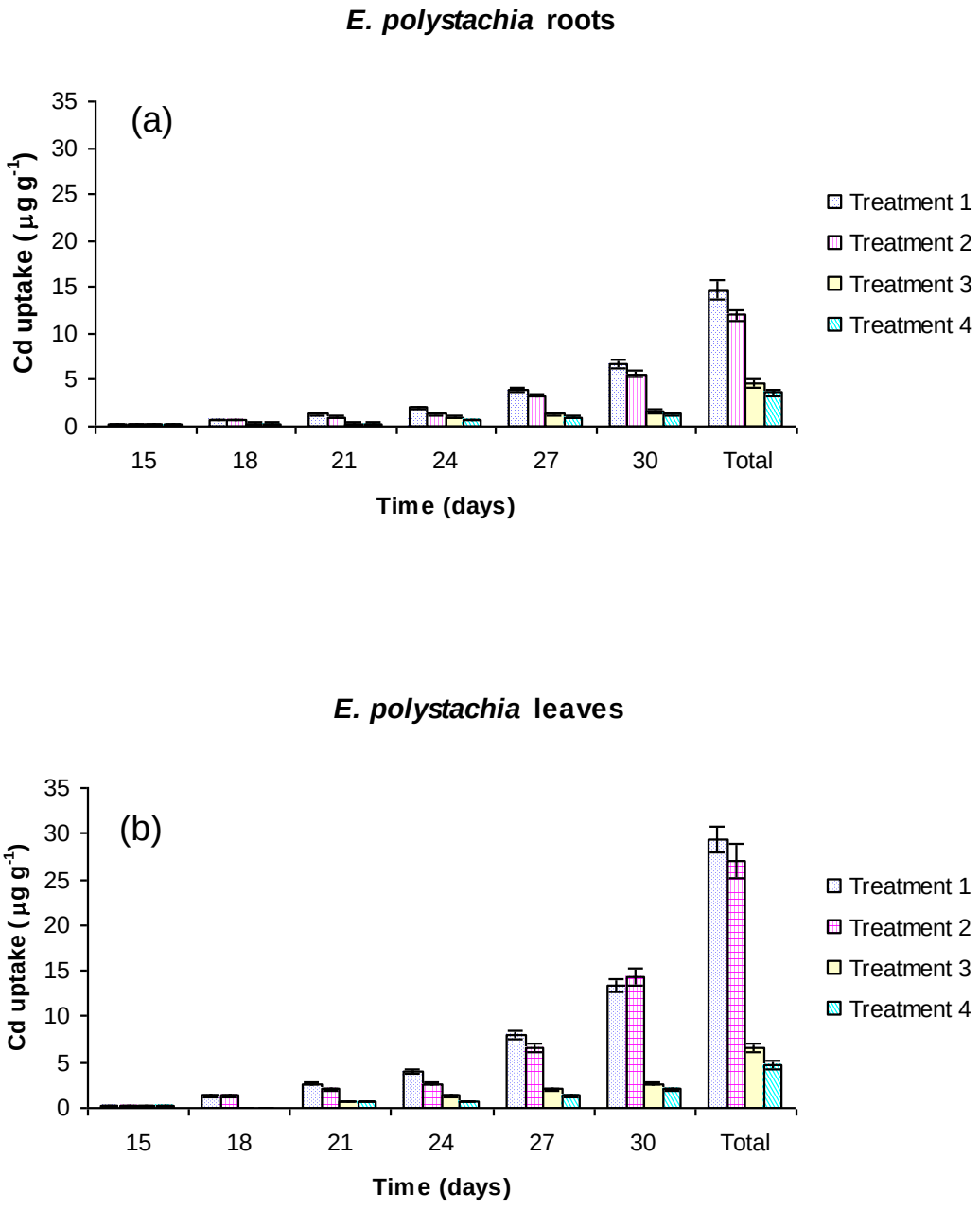



**Figure 6.18** Cadmium uptake by *E. polystachia* in: (a) roots and (b) leaves. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

### 6.3.4.2 *Triticum aestivum*

As is shown in Figure 6.19, the highest metal uptake at day 30 was found in Treatment 2 (14.8 μg $g^{-1}$) and slightly lower in Treatment 1 (14 μg $g^{-1}$), followed by Treatment 3 (3 μg $g^{-1}$) and Treatment 4 (2.5 μg $g^{-1}$). In addition, there was a significant ($p<0.005$) difference between Treatment 1 and 2. About 50% of the cadmium uptake took place on day 27 and 30.

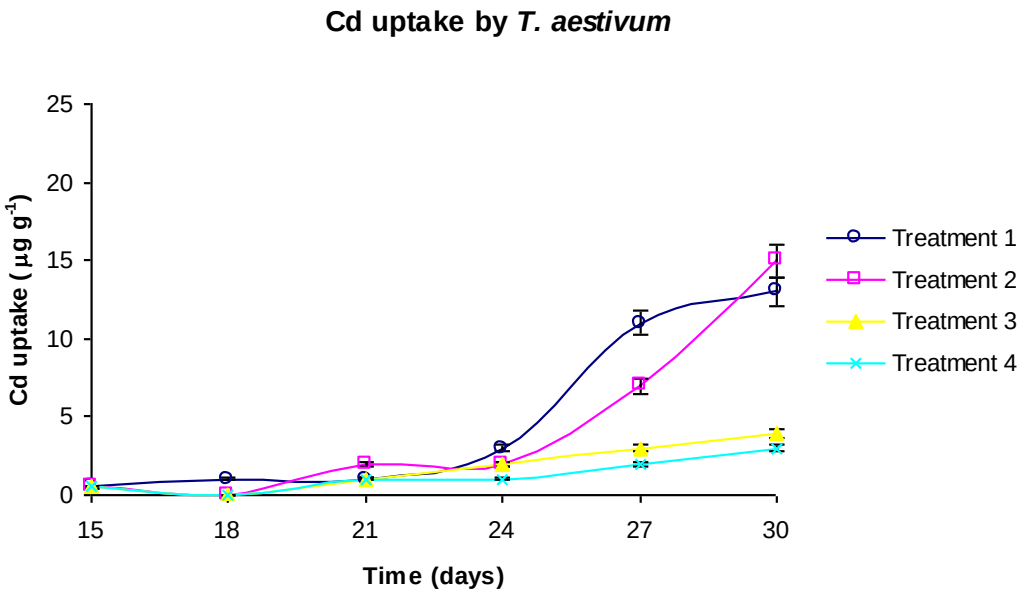



**Figure 6.19** Cadmium uptake by *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

As with the case of *E. polystachia*, *T. aestivum* was able to take up cadmium in the roots and translocated to the leaves. However, lower amounts are taken up and translocated (Figure 6.20). Maximal concentration in roots was 20 μg $g^{-1}$ and in leaves 9 μg $g^{-1}$.

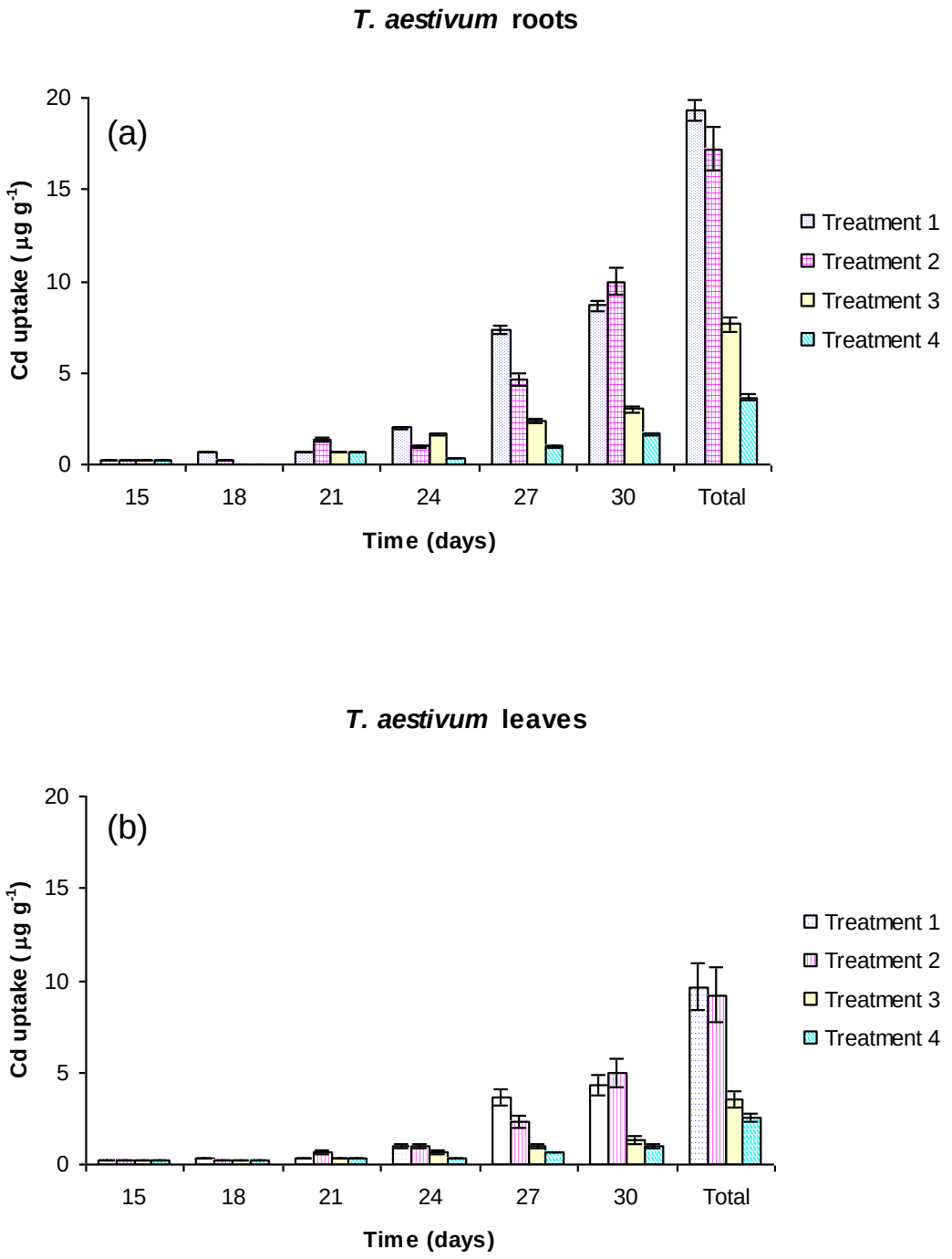



**Figure 6.20** Cadmium uptake by *T. aestivum* in: (a) roots and (b) leaves. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

The distribution of Cd within plant organs is quite variable and clearly illustrates its rapid transport from roots to tops and, in particular, to leaves (Kabata-Pendias and Pendias, 1999). When plants are grown on contaminated soil, however, Cd is very likely to also be concentrated in roots (Kabata-Pendias, 2001). This idea agrees with this finding for *T. aestivum*, but it seems to be that in *E. polystachia*, cadmium was translocated more efficiently way form the roots. So, *E. polystachia* accumulated more cadmium and was able to translocate it, while *T. aestivum* accumulated less cadmium and it largely remained in the roots. Plant species differ in plant uptake behaviour because 1) each species have different requirements, and 2) the amount and species of the available metal is different in the presence of each plant.

When plants take up metals from soil solution they may lower concentration in the immediate vicinity of the root, as with DGT (Davison *et al.*, 2000a, Zhang *et al.*, 2001). This processes occurs if the uptake step (by root/DGT) is fast, so that diffusion of metal ions from the soil solution cannot maintain the local concentration. Metal will then desorb from the solid phase to re-establish equilibrium (Davison *et al.*, 2000a). The kinetics of this re-supply from solid phase to solution has been reflected in both DGT fluxes and plant uptake. This study therefore confirms that DGT, for Cd, is a good surrogate for plant uptake, as has been showed previously (Davison *et al.*, 2000b). In addition, this study has shown that fungal bioremediation increases metal bioavailability to plants.

### 6.3.4.2 Chromium

#### 6.3.4.1 *Echinochloa polystachia*

As shown in Figure 6.21, the chromium uptake by *E. polystachia* is similar in all the treatments ($p<0.005$) and constant from day 15 to day 24. After that, there was a significant difference ($p<0.005$) between Treatment 1 and 2 with Treatments 3 and 4. The highest metal uptake was found in Treatment 1 (1.6 $\mu g\ g^{-1}$), followed by Treatment 2 (1.5 $\mu g\ g^{-1}$), Treatment 3 (0.6 $\mu g\ g^{-1}$) and Treatment 4 (0.5 $\mu g\ g^{-1}$).

During day 15 to 24, Cr uptake was <0.2 $\mu g\ g^{-1}$ for *E. polystachia*. The increased metal fluxes measured by DGT from day 15 to 24 (Figure 6.8), appear not to be available to the plant. Possibly this is a physiological or biochemical defence mechanism of the plant to limit the uptake of toxic Cr (Unterköfler *et al.*, 2001).

Figure 6.22 showed that chromium was found in roots (maximal concentration was 3.0 $\mu g\ g^{-1}$). However, none was detected in leaves (DL=0.01 $\mu g\ g^{-1}$). Similar behaviour has been reported in the literature, with higher Cr content observed in roots than in leaves or shoots, whereas the lowest concentration is in grains (Kabata-Pendias, 2001). Again, this indicates a further mechanism by the plant to localise Cr in the roots, in addition to inhibiting uptake.

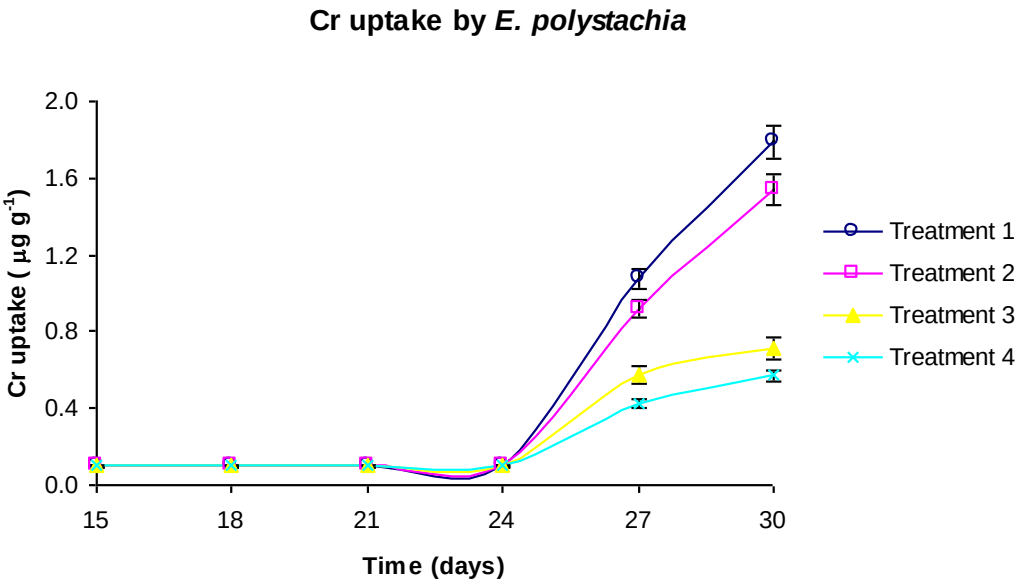



**Figure 6.21** Chromium uptake by *E. polystachia*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

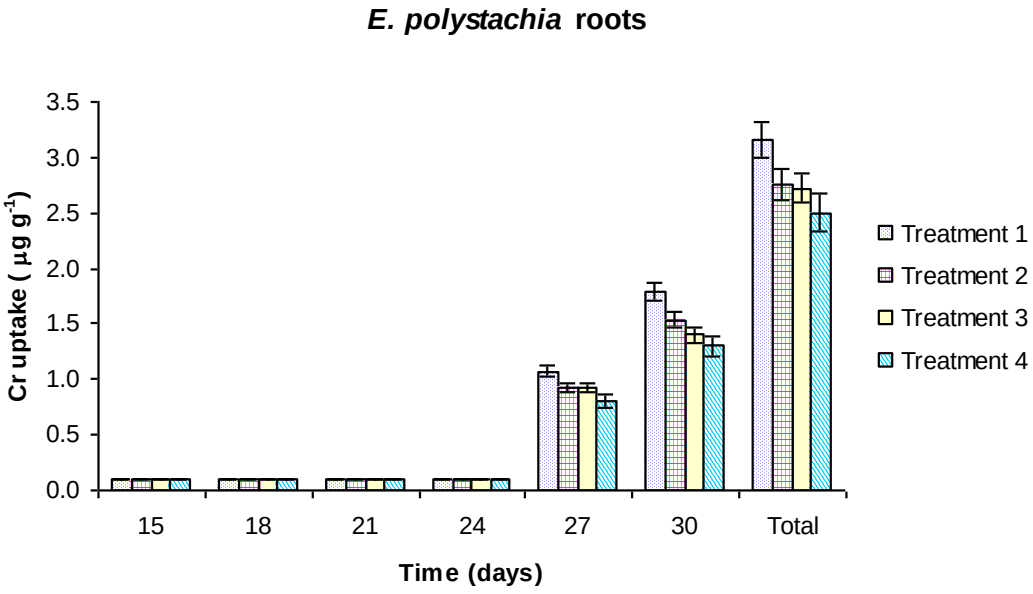



**Figure 6.22** Chromium uptake by *E. polystachia* in roots. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

Although stimulating effects of Cr on plants have been observed in several studies (Kabata-Pendias and Pendias, 1999; Kabata-Pendias, 2001), the phytotoxicity of Cr has been often reported. Initial symptoms of Cr toxicity have been observed with the addition of as little as 0.5 $\mu g\ ml^{-1}$ Cr to nutrient culture (Kabata-Pendias and Pendias, 1999). Very low Cr content in plant (1-2 $\mu g\ g^{-1}$), inhibits the growth of sensitive plant species (Kloke *et al.*, 1984). The toxic levels of Cr reported by Macnicol and Beckett (1985) are much broader and range from 1 to 10 $\mu g\ g^{-1}$ (DW). Pacha *et al.* (1988) also reported harmful effects of Cr (III) compounds on the biochemical activity of soils, including dehydrogenase and other enzymes. As the levels of Cr approach these levels, it is unsurprising that the plant appear able to delay uptake and to prevent translocation.

Taking the data together it can be seen that the removal of phenanthrene results in increased lability and mobility of chromium, which is potentially toxic. The bioremediation process therefore makes these toxic metals more available potentially to the human food chain.

#### 6.3.4.2 *Triticum aestivum*

As with *E. polystachia*, uptake by *Triticum aestivum* (Figure 6.23) showed an initial delay in Cr uptake (day 15-24), followed by a rapid metal uptake (after day 24 to day 30). Chromium uptake was similar in all the treatments ($p<0.05$) and maintained constant from day 18 to day 24 (Figure 6.23). After day 24, there was a significant ($p<0.005$) difference between Treatment 1 and 2 and Treatments 3 and 4.

The highest metal uptake was found in Treatment 1 (0.8 μg $g^{-1}$), followed by Treatment 2 (0.6 μg $g^{-1}$), Treatment 3 (0.4 μg $g^{-1}$) and Treatment 4 (0.3 μg $g^{-1}$). The maximal concentration was found at day 30. Treatment 1 and 2 were not significantly ($p<0.005$) different with Treatments 3 and 4 at day 27, but all treatments were significant ($p<0.05$) different at day 30.

As is shown in Figure 6.24, the total maximal concentration was 1.6 μg $g^{-1}$, and the main uptake was carried out from day 27 to 30. Chromium was found in the roots of *T. aestivum*, but not found in leaves with the detection limit (0.01 μg $g^{-1}$), therefore, a translocation within the plant was not detected. Cadmium concentration found in roots agrees with Zayed *et al.* (1998), who have shown that patterns of accumulation and translocation or the two Cr ions are rather identical.

The results indicate that *T. aestivum* has similar mechanisms for protecting itself against Cr toxicity. In addition, results indicated that unlike Cd, DGT is not a good surrogate for Cr plant uptake from day 15 to day 24, since DGT fluxes and concentrations (Figures 6.3 and 6.8) increased immediately after the addition of fungus in the presence of *E. polystachia* and by similar amounts in the presence of *T. aestivum*.

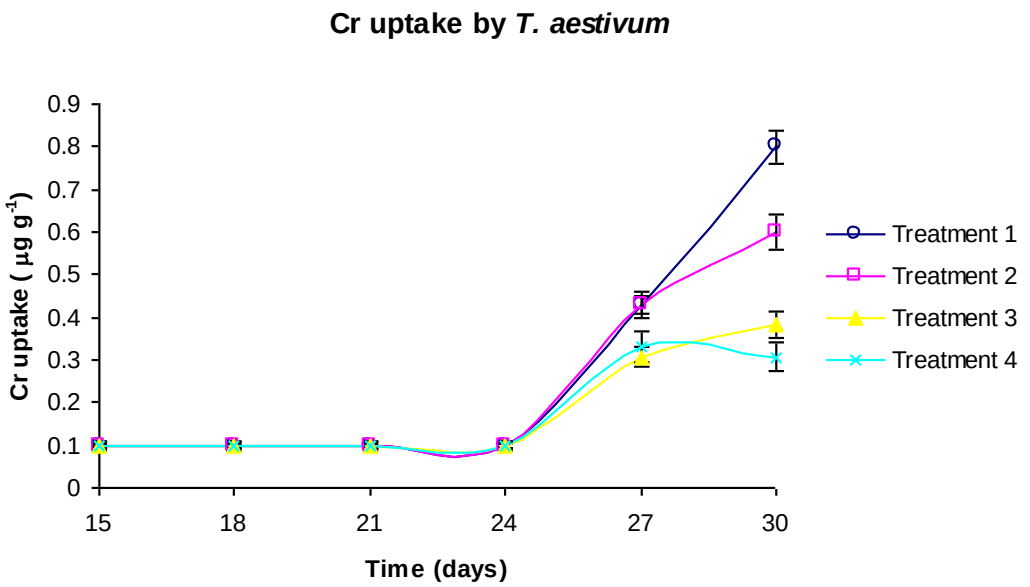



**Figure 6.23** Chromium uptake by *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

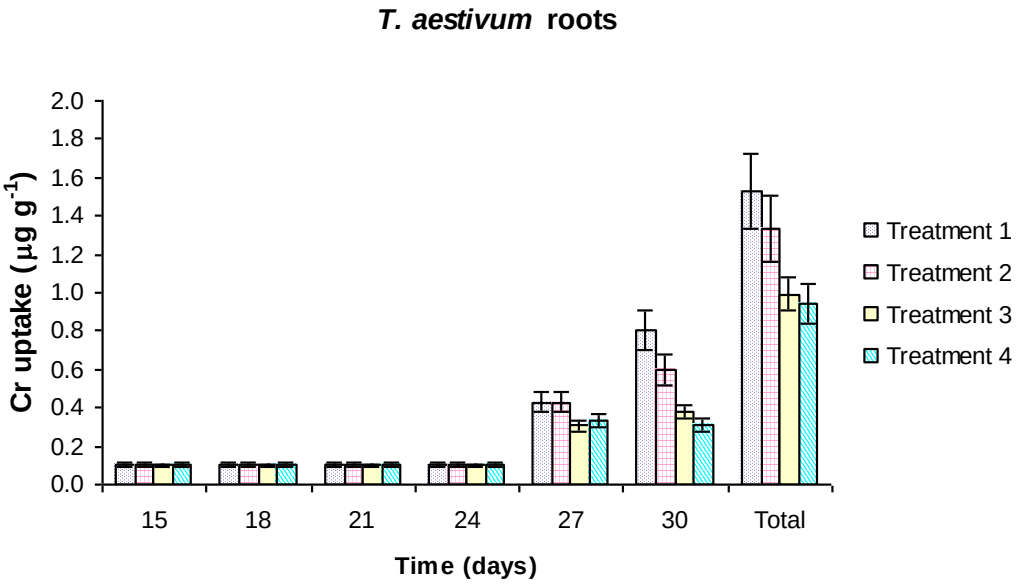



**Figure 6.24** Chromium uptake by *T. aestivum* in roots. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

### 6.3.4.3 Copper

#### 6.3.4.1 *Echinochloa polystachia*

Copper uptake by *E. polystachia* is presented in Figure 6.25. Trends show a continuous increase in metal uptake by *E. polystachia* as a function of time, becoming greatest at day 21-24. The highest metal uptake was found as with the other metals: Treatment 1, followed by Treatment 2, 3 and 4.

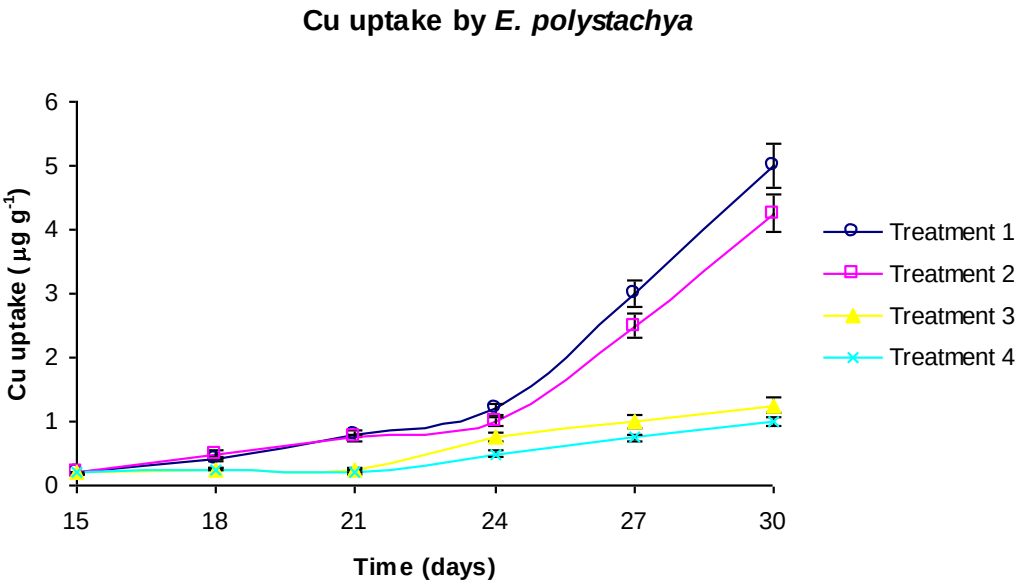



**Figure 6.25** Copper uptake by *E. polystachia*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

Copper uptake by both roots and leaves of *E. polystachia* is shown in Figure 6.26. As Cu is an essential nutrient for plants, metal uptake started immediately (day 15). The maximal copper uptake after 30 days was 7 $\mu g\ g^{-1}$ in roots and 4 $\mu g\ g^{-1}$ in leaves. Total Cu uptake was 10.5 $\mu g\ g^{-1}$ at day 30.

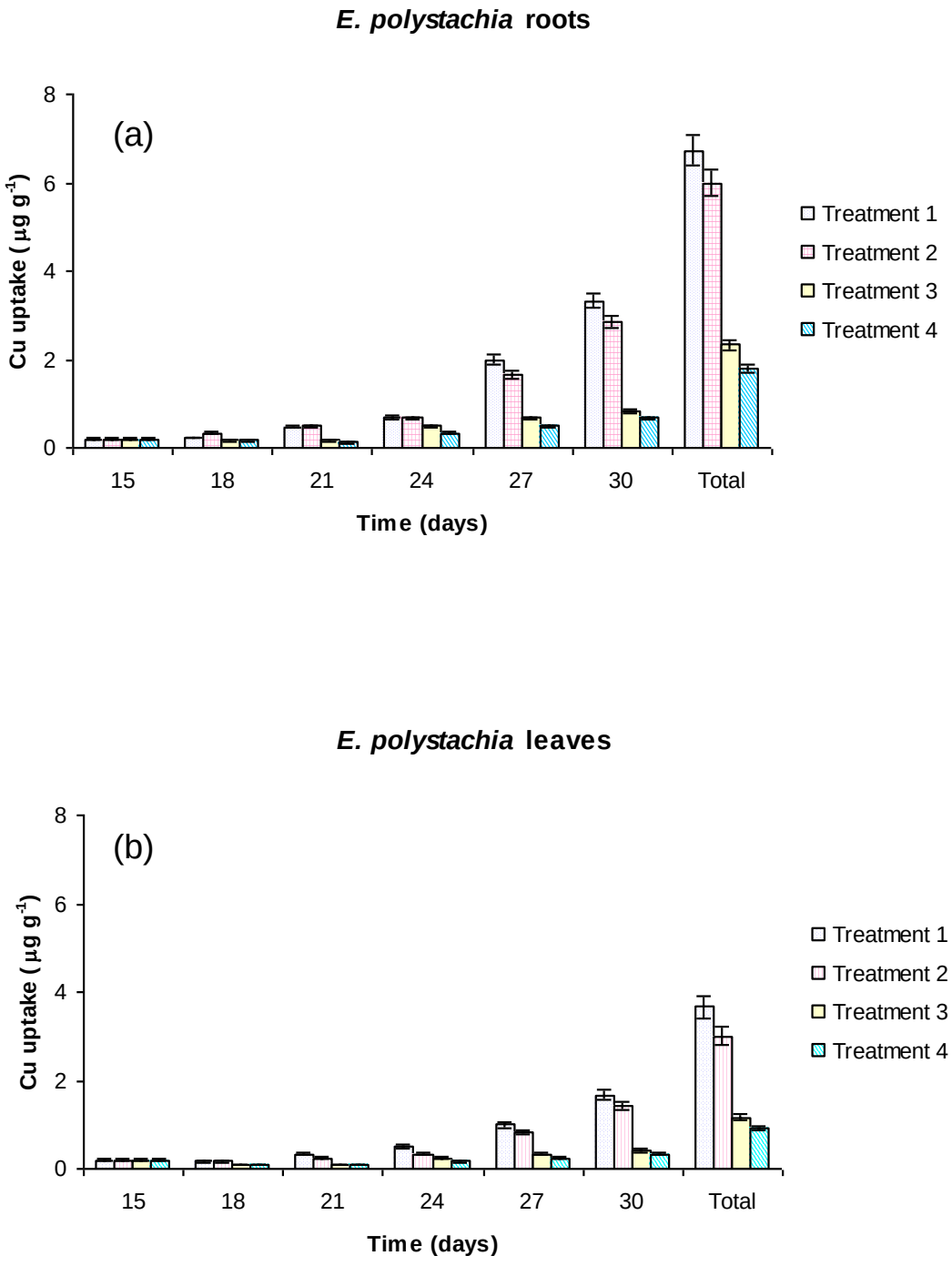



**Figure 6.26** Copper uptake by *E. polystachia* in: (a) roots and (b) leaves. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

Copper uptake by the plant is increased because of microbial activity during phenanthrene bioremoval. The trend explained above, agrees with the results obtained from metal fluxes and concentrations measured by DGT, in addition to the concentration in soil solution measured by filtration (see sections 6.3.1.3, 6.3.2.3 and 6.3.3.3). In fact, it has been previously shown that there is a relationship between the concentration of Cu measured in the growth medium and plants, especially in the toxic range (Grupe and Kuntze, 1988; Athar and Ahmad, 2002).

#### 6.3.4.2 *Triticum aestivum*

Cu uptake by *T. aestivum* (Figure 6.27) shows a continuous increase in metal uptake as a function of time. The highest total copper uptake was 4 $\mu g\ g^{-1}$ at day 30 in Treatment 1. Comparison of Treatments 1 and 2 with 3 and 4 showed that microbial activity increased metal uptake by *T. aestivum*, in line with previous metals and plants.

Figure 6.28 shows the copper taken up by the roots of *T. aestivum* and was not translocated to leaves. Although unexpected, it is known that the rate of Cu uptake by plants varies widely within species (Kabata-Pendias and Pendias, 1999). This difference is exemplified in *E. polystachia* and *T. aestivum* in this study. Moreover, the distribution of Cu within plants is highly variable. Within roots, Cu is associated mainly with cell walls and is largely immobile (Kabata-Pendias, 2001).

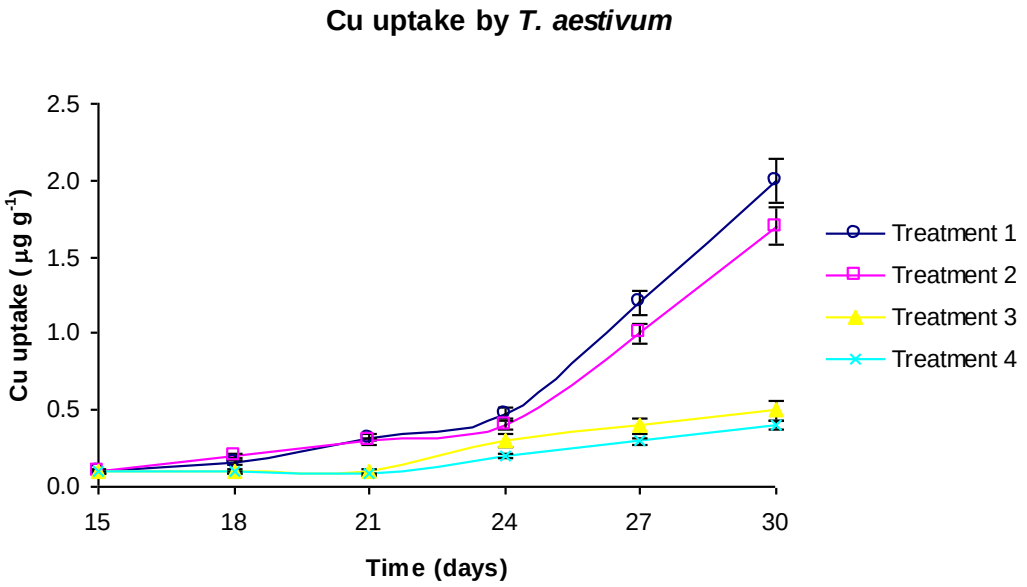



**Figure 6.27** Copper uptake by *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

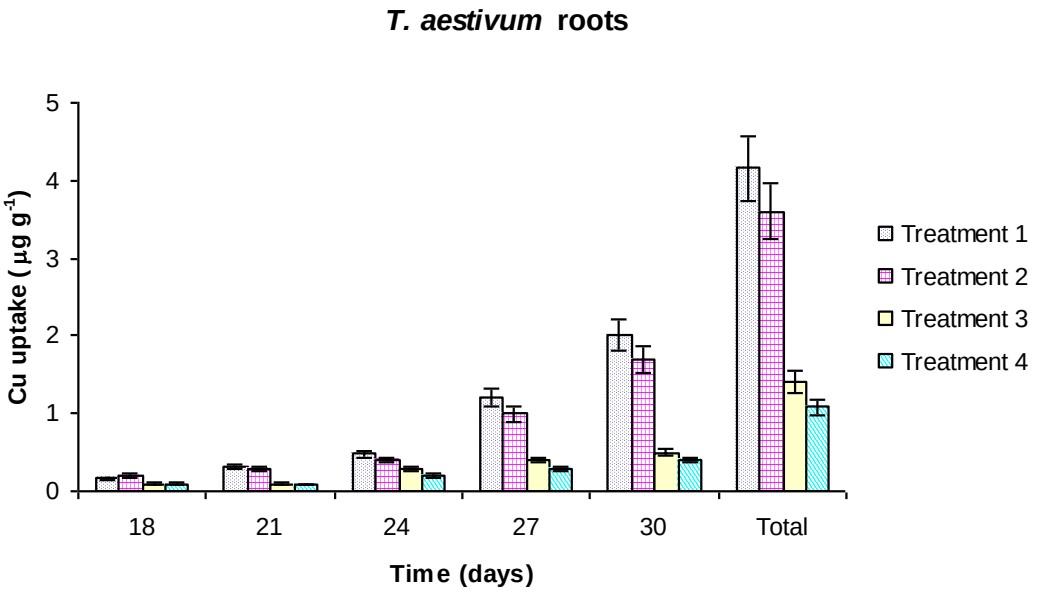



**Figure 6.28** Copper uptake by *T. aestivum* in roots. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

It is clear that, as with other metals, fungal activity increased copper uptake by plants. Riha *et al.* (1993) found that while biological activity was low in soils contaminated with PAHs and heavy metals, some bacteria in soil microflora are copper resistant (Curchill *et al.*, 1995). Likely, this varied resistance impacts metal behaviour, which is reflected on copper uptake.

#### 6.3.4.4 Nickel

##### 6.3.4.1 *Echinochloa polystachia*

Figure 6.29 showed the nickel uptake by *E. polystachia* during phenanthrene bioremoval. There were two main trends that showed an increase in metal uptake as a function of time. The first one was found in Treatments 1 and 2, with the total metal uptake of 6.5 $\mu g\ g^{-1}$ at day 30. The second one corresponds to Treatments 3 and 4, where the maximal metal uptake was 0.5 $\mu g\ g^{-1}$.

There were statistically significant differences ($p<0.05$) between Treatments 1 and 2 with Treatments 3 and 4, and between Treatments 1 and 2, and Treatments 3 and 4 ($p<0.05$). On day 18 (after 3 days of plant transplant), there was a lower nickel uptake in Treatments 3 and 4 than in Treatments 1 and 2, which continued until day 30. It is known that Ni is readily and rapidly take up by plants from soils and the adsorption is positively correlated with the soil Ni concentrations (Pankhurst *et al.*, 1998), which agrees with this study, due to the significant correlation ($p<0.005$, $r^2=0.8967$) between Ni in soil solution and Ni uptake.

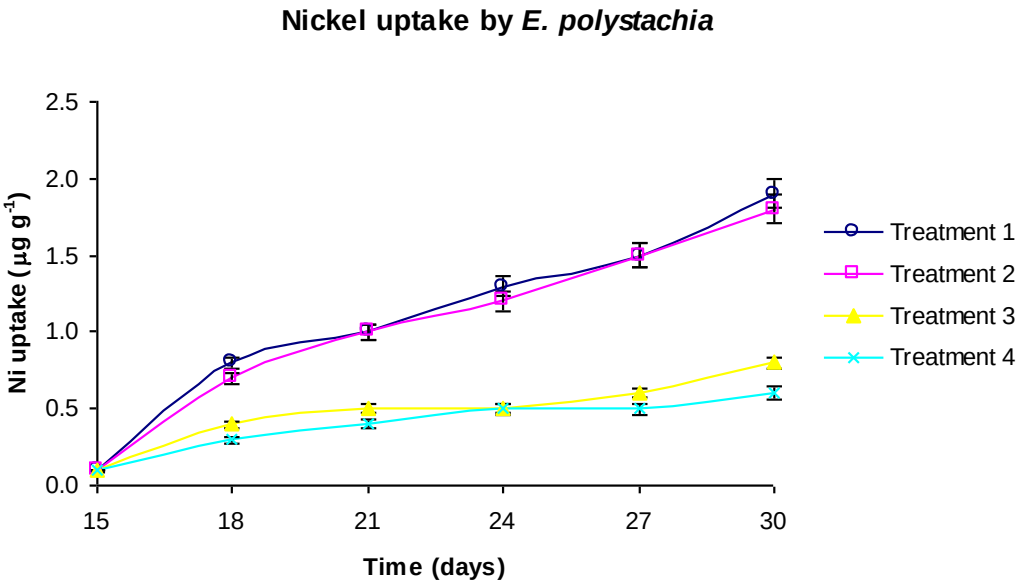
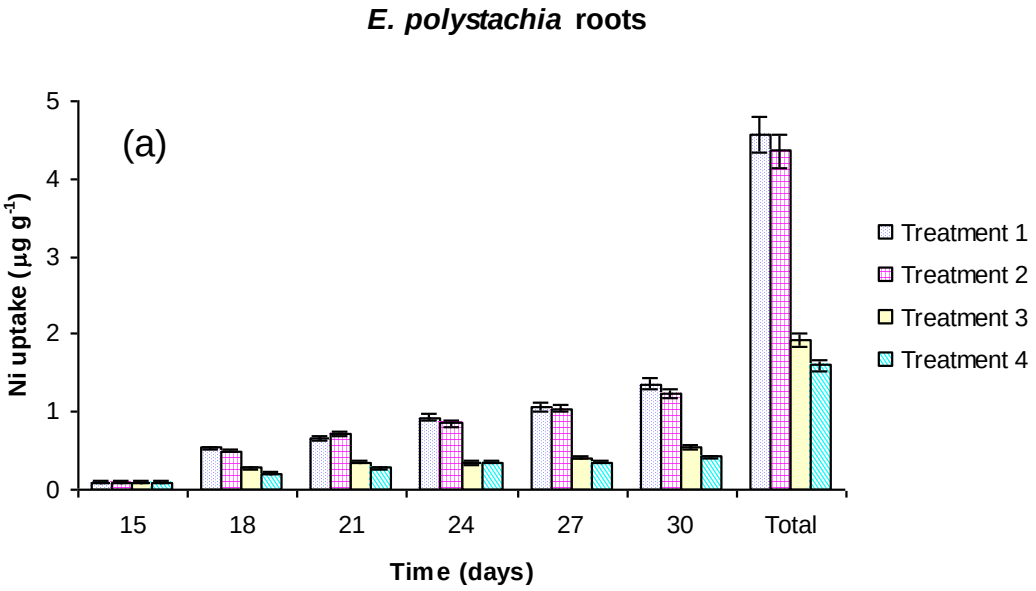



**Figure 6.29** Nickel uptake by *E. polystachia*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

The Ni uptake observed in this study possibly could be explained because of the action of plant rhizosphere, since it is known that the secretion of organic anions, and modification of soil pH by roots and fungi in the rhizosphere, may considerably decrease Ni adsorption by soil, and thus increase its bioavailability (Stauton *et al.*, 1999), as it was found in this study. Further information about nickel uptake is given in Figure 6.30. The maximal total nickel uptake was 5 μg $g^{-1}$ in roots (Figure 6.30a) and 3 μg $g^{-1}$ in leaves (Figure 6.30b), indicating a large degree of translocation.



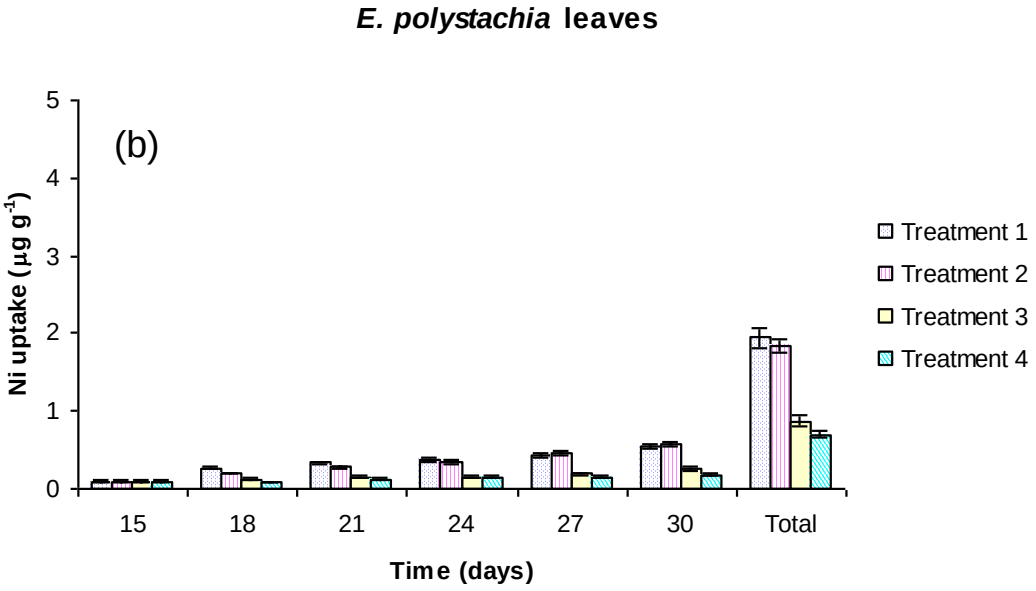




**Figure 6.30** Nickel uptake by *E. polystachia* in: (a) roots and (b) leaves. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

### 6.3.4.2 *Triticum aestivum*

According to the results of Figure 6.31, nickel uptake by *T. aestivum* was higher than by *E. polystachia*. This is the only metal in which *T. aestivum* showed higher metal uptake. Although this result could be unexpected, due to the lower plant biomass on a dry basis of *T. aestivum* comparing with *E. polystachia*, the explanation is based on the type of plant: *T. aestivum* is very resistant to Ni (Kabata-Pendias, 2001). In section 6.3.1.4, it was shown that DGT fluxes were higher in the presence of *T. aestivum* compared with *E. polystachia*. This suggests that Ni uptake by *T. aestivum* will be higher.

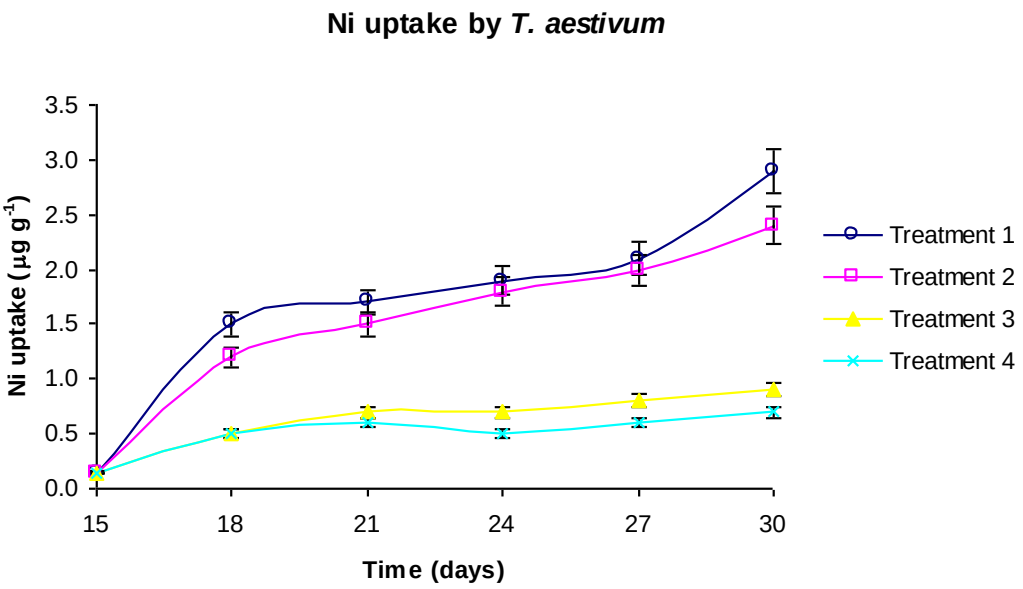



**Figure 6.31** Nickel uptake by *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

As was previously observed in DGT concentration (sections 6.6.3), increased microbial activity mobilises more potentially bioavailable nickel species. Then plants take up Ni and can translocate to the leaves in *T. aestivum* (Figure 6.32).

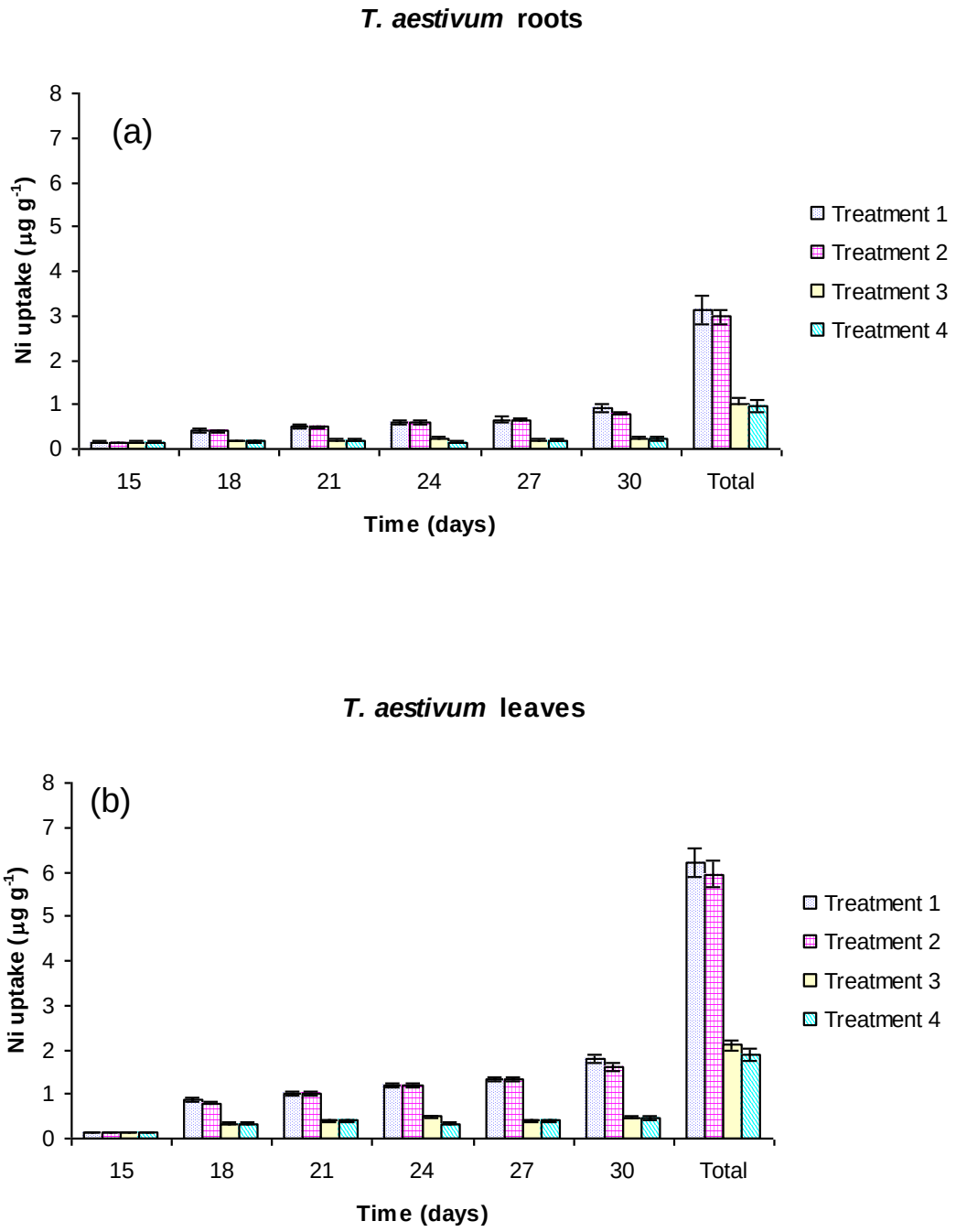



**Figure 6.32** Nickel uptake by *T. aestivum* in: (a) roots and (b) leaves. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

### 6.3.4.5 Lead

#### 6.3.4.1 *Echinochloa polystachia*

Unlike Ni, but similar to Cr, trend the lead uptake by *E. polystachia* (Figure 6.33) is low and similar in all the treatments ($p<0.005$) and constant from day 15 to day 24. After that, there was a significant difference ($p<0.005$) between Treatment 1 and 2 with Treatments 3 and 4. The highest metal uptake was found in Treatment 1(2.8 $\mu g\ g^{-1}$), followed by Treatments 2 (2.3 $\mu g\ g^{-1}$), 3 (0.7$\mu g\ g^{-1}$) and 4 (0.6 $\mu g\ g^{-1}$). The comparison between Treatments 1 and 2 with Treatments 3 and 4 showed that lead uptake by *E. polystachia* increases significantly ($p<0.005$) due to the microbial activity.

Although Pb occurs naturally in all plants, it has not been shown to play any essential roles in their metabolism. Kabata-Pendias and Pendias (1999) concluded that if Pb is necessary for plants its concentration at the level of 2 to 6 ppb should be sufficient. This is one of the reasons why Pb has recently received much attention as a major chemical pollutant of the environment and as an element toxic to plants.

Although even a very low Pb concentration may inhibit some vital plant processes, Pb poisoning has seldom been observed in plants growing under field conditions. Kabata-Pendias (2002) discussed that problem and suggested two possible explanations: 1) the relatively low Pb concentration in soil and 2) a low Pb availability. Since lead bioavailable concentrations are increased because of microbial activity to remove phenanthrene, there is a

major risk from lead if any bioremediation strategies do not consider the change in metal behaviour observed in this study. However it appears that the plants have certain resistance mechanisms as with Cr. The plant can delay uptake and prevent translocation.

Lead was found in *E. polystachia* roots and the total uptake was 6 $\mu g\ g^{-1}$ at day 30, but was not detected in leaves (Figure 6.34). When Pb is present in soluble forms in nutrient solutions, plant roots are able to take up great amounts of this metal, the rate increasing with increasing concentration in the solutions and with time. The translocation of Pb from roots to tops is greatly limited, and as Kabata-Pendias (2001) described, only 3% of the Pb in the root is translocated to the shoot.

The degree to which soil Pb is available to plants is of great environmental concern. Zimdahl and Koeppe (1987) showed that despite several statements in the literature on the slight effect of soil Pb on concentrations of Pb in plant tissues, plants are able to take up Pb from soils to a limited extent. Apparently, most of the Pb in soil is unavailable to plant roots. However, in this study it has been demonstrated that microbial activity increased lead uptake by plant roots.

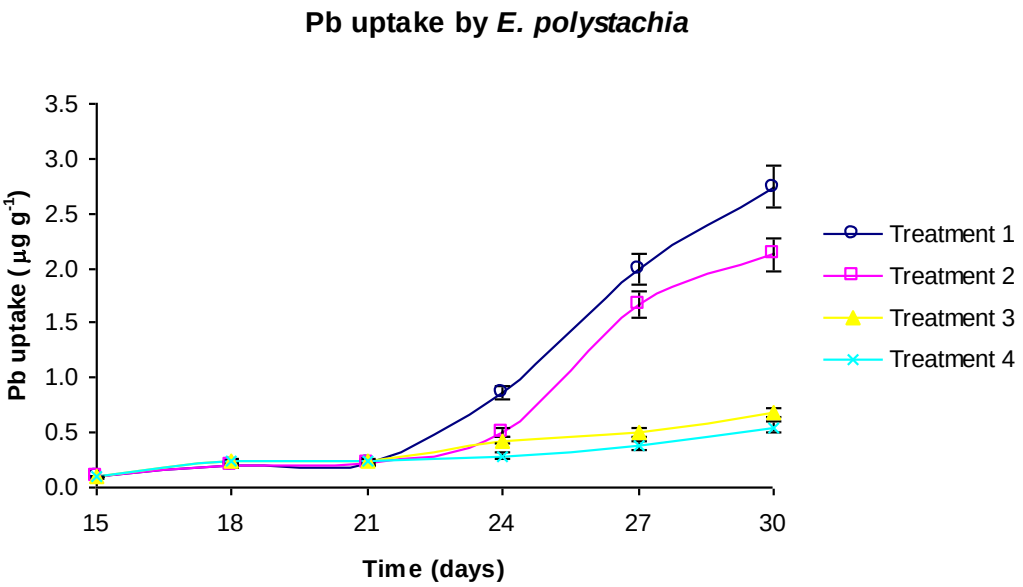



**Figure 6.33** Lead uptake by *E. polystachia*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

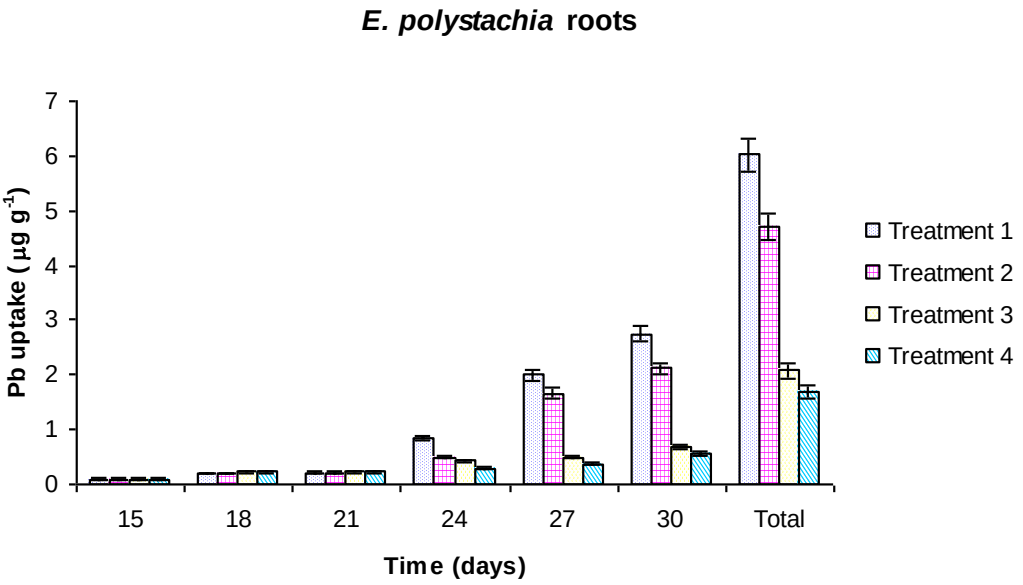



**Figure 6.34** Lead uptake by *E. polystachia* in roots. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

### 6.3.4.2 *Triticum aestivum*

Figure 6.35 showed lead uptake by *T. aestivum* as a function of time. As with *E. polystachia*, lead uptake is similar in all the treatments ($p<0.005$) and constant from day 18 to day 24. There were statistically significant ($p<0.005$) differences between all treatments at day 30. Although metal uptake was found in all treatments, the uptake is higher by at least 3 fold in Treatments 1 (0.7 $\mu g\ g^{-1}$) and 2(0.57 $\mu g\ g^{-1}$) compared to Treatment 3 (0.17 $\mu g\ g^{-1}$) and 4 (0.12 $\mu g\ g^{-1}$).

Lead was found in the roots of *T. aestivum* (Figure 6.36), with 1.5 $\mu g\ g^{-1}$ maximal lead uptake at day 30. Lead was not detected in leaves, as with *E. polystachia*. This partially disagrees with the report that only a small fraction (0.2-1%) of lead in lead contaminated soil appears to be in water-soluble form (Ma *et al.*, 1983), due to Pb was found in plant roots. However, bioremediation appears to make Pb soluble and therefore available for root uptake. However, plant mechanisms prevented translocation, as with Cr.

Comparison between treatments shows that the presence of *Penicillium frequentans* increased the uptake of lead. Changing soluble lead concentrations *in situ* alter the ecology of the soil (Roane and Kellogg, 1996), causing potential problems in soil microorganisms to survive.

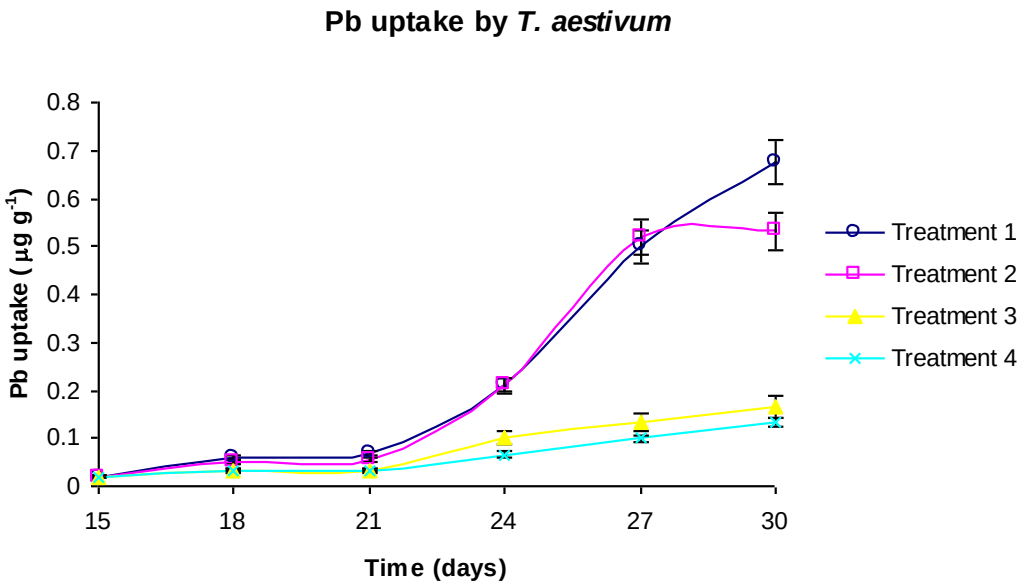



**Figure 6.35** Lead uptake by *T. aestivum*. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

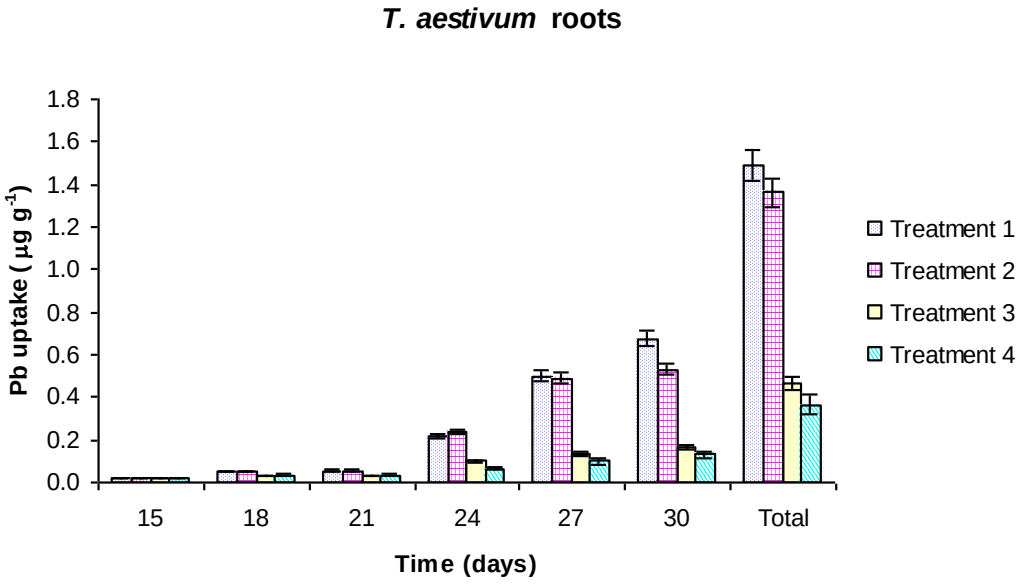



**Figure 6.36** Lead uptake by *T. aestivum* in roots. Treatment 1= Soil + Fungi + Phenanthrene + Plant, Treatment 2= Soil + Fungi + Plant, Treatment 3= Soil + Phenanthrene + Plant and Treatment 4= Soil + Plant. Treatment 5 is omitted because it only contained soil. T= 28°C, C:N= 40.

The results of Cr and Pb in plant uptake by *E. polystachia* and *T. aestivum* showed similar mechanisms for protecting itself against Cr and Pb toxicity, delaying metal uptake. DGT is not a good surrogate for Cr (from day 15 to day 24) and Pb (from day 15 to day 21) biouptake. This is because a) DGT fluxes (Figures 6.3 and 6.6) and concentrations (Figures 6.8 and 6.11) increased immediately after the addition of fungus in the presence of plants, and b) metal uptake by both plants was delayed until day 21 and 24.

## 6.4 Differences in non-sterilised soil with or without fungi and phenanthrene in the presence/absence of plant

Although all data show essentially the same trends in the presence of either type of plant, comparison of metal behaviour under different treatments (*i.e.* with or without phenanthrene and fungi) reveals differences in magnitude of metal fluxes and concentrations in soil solution. Moreover, comparison between non-sterilised soils in the presence or absence of plants reveals differences on metal impact.

### 6.4.1 Comparison of the presence of fungi (Treatments 1 and 2) or absence of fungi (Treatments 3, 4 and 5)

Comparison between Treatments 1 and 2 (in presence of fungi) with Treatment 3, 4 and 5 (in absence of fungi) showed that the presence of fungi increases the metal solid to solution flux, increases the concentration of metal in soil solution and increases plant uptake of metal. This hold for all

metals, although in some cases metal uptake was delayed. Since it was previously mentioned that microbial activity either by fungus or mixed fungus and native microflora resulted in an increased pool of potentially bioavailable and toxic metal species, these results confirm the behaviour of metals as a side effect of the bioremediation process by bioaugmentation.

It was shown that while the fungal bioremediation reduces the concentration of phenanthrene, it also changes the chemistry and bioavailability of toxic trace metals. These changes must be kept in mind when assessing the best option for the remediation of contaminated land.

### 6.4.2 Comparison of phenanthrene presence/absence in the presence of plant and fungi (Treatments 1 and 2)

Higher metal fluxes and concentrations in non-sterilised soil were found with the addition of phenanthrene in the presence of both fungi and plant than in the absence of phenanthrene. This could be attributed to the fact that metal mobilisation is due to the microbial activity from both fungi and presumably microorganisms associated to the plant rhizosphere to utilise phenanthrene as a carbon and energy source, which is consistent with the results of phenanthrene bioremoval (section 6.2).

When soil is not contaminated with phenanthrene (Treatments 2), the presence of fungi by itself mobilise more metal than the presence of the plant. However, only 8-10% of phenanthrene removal is higher in the

presence of fungi than in its absence. This result confirms phytoremediation and not bioaugmentation using *P. frequentans* will be a suitable technique to obtain a considerable phenanthrene removal, while minimising the increase in pool of potentially bioavailable and toxic metal species.

### 6.4.3 Comparison of phenanthrene presence/absence in the presence of plant and absence of fungi (Treatments 3 and 4) in non-sterilised soil (Treatment 5)

Higher metal mobilisation and concentrations were found with the addition of phenanthrene in the presence of plant than in the lack of phenanthrene (Treatments 3 and 4). Because all treatments do not contain fungi, it is clear that the presence of plants also impacted on metal behaviour, but to a much lower degree than fungi. Both plant and presumably plant rhizosphere act on phenanthrene removal and also mobilise metal.

Comparison between Treatment 3 and 4 with non-sterilised soil (Treatment 5) showed that metal lability and speciation change during the phytoremediation process. The removal of phenanthrene by native soil microflora and plants resulted in an increased pool of potentially bioavailable and toxic metal species. However, this impact is significantly lower than in the presence of fungi.

## 6.5 Bioremediation and metal behaviour considerations

The main purpose of the bioremediation process is to enhance phenanthrene removal. Comparison between Treatments 1 and 3 showed that the presence of *P. frequentans* with either plant increased the phenanthrene removal (10% in presence of *E. polystachia* and 8% in presence of *T. aestivum*). This suggested a slight synergism between *P. frequentans* and microorganisms associated with the plant rhizosphere. However, comparison between all the experimental conditions tested showed that the highest total phenanthrene removal (75-77%) was in Treatment 1 (S + F + P + Pl), followed by Treatment A (S + F + P) (73%) and Treatment 3 (S + P + Pl) (67%). Therefore, plants alone remove a substantial fraction of phenanthrene, although this fraction is lower than when the soil is treated with fungus.

Although phenanthrene bioremoval is enhanced using *P. frequentans* (Chapter 4) or mixed fungus and native microflora (Chapter 5), microbial activity resulted in a significantly increased pool of potentially bioavailable and toxic metal species.

Although a relatively short timespan is acknowledged, the impact of microbial activity on metal behaviour is evident. In order to consider what might occur over longer timespans further bioremediation process that takes longer will be helpful.

Comparison of all treatments showed that *P. frequentans* inoculum has the highest impact on metal mobilisation with plants having a much-reduced impact on metal mobilisation.

For the above reasons, phytoremediation seems to be the best option for both phenanthrene removal and to prevent the biouptake of toxic metals. Phytoremediation of PAHs is an emerging technology that employs the use of plants for the cleanup of soil (Rugh *et al.*, 2000). Phytoremediation is considered to be a more ecologically compatible alternative to standard engineering-based approaches for site restoration. Naturally occurring plants have been shown to be effective for treatment of metals (Lasat, 2002). Plants can provide large amount of biomass as a sink for the accumulation of metals and appropriate metabolic activity for the degradation of organic contaminants such as phenanthrene (Rugh *et al.*, 2000). Furthermore, plants can work synergistically with native soil microflora as shown in this study. However, progress in the field of phytoremediation is precluded by limited knowledge of plant remedial mechanisms and the poorly understood of phytoremediation effects on agricultural practices (Lasat, 2002). Further work is needed to establish the range of conditions within which phytoremediation is a good technique to apply for PAHs bioremoval as well as minimise its impact on metal behaviour.

## 6.6 Conclusions

The highest total phenanthrene removal (75-77%) was in Treatment 1 (S + F+ P + Pl), followed by Treatment A (S + F + P) (73%) and Treatment 3 (S + P + Pl) (67%). Presumably, phenanthrene removal is enhanced with plants due to the presence of major number of microorganisms associated to the plant rhizosphere.

Microbial activity increased metal fluxes and concentration in pore waters. Treatments 1 and 2 (in the presence of fungi) showed the highest values in both metal fluxes and DGT concentrations. Treatments 3 and 4 showed the influence of plants, which was lower. Results in Treatments 5 were comparable with the previous experiments with non-sterilised soil (Chapter 5).

The DGT technique is confirmed as a good surrogate for metal biouptake, in most cases. Metal uptake by plants corresponds to the metal behaviour using DGT, except in the case of Cr and Pb from day 15 to 24. In these two cases, plants appeared able to delay metal uptake, and this behaviour was not mimicked by DGT. Trends in fluxes are the same in DGT concentration.

Soil solution concentration measured by filtration in Cd and Cr showed a bimodal distribution, which was associated to the pathway of breaking down the solid soil to smaller, ‘solution-phase’ material leading to a peak in soil solution concentration. However, the Cu and Ni behaviour, without peaks,

and Pb, which showed both unimodal and bimodal distributions, question this idea. Further work is required to explain the metal behaviour in soil solution by filtration, including a higher sampling frequency. However, the behaviour of Cu, Ni and Pb may be explained by microbial activity and/or physicochemical processes (*e.g.* binding and desorption) in soil.

It is clear that fungal activity increased metal uptake by plants. Higher metal uptake was detected in *E. polystachia* compared with *T. aestivum*, except in the case of nickel. Moreover, metal translocation from root to leaves was detected in Cd, Cu and Ni. Overall, the results suggest that phytoremediation and not bioaugmentation using *P. frequentans* will be a suitable alternative to obtain a considerable (67%) phenanthrene removal, while minimising the increase in pool of potentially bioavailable and toxic metal species.

# CHAPTER 7

# CONCLUSIONS AND FURTHER WORK

The work presented in the previous chapters has discussed the effect of microbial activity on metal behaviour. Particular attention was paid to the increased pool of potentially bioavailable and toxic metal species and greater plant uptake, caused by bioremediation.

Greatest phenanthrene removal was found in the presence of both *Penicillium frequentans* and native microflora, which appears to act synergistically. It was found that the removal of phenanthrene either by fungus or mixed fungus and native microflora affected metal behaviour. In particular, bioremediation increased solid-soil fluxes and concentrations of labile metal complexes. These results highlight the behaviour of metals as a side effect of the bioremediation process.

Phenanthrene removal was enhanced with the use of plants. The highest total phenanthrene removal (75-77%) was in Treatment 1 (S + F + P + Pl), followed by Treatment A (S + F+ P) (73%) and Treatment 3 (S + P + Pl) (67%). Presumably, phenanthrene removal is enhanced with plants due to the presence of microorganisms associated to the plant rhizosphere.

It is clear that fungal activity increased metal uptake by plants. Higher metal uptake was detected in *E. polystachia* compared with *T. aestivum*, except in the case of nickel. Moreover, metal translocation from root to leaves was

detected in Cd, Cu and Ni. Overall, the results suggest that phytoremediation and not bioaugmentation using *P. frequentans* is a better alternative to obtain a considerable phenanthrene removal, while minimising the increase in pool of potentially bioavailable and toxic metal species.

The DGT technique is confirmed as a good surrogate for metal biouptake in general. Metal uptake by plants corresponds to the metal behaviour using DGT, except in the case of Cr (from day 15 to 24) and Pb (from day 15 to 21), where biological mechanisms came into play. Trends in fluxes are the same in DGT concentration.

Higher metal uptake was detected in *E. polystachia* than *T. aestivum*, except in the case of nickel, possibly due to the capacity of *T. aestivum* to be more resistant to nickel than *E. polystachia*. Moreover, metal translocation from root to leaves was detected in some cases (Cd, Cu and Ni).

The objectives of this work (section 1.2) have largely been met. The behaviour of trace metal (cadmium, copper, chromium, nickel and lead) during the phenanthrene bioremoval in soil was described fully. The hypothesis of this thesis is accepted and has been proven. However, further work is needed in various areas related to this research. Further research areas included the following:

1) The study of other types of soils and PAHs, using other bioremediation techniques is needed to optimise culture conditions as well as PAHs

removal kinetics. Describe metal behaviour in soil during the bioremoval process, considering different type of plants.

2) There remains no single method for the determination of metal behaviour, which is without limitations *e.g.* the production of artefacts. A deeper knowledge of the DGT technique in order to ensure a more comprehensive measurement is needed. The precise mechanism for the increase in solid-solution flux is still not well understood.

3) Further work is needed to explain the metal behaviour in soil solution by filtration. The behaviour of Cu, Ni and Pb may be explained by microbial activity and/or physicochemical processes (*e.g.* binding and desorption) in soil.

4) Further work is needed to establish the range of conditions within which phytoremediation is a good alternative to apply for PAH bioremoval, while minimising the impact on potentially bioavailable and toxic metal species.

## REFERENCES


Abrams, M. & Jarrell, W. (1992). Bioavailability Index for P using Ion Exchange resin impregnated Membranes, *Soil Sci. Soc. Am. J*, **56**, 1532-1537.

Alexander, M. (1982). Most Probable Number Method for Microbial Populations. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 815-816.

Alexander, M. (1994). *Biodegradation and Bioremediation*. San Diego, California: Academic Press. 302 pp.

Al-Hadhrami, M. N., Lappin-Scot, H. M. & Fisher, P. J. (1996). Effects of the addition of organic carbon sources on bacterial respiration and *n*-alkane biodegradation of Omani crude oil. *Mar. Pollut. Bull.*, **32**, 4, 351-357.

Alloway, B. J. & Jackson, A. P. (1991). The behaviour of heavy metals in sewage-sludge amended soils, *Sci. Tot. Env.*, **100**, 151-176.

Almås, Å., Singh, B. R. & Salbu, B. (1999). Mobility of $^{109}$Cd and $^{65}$Zn in soil influence by equilibration time, temperature and organic matter, *J Environ. Qual.*, **28**, 1742.

Amady, A., Abbey, S. D. & Nima, A. (1996). Chronic effects of oil spills on soil properties and microflora of a rainforest ecosystem in Nigeria. *Water Air Soil Pollut*., **86**, 1-4, 1-11.

Andersen, S. & Engelstad, F. (1993). Application of factorial designs: estimating the pollution potential inferred from changes in soil water chemistry, *Soil Environ.*, **1**, 227-230.

Anderson, T. (1997). Development of a phytoremediation handbook: Consideration for enhancing microbial degradation in the rizhosphere [WWW document] http://es.epa.gov/ncerqa/ru/index.html

Andersson, B. E. & Henrysson, T. (1996). Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi, *Appl. Microbiol. Biotechnol.,* , **46**, 647-652.

AOAC (1970). *Official Methods of Analysis*, Washington, DC: Association of Official Analytical Chemists.

APHA, AWWA & WPCF (1995). *Standard Methods for the examination of Water and Wastewater***,** Washington, DC: American Public Health Association.

Apte, S. & Batley, G. E. (1995). Trace Metal Speciation of Labile Chemical Species in natural waters and sediments: Non-Electrochemical Approaches. In *Metal Speciation and Bioavailability in Aquatic Systems,* (A. Tessier & D. Turner, Eds.), England: John Wiley and Sons Ltd., 259-306.

ARPEL (2002). The gateway to oil and gas world in the Latin America region. Annex 1, p. 107.

Arora, H. S., Cantor, R. R. & Nemeth, J. C. (1982). Land treatment: available and successful method of treating petroleum industry wastes, *Environ. Int.*, **7**, 285-291.

Athar, R. & Ahmad, M. (2002). Heavy metal toxicity: Effect on plant growth and metal uptake by *T. aestivum*, and on free living *Azotobacter*, *Water Air Soil Pollut.*, **138**, 1-4, 165-180.

Atlas, R. M. & Bartha, R. (1981). *Microbial Ecology, Fundamentals and Applications*. Reading, MA: Addison-Wesley, 560 pp.

Atlas, R. M. & Bartha, R. (1993). *Microbial Ecology: Fundamentals and Applications*, Redwood City, CA: Benajmin/Cummings.

Atlas, R. M. & Cerniglia, C. E. (1995). Bioremediation of petroleum pollutants, *Bioscience*, **45**, 5, 332-338.

Barber, S. A. (1984). *Soil nutrient bioavailability*. NY: John Wiley & Sons, 22.

Bartlett, R. J. (1999). Characterising soil redox behaviour. In *Soil Physical Chemistry*, (D. L. Sparks, Ed.), Boca Raton, FL: CRC Press, 371.

Battelle (1998). *Environmental updates. Finding the Facts in Southern Mexico*. Battelle Memorial Institution.

Beaty, R. & Kerber, J. (1993). Introduction to Graphite Furnace Atomic Absorption. In *Concepts, Instrumentation and Techniques in Atomic Absorption Spectrophotometry*, USA: The Perkin Elmer Corporation.

Beeby, A. & Richmond, L. (2003). Do the soft tissues of *Helix aspersa* serve as a quantitative sentinel of predicted free lead concentrations in soils?, *App. Soil Ecol.*, **22**, 2, 159-165.

Bezale, l. L., Hadar, Y., Fu, P. P., Freeman, J. P. & Cerniglia, C. E. (1996). Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*, *Appl. Environ. Microbiol.,* **62**, 7, 2547-2553.

Bodek, I., Lyman, W., Reehl, W. F. & Rosenblatt, D. H. (Eds.) (1988). *Environmental Inorganic Chemistry*, NY: Pergamon Press.

Boekhold, A. E., Temminghoff, E. J. M. & Van der Zee, E. A. T. (1993). Influence of electrolyte composition and pH on cadmium sorption by an acid sandy soil, *Soil Sci.*, **44**, 85.

Bossert, I. D. & Bartha, R. (1986). Structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil, *Bull. Environ. Contam. Toxicol.*, **37**, 490-495.

Bouyoucos, G. J. (1962). Hydrometer improved method for making particles size analysis of soil. Agron. J, 54, 464-465.

Bremner, J. M. & Mulvaney, C. S. (1982). Nitrogen-Total. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 595-616.

Brüggeman, J., Dörfner, H. H., Hecht, H., Kumpulainen, J. T. & Westermair, T. (1996). Status of trace elements in staple foods from Germany 1990-1994. In *Trace Elements, Natural Antioxidants and Contaminants in European Foods and Diets*, (J. T. Kumpulainen, Ed.), FAO/REU Techn. Ser., **49**, 5.

Bryan, C. W. & Langston, W. J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: A review, *Environ. Pollut.*, **76**, 89.

Buffle, J., Perret, D. & Newman, M. (1992). The use of filtration and ultrafiltration for size fractionation of aquatic particles, colloids and macromolecules. In *Environmental Particles*, (J. Buffle & H. P. van Leeuwen, Eds.), Florida: Lewis Publishers.

Bumpus, J. A. (1993). Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium, Appl. Environ. Microbiol*., **55**, 154-158.

Campbell, P. G. C. (1995) Interactions between Trace Metals and Aquatic Organisms: A Critique of the Free-ion Activity Model. In *Metal Speciation and Bioavailability in Aquatic Systems*, (A. Tessier & D. Turner, Eds.), England: John Wiley and Sons Ltd., 45-102.

Cerniglia, C. E. & Heitkamp, M. A. (1989). Microbial degradation of polycyclic aromatic hydrocarbons in the aquatic environment. In *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment, (*U. Varanasi, Ed.), Florida: CRC Press, 41-68.

Cerniglia, C. E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons, *Adv. Appl. Microbiol*., **30**, 31-71.

Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons, *Biodegradation*, **3**, 351-368.

Chang, L., Davison, B., Zhang, H. & Kelly, M. (1998). Performance characteristics for the measurement of Cs and Sr by diffusive gradients in thin-films (DGT), *Anal. Chim. Acta*, **368**, 243.

Chávez-Gomez B., Quintero-Ramirez, R. Esparza-Garcia, F. Mesta-Howard, A. Zavala-Díaz de la Serna, A, Hernández-Rodríguez, C., Guillén, T., Poggi-Varaldo, H. M., Barrera-Cortés, J. & Rodríguez–Vázquez, R. (2003). Removal of phenanthrene from soil by co-cultures of bacteria and fungi pregrown on sugarcane bagasse pith. Bioresource Tech., 89, 177-183.

Chen, Y. W. (1993). Physicochemical Characterization of Colloids in River Waters and Studies of Experimental Conditions. Ph. D. Thesis. University of Geneva. p. 29.

Christensen, T. H. & Tjell, J. Ch. (1991). Sustainable management of heavy metals in agriculture, example: cadmium. In *Heavy metals in the environment* (S. E. Lindberg & T. C. Hutchinson, Eds.), Edinburgh, UK: CEP Consl.,1, 40.

Churchill, S. A., Walters, J. V. & Curchill, P. F. (1995). Sorption of heavy metals by prepared bacterial cell surfaces, *J Environ. Eng.*, **121**, 706-711.

CNA (1989). Manual de clasificación cartográfica e interpretación de suelos con base en el sistema de clasificación de suelos, Mexico: CNA.

Commission Decision (2001). The European eco-label for soil improvers and growing media. Commission Decision 2001/689/EC [WWW document] http://europa.eu.int/comm/environment/ecolabel/pdf/fiches/fact_soil_2002.pdf

CONCAWE (1980). Report No. 3/80. The Hague, Netherlands.

Cortés-Espinosa, D., Fernández-Perrino, F. Loera, O., Arana-Cuenca, A. & Rodríguez-Vázquez, R. (2003). Removal of phenanthrene in solid culture by non-ligninolytic fungi isolated from sugarcane bagasse. *Lett. Appl. Microbiol.* (submitted).

Dane, J. H., & Topp, G. C. (Eds.) (2002). Methods of Soil Analysis, Part 4, Physical Methods, Madison, WI: Soil Science Society of America, Soil Science Society of America Book Series Number 5, p. 1692.

Davies, B. E., Lear, J. M. & Lewis, N. J. (1987). Plant availability of heavy metals in soils. In *Pollutant transport and fate in ecosystems*, (P. J. Coughtry, M. H. Martin & M. H. Unsworth, Eds.), England: Blackwell Scientific Publishers, 267-275.

Davison, W., Grime, G. W., Morgan, J. A. & Clarke, K. (1991). Trace metals in natural waters using DET, *Nature*, **352**, 323-325.

Davison, W. & Zhang, H. (1994). *In situ* speciation measurements of trace components in natural waters using thin-films gels, *Nature*, **367**, 546-548.

Davison, W., Zhang, H. & Grime, G. W. (1994). Performance characteristics of gel probe used for measuring pore waters, *Environ. Sci. Technol.*, **28**, 1623.

Davison, W. & Hutchinson, W. (1997). An assessment of the feasibility of using DGT procedures to measure trace metals and radionuclides in rivers, Environment Agency, R & D Technical Report, 92.

Davison, W. Fones, G. Harper, M. Teasdale, P. & Zhang H. (2000a). Dialysis, DET and DGT: *In situ* Diffusional Techniques for Studying Water, Sediments and Soils. In *In situ monitoring of aquatic systems: chemical analysis and speciation*, (J. Buffle & G. Horvai, Eds.), England: John Wiley & Sons, 495-569.

Davison, W., Hooda, P., Zhang, H., & Edwards, A. (2000b). DGT measured fluxes as surrogates for uptake of metals by plants. *Adv. Environ. Res.*, **4**,550-555.

Donahue, R. L., Miller, R. W. & Schickluna, J. C. (1977). *Soils: an introduction to soils and plant growth*. Englewood Cliffs, New Jersey: Prentice Hall.

Dosoretz, C. G., Chen, H. C. & Grethlein, H. E. (1990). Effect of environmental conditions on extra cellular protease activity in ligninolytic cultures of *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol*., **56**, 2, 395-400.

Douglas, G. S., Prince, R. S., Butler, E. L. & Steinhauer, W. G. (1994). The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. In *Hydrocarbon Bioremediation*, (R. E. Hinchee, B. C. Alleman, R. E. Hoeppel & R. N. Miller, Eds.), Boca Raton, FL: CRC Press, 219-236.

Echevarria, G., Morel, J. L., Fardeus, J. C. & Leclerc-Cessac, E. (1998). Assessment of phytoavailability of nickel in soils, *J Environ. Qual.*, **27**, 1064.

Edwards, R. (1996). Toxic Sludge Flows Through the Andes, *New Scientist* (Nov. 23), 13-23.

Escudero-Uribe, H. (2003). Determinación de la acumulación de los hidrocarburos polinucleoaromáticos en el pasto aleman *Echinochloa polystachia*, durante el proceso de fitorremediación. M.Sc. thesis. Universidad del Noroeste, Tampico Tamaulipas, Mexico.

Fernández-Sánchez J. M., Rodríguez-Vázquez, R. Ruiz-Aguilar, G. & Alvarez, P. (2001). PCB biodegradation in aged contaminated soil: interactions between exogenous *Phanerochaete chrysosporium* and indigenous microorganisms, *J Environ. Sci.*, **36**, 7,1145-1162.

Field, J. A., de Jong, E., Feijoo-Costa, G. F. & de Bont, J. A. M. (1993). Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics, *Trends Biotehcnol.*, **11**, 2, 44-49.

Folk, R. L. (1969). *Petrología de las rocas sedimentarias*. Instituto de Geología, UNAM, México.

Forstner, U. (1987). Metal speciation in solid wastes-factors affecting mobility. In *Speciation of metals in water, sediment and soil systems*, (L. Landner, Ed.), Germany: Springer-Verlag, 13-42.

García, E. (1988). *Modificaciones al sistema de clasificación climática de Köppen para adaptarlo a las condiciones de la República Mexicana*, Mexico: Instituto de Geofísica, UNAM, 13-27.

George, E. & Neufeld, R. (1989). Degradation of flourene in soil by fungus *Phanerochaete chrysosporium*, *Biotechnol. Bioeng*.,**. 33**, 1306-1310.

Gibson, D. & Subramanian, D. (1984). Microbial degradation of aromatic hydrocarbons. In *Microbial Degradation of Organic Compounds*, (D. Gibson, Ed.), New York: Mercel Dekker, 181-252.

Godbout, J. G. Comeau, Y. & Creer, C. W. (1995). Soil characteristics effects on introduced bacterial survival and activity. In *Bioaugmentation for site remediation. Pap. 3rd Int. In Situ On-Site Bioreclam. Symp.* (R. E. Hinchee, J. Fredrickson & B. C. Alleman, Eds.), Columbus, OH: Battelle Press, 115-120.

GPA (2002). Heavy metals. GPA Coordination Office, United Nations Environment Programme, The Netherlands [WWW document] http://www.gpa.unep.org/pollute/metals.htm

Grahn, O. & Hakanson, L. (1987). Case studies on metal distribution and uptake in biota. In *Speciation of metals in water, sediment and soil systems*, (L. Landner, Ed.), Germany: Springer-Verlag, 127-144.

Grupe, M. & Kuntze, H. (1987). Zur Ni-Mobilität einer geogen belasteten Braunerde, *Mitt. Disch. Boden-kundl. Gesellsch.*, **55**, 333.

Hammel, K. E., Gai, W. Z., Green, B. & Moen, M. A. (1992). Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*, *Appl Environ Microbiol.*, **58**, 6,1832-1838.

Hammel, K. E. (1995). Extracellular free radical biochemistry of ligninolytic fungi, *New J Chem.*, **20**, 195-198

Han, F. X., Kingery, W. L. & Selim, H. M. (2001). Accumulation, redistribution, transport and bioavailability of heavy metals in waste-amended soils. In *Trace Elements in Soil. Bioavailability, Flux, and Transfer*, (K. Iskandar & M. B. Kirkham, Eds.), Boca Raton, Florida: CRC Press, 145-173.

Harper, M. P., Davison, W., Tych, W. & Zhang, H. (1998). Kinetics of metal exchange between solids and solutions in sediments and soils interpreted from DGT measured fluxes, *Geochim. Cosmochim. Acta,* **62**, 2757.

Hausenbuiller, R. L. (1981). *Soils science. Principles and practices*. Dubuque, Iowa: WCB Company.

Helalia, A. M. (1993). The relation between soil infiltration and effective porosity in different soils, *Agricult. Water Manag.*, **24**, 1, 39-47.

Hofrichter M. & Scheibner, K. (1993). Utilization of aromatic compounds by the *Penicillium* strain BI 7/2. *J Basic Microbiol*., **33**, 4, 227-232.

Hofrichter, M., Bublitz, F. & Fritsche, W. (1995). Cometabolic degradation of o-cresol and 2,6-dimethylphenol by *Penicillium frequentans* Bi-7/2, *J Basic Microbiol.*, **35**, 5, 303-313.

Holman, H. Y. & Tsang, Y. W. (1995). Effects of soil moisture on biodegradation of petroleum hydrocarbons. In *In situ Aeration: Air Sparging, Bioventing, Relat. Rem. Processes. Pap 3rd Int. In situ On-*

*site Bioreclamation Symposium*, pp. 181-187. (R. E. Hinchee, J. Fredrickson & B. C. Alleman, Eds.), Columbus, Ohio: Battelle Press.

Hooda, P. S. & Alloway, B. J. (1993). Effects of time and temperature on the bioavailability of Cd and Pb from sludge-amended soils, *J Soil Sci.*, **44**, 97-110.

Hooda, P. S. & Alloway, B. J. (1994). The plant availability and DTPA extractability of trace-metals in sludge-amended soils, *Sci. Tot. Environ.*, **149**, 39-51.

Hooda P. S., H. Zhang, W. Davison & Edwards, A. C. (1999). Measuring bioavailable trace metals by DGT: soil moisture effects on its performance in soils, *Eur. J Soil Sci.*, **50**, 2, 285-294.

Hornick, S. B., Fisher, R. H. & Paolini, P. A. (1983). Petroleum Wastes. In *Land Treatment of Hazardous Wastes*, (J. F. Parr, P. B. Marsh & J. M. Kla, Eds.), Park Ridge, NJ: Noyes Data Corporation, 321-337.

Horowitz, A. J., Lum, K. R., Grabarino, J. R., Hall, G. E. M., Lemieux, C. & Demas, C. R. (1996). Problems associated with using filtration to define dissolved trace elements concentrations in natural water samples, *Environ. Sci. Technol.*, **30**, 954-963.

Hudson, R. J. M. & Morel, F. M. M. (1990). Iron transport in marine phytoplankton: kinetics of cellular and medium coordination reactions, *Limnol. Oceanogr*. **35,** 1002–1020.

IMP (1999). Programa ambiental de la región sur de PEMEX (PARS). Primera etapa, Mexico: IMP, Subdirección de Protección Ambiental, 12-36.

JRB Associates, Inc. (1984). *Summary report: Remedial response at Hazardous Waste Sites*, Cincinnati, OH: Municipal Environmental Research Laboratory PB 85-124899.

Kabata-Pendias, A. & Pendias, H. (1999). *Biogeochemistry of trace elements*, Warsaw: Wyd. Nauk PWN, 1-400.

Kabata-Pendias, A. (2001). *Trace Elements in Soils and Plants*, Boca Raton, FL: CRC Press, 1-486.

Karimi-Lotfabad, S., Pickard, M. & Gray, M. (1996). Reactions of polynuclear aromatic hydrocarbons on soil, *Environ. Sci. Technol.*, **30**, 4, 1145-1151.

Kashem, A. & Singh, B. R. (2001). Solid phase speciation of Cd, Ni and Zn in contaminated and noncontaminated tropical soils. In *Trace elements in soil. Bioavailability, flux and transfer*, (I. K. Iskandar & M. B. Kirkham, Eds.), Boca Raton, FL: Lewis Publishers, 213-228.

Kirk, T. K. (1980). Physiology of lignin metabolism by white-rot fungi. In *Lignin biodegradation: microbiology, chemistry, and potential applications*, Volume 2, pp. 56-57. (T. K Kirk, T. Higuchi & H. Chang, Eds.), Boca Raton, Florida: CRC press.

Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Lida, T., Hayashi, H. & Takizawa, N. (1994). Cloning and characterization of a chromosomal gene cluster, path, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82, *J Bacteriol.*,**176**, 8, 2439-2443.

Knight, B. & McGrath, S. P. (1995). A method to buffer the concentrations of free Zn and Cd ions using a cation-exchange resin in bacterial toxicity studies, *Environ. Toxicol. Chem.*, **14**, 2033-2039.

Kokle, A., Sauerbeck, D. R. & Vetter, H. (1984). The contamination of plants and soils with heavy metals and the transport of metals in terrestrial food chains. In *Changing Metal Cycles and Human Health*, (J. O. Nriagu, Ed.), Dahlem, Konferenzen, Berlin: Springer-Verlag, 113.

Krishnamaturi, G. S. R. & Naidu, R. (2002). Solid-solution speciation and phytoavailability of copper and zinc in soils. *Environ. Sci. Technol.*, **36**, 2645-2651.

Laborda, F., Monistrol, M., Luna, I. & Fernández, M. (1999). Process of liquefaction-solubilization of Spanish coal by microorganisms, *Appl. Env. Microbiol*,. **52**, 49-56.

LaGrega, M. D., Buckingham, P. L. & Evans, J. C. (1996). *Gestión de residuos tóxicos. Tratamiento, eliminación y recuperación de suelos*. Spain: McGraw Hill, 722-723.

Larsen, P. B., Dagenhardt, J., Tai, C., Stenzler, L. M., Howell, S. H. & Kochian, L. V. (1998). Aluminium-resistant *Arabidopsis* mutants that exhibit altered patterns of aluminium accumulation and organic acid release from roots, *Plant Physiol.*, **117**, 19-27.

Lasat, M. M. (2002). The use of plants for the removal of toxic metals from contaminated soil. American Association for the Advancement of Science. EPA publication, 33.

Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. & Moore, M. (1995). The oxidation of pyrene and benzo(a)pyrene by non-basidiomycete soil fungi, *Can. J Microbiol.*, **41**, 6, 477-488.

Lead, J. R., Davison, D., Hamilton-Taylor, J. & Buffle, J. (1997). Characterizing Colloidal Material in Natural Waters, *Aquatic Geochem*., **3**, 213-232.

Lead, J. R., Hamilton-Taylor, J. & Davison, D. (1998). The effect of sequential extractions of suspended particulate matter on trace metal sorption and microbial cell stability, *Sci. Tot. Environ.*, **209**, 193-199.

Lead, J. R., Hamilton-Taylor, J., Davison, W. & Harper, M. (1999). Trace metal sorption by natural particles and coarse colloids, *Geochim. Cosmochim. Acta*, **63**, 1661-1670.

Lead, J. R., Starchev, K. & Wilkinson, K. J. (2003). Diffusion coefficients of humic substances in agarose gel and in water, *Environ. Sci. Technol.*, **37**, 482-487.

Lena, Q. M. & Gade, N. R. (1997). Chemical fractionation of cadmium, copper, nickel, and zinc in contaminated soils, *J Env. Qual.*, **26**, 259.

Li, Y. H. & Gregory, S. (1974). Diffusion of ions in seawater and in deep-sea sediments, *Geochim. Cosmochim. Acta*, **38**, 703.

Loehr, R. C. (1992). Bioremediation of PAH compounds in contaminated soil. In *Hydrocarbon Contaminated soils and Groundwater. Proc. 1st. Annu. West Coast Conf. Hydrocarbon Contam. Soil Groundwater*, Volume 1, (E. J. Calabrese & P. T. Kostechi, Eds.), Newport Beach, Chelsea, MI: Lewis Publisher, 213-222.

Lombi, E., Zhao, F. J., Dunhma, S. J. & McGrath, S. P. (2001). Phytoremediation of heavy metal-contaminated soils: Natural hyperaccumulation versus chemically enhanced phytoextraction, *J Environ. Quality*, **30**, 6, 1919-1926.

Lorenz, S. E., Hamon, R. E., McGrath, S. P., Holm, P. E. & Christensen, T. H. (1994). Application of fertilizer cations affects cadmium and zinc concentrations in soil solutions and uptake by plants, *Eur. J Soil Sci.*, **45**, 159-165.

Ma, W., Edelman, T., van Beersum, I. & Jans, T. (1983). Uptake of cadmium, zinc, lead and copper by earthworms near a zinc-smelting complex: influence of soil pH and organic matter, *Bull. Environ. Contam. Toxicol*., **30**, 424.

Macksimiec, W. & Baszynski, T. (1996). Chlorophyll fluorescence in primary leaves of excess Cu-treated runner bean plants depends on their growth stages and the duration of Cu-action, *J Plant Physiol.*, **149**, 196.

Macnicol, R. D. & Beckett, P. H. T. (1985). Critical tissue concentrations of potentially toxic elements, *Plant soil*, **85**, 107.

Mahmood, S. K. & Rao, P. R. (1993). Microbial abundance and degradation of polycyclic aromatic hydrocarbons in soil, *Bull. Environ. Contam. Toxicol.*, **50**, 486-491.

Manilal, V. & Alexander, M. (1991). Factors affecting the microbial degradation of phenanthrene in soil, *Appl. Microbiol. Biol.*, **35**, 3, 401-405.

Maxwell, C. R. & Baqai, H. A. (1995). Remediation of petroleum hydrocarbons by inoculation with laboratory-cultured microorganisms. In *Bioaugmentation for Site Remediation,* (R. E. Hinchee, J. Fredrickson, & B. C. Alleman, Eds.), Pap. 3rd Int. In situ On-Site Bioreclam. Symp., Columbus, Ohio: Battelle Press, 129-138.

McGrath, S. P. (1998). Phytoextraction for soil remediation. In *Plants that hyperaccumulate heavy metals*, (R. R. Brooks, Ed.), UK: CAB International, 261.

McGrath, S. P., Dunham, S. J. & Atkin, R. K. (1998). An extracting science. *Chem. Ind.,* **22**, 915-918.

McInerney, M. J., Weirick, E. W., Sharma, P. K. & Knapp, R. M. (1993). Non-invasive methodology to study the kinetics of microbial growth and metabolism in subsurface porous material. *Dev. Petrol. Sci*., **39**, 151-157.

McLaughlin, M. L., Palmer, L. T., Tiller, K. G., Beech, T. A. & Smart, M. K. (1994). Increased soil salinity causes elevated cadmium concentrations in field-grown potato tubers, *J Environ. Qual.,* **23**, 1013.

Mellum, H. K., Arnesen, A. K. M. & Singh, B. R. (1998). Extractability and plant uptake of heavy metals in alum shale soils, *Commun. Soil Sci. Plant Anal.*, **29**, 1183.

Meulenberg, R., Rijhaams, H., Doddema, H. & Field, J. (1997). Partially oxidised polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability, *FEMS Microbiol. Lett.*, **152**, 45-49.

Mishra, S. P. & Chaudhury, G. R. (1996). Biosorption of heavy metal ions by *Penicillium* sp.-a case study, *Trans. Indian Inst. Met.*, **49**, 1-2, 85-87.

Mitchell, D. A. (1992). Biomass Determination in Solid-State Cultivation. In *Solid Substrate Cultivation*, (H. Doelle, D. A. Mitchell & C. E. Rolz, Eds.), London, Elsevier, 55.

Miteva, E., Maneva, S. V., Bojinova, P. & Ivanova, J. (1997). Accumulation of trace elements in crops cultivated on polluted soil after herbicide treatment. In *Contaminated soils* (R. Prost, Ed.), Paris: INRA, 33-43.

Molas, J. (1997). Range of tolerance limits and toxicity symptoms of ionic and chelated nickel forms in cabbage plants (*Brassica oleracea* L.), *Z. Probl. Post. Nauk Roln*., **448b**, 203.

Montgomery, D. C. (2000). *Design and analysis of experiments*. USA: John Wiley and Sons Inc.

Morel, F. M. M. & Hering, J. G. (1993). *Principles and Applications of Aquatic Chemistry,* New York: Wiley-Interscience.

Morrison, G. M. P. (1987). Bioavailable metal uptake rate determination in polluted waters by dialysis with receiving resins, *Env. Technol. Lett.,* **8**, 393-402.

Müncnerova, D. & Augustin, J. (1994). Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons: a review, *Biores. Technol.*, **48**:97-106.

National Research Council (1993). *In situ Bioremediation. When does it work?* Washington, DC: Water Science and Technology Board, Com. Eng. Tech. Systems, National Academy of Sciences, National Academy Press.

Nelson, D. W. & Sommers, L. E. (1982). Total Carbon, Organic Carbon and Organic Matter. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 539-579.

Nishioka, M., Chang, H. C. & Lee, M. L. (1986). Structural characteristics of polycyclic aromatic hydrocarbon isomers in coal tars and combustion products, *Env. Sci. Technol.*, **20**, 10, 1023-1027.

Niyazova, G. A. & Letunova, S. V. (1981). Microelements accumulation by soil microflora at the conditions of the Sumsarsky lead zinc biogeochemical province in Kirghizya, *Ecologiya*, **5**, 89.

Nriagu, J. O. (1996). A History of Global Metal Pollution, *Science*, **272**, 5259, 223.

Odzak, N., Kistler, D., Xue, H. & Sigg, L. (2002). *In situ* trace metal speciation in a eutrophic lake using the technique of diffusion gradients in thin films (DGT), *Aquat. Sci*., **64**, 292-299.

Olsen, S. R. & Sommers, L. E. (1982). Phosphorous. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 404-407.

Ouyang, J. (2002). Phenanthrene pathway. The University of Minnesota Biocatalysis/Biodegradation Database, University of Minnesota [WWW document] http://umbbd.ahc.umn.edu/pha2/pha2_image_map.html

Pacha, J., Kędziorska, M., Krupa, G., Orzel, M. Polańska, J. & Schikora, G. (1988). Activity of selected enzymes in soil subjected to the action of trivalent chromium compounds, *Acta Biology Soil*., **9**, 38.

Padley, A., Soccol, C. R., Nigan, P. & Soccol, V. T. (2000). Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Biores. Technol.,* **74**, 69-80.

Pankhurst, C. E., Rogers, S. L., & Gupta, V. V. S. R. (1998). Microbial parameters for monitoring soil pollution. In *Environmental Biomonitoring: The Biotechnology Ecotoxicology Interface*, (J. M. Lynch & A. Wiseman, Eds.), Cambridge Univ. Press, 4.

Papendick, R. I. & Campbell, G. S. (1981). Theory and measurement of water potential. In *Water Potential Relations in Soil Microbiology*: Proc. of a Symposium of the Soil Science Society of America, (J. F. Parr, W. R. Gardner & L. F. Elliott, Eds.), Madison, WI: Soil Science Society of America, 1-22.

Parkinson, D. (1982). Filamentous Fungi. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 949-968.

Parr, J. F., Sikora, L. J., & Buges, W. D. (1983). Factors affecting the degradation and inactivation of waste constituents in soils. In *Land Treatment of Hazardous Wastes*, (F. J. Parr, P. B. Marsh & J. M. Kla, Eds.), Noyes Data Corp., Park Ridge, NJ, 20-49, 321-337.

Pattee, O. H. & Pain, D. J. (2003). Lead in the Environment. In *Handbook of Ecotoxicology*, (D. J. Hoffman, B. A. Rattner, G. A. Burton Jr. & J. Cairns Jr., Eds.), USA: Lewis Publishers, 373-408.

Pérez-Armendariz B., Rodríguez-Vázquez, R., Loera-Corral, O. Fernández-Linares, L. & Esparza-García, F. (2003). Biostimulation of microorganisms from sugarcane bagasse pith for weathered hydrocarbon removal from soil**,** *Letters Appl. Microbiol.* (submitted).

Pickering, W. F. (1998). Metal ion speciation. Soils and sediments, *Ore Geol Rev.*, **1**, 83.

Ponizovsky, A. & Mironenko, E. (2001). Speciation and sorption of lead (II) in soils. In *Trace Elements in Soil. Bioavailability, Flux, and Transfer*, (K.

Iskandar & M. B. Kirkham, Eds.), Boca Raton, Florida: CRC Press, 261-279.

PROFEPA (2002). Critrios internos de restauración de suelos contaminados por metales pesados, PROFEPA, Mexico.

Ramos, L., Hernández, L. M. & González, M. J. (1994). Sequential fraction of copper, lead, cadmium and zinc in soils from or near Donana National Park, *J Environ. Qual.*, **23**, 50.

Rhoades, J. D. (1982). Cation Exchange Capacity. In *Methods of soil analysis. Part 2 Chemical and microbiological properties*, (A. L. Page, R. H. Miller & D. R. Keeney, Eds.), USA: American Society of Agronomy and Soil Science Society of America, 149-157.

Riha, V., Nymburska, K., Tichy, R. & Triska, J. (1993). Microbiological, chemical and toxicological characterization of contaminated sites in Czechoslovakia, *Sci. Total Environ.*, Suppl. Pt. **1**, 185-193.

Riser-Roberts, E. (1998) *Remediation of Petroleum Contaminated Soils. Biological, Physical and Chemical Processes*, Boca Raton, Florida: CRC Press, 115-198.

Roane, T. M. & Kellogg, S. T. (1996). Characterization of bacterial communities in heavy metal contaminated soils, *Can. J Microbiol.*, **42**, 593-603.

Rodríguez-Vázquez, R. (1993). Evaluation of pre-treatments of biomass to increase enzyme accessibility and comparison with brown rot decay mechanism. Ph.D. thesis. Colorado State University.

Rodríguez-Vázquez, R., C. Montalvo, L. Dendoven, F. Esparza, L. Fernández-Linares (1999a). Degradation of benzo(a)pyrene in soil by white rot fungi. In *Bioremediation of Nitroaromatic and Haloaromatic Compounds*, (B. Alleman & A. Leeson, Eds.), Battelle Press., 93, 98, 173-178.

Rodríguez-Vázquez, R., Cruz-Córdova, T. Fernández-Sánchez, J. M., Roldán-Carrillo, T., Mendoza-Cantú, A., Saucedo-Castañeda, G. & Tomasini-Campocosio, A. (1999b). Use of Sugarcane Bagasse Pith as Solid Substrate for *Phanerochaete chrysosporium* Growth, *Folia Microbiol.*, **44**, 2, 213-218.

Rogers, J. A., Tedaldi, D. J. & Kavanaugh, M. C. (1993). A screening protocol of bioremediation of contaminated soil, *Environ. Progress*, **12**, 146-156.

Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E. & Ron, E. Z. (1992). Petroleum bioremediation-a multiphase problem. *Biodegradation*, **3**, 337-350.

Ross, S. M. (1994). Retention, transformation and mobility of trace metals in soils. In *Toxic Metals in Soil-Plant systems*, (S. M. Ross, Ed.), England: John Wiley & Sons, 27-61.

Rugh, C., Wood, T., Redwine, S., Settavongsin, R., Susilawati, E., Wan, C., & Kinder, S. (2000). Design and construction of phytoremediation demonstration facility [WWW document] http://www.css.msu.edu/phytoremediation/poster2000.html

Ruiz-Duenas, F., Guillen, F., Camarero, F., Perez-Boad, M., Martinez, M. & Martinez, A. (1999). Regulation of peroxidase transcript levels in liquid cultures of the ligninolytic fungus *Pleurotus eryngii*, *Appl. Environ. Microbiol.*, **65**, 4458-4463.

Ryan, R. G. & Dhir, V. K. (1993). The effect of soil particle size on hydrocarbon entrapment near a dynamic water table, *Hydrocarbon Contam. Soils Groundwater*, **3**, 213-235.

Sack, U., Heinze, T. M., Deck, J., Cerniglia, C. E., Cazau, M. C. & Fritsche, W. (1997). Novel metabolites in phenanthrene and pyrene transformation by *Aspergillus niger*, *Appl. Environ. Microbiol*., **63**, 7, 2906-2909.

Salbu, B., Krekling, T. & Oughton, D. H. (1998). Characterisation of radioactive particles in the environment, *Analyst*, **123**, 843.

Samanta, S. K., Singh O. V. & Jain, R. K. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation, *Trends Biotechnol.*, **20**, 243.

Saucedo, C. J., Trejo, H. M. & Lonsane, B. K. (1994). On line-automated monitoring and control systems for $CO_2$ and $O_2$ in aerobic and anaerobic solid-state fermentations, *Proc. Biochem.*, **29**, 13-24.

Scally, S, Davison, W & Zhang, H. (2003). *In situ* measurements of dissociation kinetics and labilities of metal complexes in solution using DGT, *Env. Sci. Technol.*, **37**, 7, 1379-1384.

Sigg, L. (1994). Regulation of trace elements in lakes: the role of sedimentation. In Chemical and biological regulation of aquatic systems, (J. Buffle & R. R. De Vitre, Eds.), Boca Raton, Florida: Lewis, 175-195.

Simon, L., Prokisch, J., Kovács, B. & Györi, Z. (1988). Phytoextraction of heavy metals from a galvanic mud contaminated soil. In *Soil Pollution*, (G. Filep, Ed.), Debrecen: Agr. Univ., 289.

Sims, R. & Bass, J. (1984). *Review of In-Place Treatment Techniques for Contaminated Surface Soils, Vol. 1: Technical Evaluation*, Washington, DC: Environmental Protection Agency, EPA-540/2-84-003a.

Singh, S. P., Tack, F. M. & Verloo, M. G. (1998). Heavy metal fractionation and extractability in dredge sediment derived surface soils, *Water Air Soil Pollut.*, **102**, 313.

Smith, T. (1992). *Heavy metals, from a primer of environmental toxicology*, USA: Health Sciences bookstall, 142-150

Solanas, A. M., Pares, R., Bayona, J. M. & Aalbaiges, J. (1984). Degradation of aromatic petroleum hydrocarbons by pure microbial cultures, *Chemosphere*, **13**, 593-601.

Sommers, L. E., Gilmore, C. M., Wildung, R. E. & Beck, S. M. (1981). The effect of water potential on decomposition processes in soils. In *Water Potential Relations in Soil Microbiology*, SSSA Special Publication No. 9, Madison, WI: Soil Science Society of America, 97-117.

SSDS-USDA (1993). *Soil survey*. Handbook No. 18, Washington: USDA.

Stauton, S., Bonafos, B. & Leclerc-Cessac, E. (1999). Possible effects of root action in the rizhosphere on the adsorption of trace amounts of nickel by various soils, Proc. 5$^{th}$ Int. Conf. Biogeochemistry of Trace Elements, Vienna, July 11-15, 46.

Streebin, L. B., Robertson, J. M., Callender, A. B., Doty, L. & Bagawandoss, K. (1984). Closure evaluation for petroleum residue land treatment. Washington, DC: Environmental Protection Agency, EPA-600/2-84-162.

Studenikina, T. A. (1999). Interaction of Copper (II) with Soil Components under Influence of Organic Matter Dissolved in Soil Solution, Ph.D. thesis, Moscow Univ., 21.

Sutherland, J. B., Selby, A. L., Freeman, J. P., Evans, F. E. & Cerniglia, C. E. (1991). Metabolism of phenanthrene by *Phanerochaete chrysosporium*, *Appl. Environ. Microbiol.*, **57**, 3310-3316.

Sutherland, J. B. (1992). Detoxification of polycyclic aromatic hydrocarbons by fungi, *J Ind. Microbiol.*, **9**:53-62.

Tessier, A. Buffle, J. & Campbell, G. C. (1994). Uptake of trace metals by aquatic organisms. In *Chemical and Biological regulation of Aquatic Systems* (J. Buffle & R. R. De Vitre, Eds.), Boca Raton, FL: Lewis Publishers, 197-230.

Trzesicka-Mlynarz, D. & Ward, O. P. (1995). Degradation of Polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures, obtained from PAH-contaminated soil, *Can. J Microbiol*., **41**, 470-476.

Turco, R. F. & Sadowsky, M. (1995). The microflora of bioremediation. In *Bioremediation: Science and Application*. SSSA Special Pub. 43, Madison, WI: Soil Science Society of America, American Society of Agronomy and Crop Science Society, 87-102.

Unterköfler, J., Wenzel, W. W., Adriano, D. C., Wieshammer, G., Sommer, P., Fitz, W. (2001). Integrated phytoextraction and physico-chemical mobilization- a new approach to remediate contaminated soils. In *Proceedings of the International Conference on the Biogeochemistry of Trace elements*, (International Society for Trace Element Biogeochemistry, Ed.), Guelph, p. 411.

USEPA (1985). *Handbook for Remedial Action at Waste Disposal Sites*, Washington, DC: Environmental Protection Agency, EPA/625/6-85/006.

USEPA (1994). SITE Demonstration Bulletin: *In situ* verification, Geosafe Corp. Cincinnati, OH: Environmental Protection Agency.

USEPA (2002). Metal soil criteria [WWW document] http://www.ecosite.co.uk/depart/eubkinfo/soilimp.htm

van Leeuwen, H. P. (1999). Metal speciation dynamics and bioavailability: Inert and labile complexes, *Env. Sci. Technol.*, **33**, 3743-3748.

Vecchioloi, G. I., Del Panno, M. T. & Painceira, M. T. (1990). Use of selected autochthonous soil bacteria to enhance degradation of hydrocarbons in soil, *Environ. Pollut.*, **67**, 249-258.

Wagner, G. J. (1993). Accumulation of cadmium in crop plants and its consequences to human health, *Adv. Agron.*, **51**, 173-212.

Wallace, A., Mueller, R. T. & Wood, R. A. (1990). Arsenic phytotoxicity and interactions in bush bean plants grown in solution culture, *J Plant Nutr.*, **2**, 51.

Wang, P., Erfu, Q., Li, Z. & Shuman, L. M. (1997). Fractions of availability of nickel in loessial soil amended with sewage or sewage sludge, *J Environ. Qual.*, **26**, 795.

Webb, J. A. & Keough, M. J. (2002). Measurement of environmental trace-metal levels with transplanted mussels and diffusive gradients in thin films (DGT): a comparison of techniques, *Marine Poll. Bull.*, **44**, 3, 222-229.

Wenzel, W. W., Fitz, W., Puschenreiter, M., Sommer, P., Schnepf, A., Unterköfler, J. (2001). Rhizosphere processes involved in phytoextraction of metal-contaminated soils. In *Proceedings of the International Conference on the Biogeochemistry of Trace elements*, (International Society for Trace Element Biogeochemistry, Ed.), Guelph, p. 289.

WHO (2001). Environmental Health Criteria 202: Selected non-heterocyclic polycyclic aromatic hydrocarbons [WWW document] http://www.who.int/pcs/ehc/corrigenda/202.html

Wilson, S. C. & Jones, K. C. (1993). Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review, *Environ. Pollut.*, **81**, 229-249.

Woźny, A. (1998). Lead in plants. In *Lead in the Environment-Ecological and Analytical Problems*, (A. Kabata-Pendias & B. Szteke, Eds.), PAN Kom. Nauk, **21**, 171.

Wunder, T., Kremer, S., Sterne, C. & Anke, H. (1994). Metabolism of the polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK 9317, *Appl. Microbiol. Biotechnol.*, **42**, 636-641.

Wunderwald, U., Hofrichter M., Kreisel G. & Fritsche, W. (1997). Transformation of difluorinated phenols by *Penicillium frequentans* Bi 7/2. *Biodegradation*, **8**, 6, 379-385.

Yuan, S. Y., Wei, S. H. & Chang, B. V. (2000). Biodegradation of PAHs by a mixed culture, *Chemosphere*, **41**, 1463-1468.

Zayed, A., Lytle, C. M., Qian, J. H. & Terry, N. (1998). Chromium accumulation, translocation and chemical speciation in vegetable crops, *Planta*, **206**, 284.

Zhang, H. & Davison, W. (1995). Performance Characteristics of Diffusion Gradients in Thin Films for the *in situ* Measurement of Trace Metals in aqueous Solution, *Analytical Chem.*, **67**, 19, 3391-3400.

Zhang H., Davison W., Millera, S. & Tycha, W. (1995). *In situ* high resolution measurements of fluxes of Ni, Cu, Fe, and Mn and concentrations of Zn and Cd in porewaters by DGT, *Geochim. Cosmochim. Acta*, **59**, 20, 4181-4192.

Zhang, H., Davison, W., Knight B. & McGrath, S. P. (1998). *In situ* measurements of solution concentrations and fluxes of trace metals in soils using DGT, *Environ. Sci. Technol.*, **32**, 704-710.

Zhang, H. & Davison, B. (1999). Diffusional characteristics of hydrogels used in DGT and DET techniques, *Anal. Chim. Acta*, **398**, 329-340.

Zhang, H., Zhao, F. J., Sun, B. Davison, W. & McGrath, S. P. (2001). A New Method to Measure Effective Soil Solution Concentration Predicts Copper Availability to Plants, *Environ. Sci. Technol.*, **35**, 2602-2607.

Zhou, E. & Crawford, R. L. (1995). Effects of oxygen, nitrogen and temperature on gasoline biodegradation in soil, *Biodegradation*, **6**: 127-140.

Zimdahl, R. L. & Koeppe, D. E. (1987). Uptake by plants. In *Lead in the Environment*, (W. R. Bogges and B. G. Wixson, Eds.), Report NSF, National Science Foundation, Washington, D.C. 99.

Zitrides, T. (1983). Biodecontamination of spill sites, *Pollut. Eng.*, **15**, 25-27.