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Changes of chromium behavior in soil during phenanthrene removal by *Penicillium frequentans*

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Abstract

Soil contamination due to polycyclic aromatic hydrocarbons is often associated with the presence of high levels of potentially toxic metals. Bioremediation is an important option for the clean up of this type of contamination. Changes of chromium fluxes and concentrations during the phenanthrene removal by *Penicillium frequentans* in soil were investigated. During the bioremediation process, changes in chromium behavior were monitored by Diffusive Gradients in Thin-films (DGT) and by filtration in both sterilized and non-sterilized soils. DGT provided absolute data on fluxes from the solid phase and relative trends of concentrations of the most labile metal species. Filtration provided data on the concentrations of Cr in the solution phase. Together the data provided information about the physical and chemical metal behavior. Results showed that the highest phenanthrene removal was observed in non-sterilized soil (which included the autochthonous microorganisms and *P. frequentans* inoculum), with a phenanthrene removal of 73 ± 3.2%. However, in all cases microbial activity increased chromium fluxes and chromium soil solution concentration. The bioremediation of soil by *P. frequentans* increased the lability and mobility of chromium in soil, with potential consequences for plant uptake and for increased movement of metals into the human food chain.

Introduction

Environmental pollution with polycyclic aromatic hydrocarbons (PAHs) has attracted much attention in recent years, because carcinogenic substances may be formed during biodegradation of PAHs in humans and microorganisms. Although human genotoxicity and carcinogenicity of phenanthrene is questionable (WHO 2001), analoges of its structure are found in carcinogenic PAHs (Cerniglia 1984). In addition, as phenanthrene is the smallest PAH to have a bay-region and a K-region, it is often used as a model substrate for studies on the carcinogenic PAHs (Samanta *et al.* 2002).

Biodegradation of PAHs has many benefits due to the removal of these contaminants (Samanta *et al.* 2002). However, a major unknown is the subsequent behavior of pollutants, such as heavy metals after the bioremediation process. High levels of metals are known to be toxic for soil microflora (Burkhardt *et al.* 1993; Riser-Roberts 1998; Sokhn *et al.* 2001), while it is acknowledged that solid–solution fluxes and chemical speciation are of overriding importance in determining uptake and toxicity (Hooda *et al.* 1999; Davison *et al.* 2000a, 2000b).

It is known that Cr behavior is affected by soil microorganisms (Endo & Siluer, 1995; Cifuentes *et al.* 1996). For instance, many microorganisms exhibit resistance to deleterious effects of Cr. Additionally, chromate may be microbially reduced to Cr(III). However, there is a lack of understanding of the influence of microbial

activity, especially fungal activity, on Cr speciation and solid–solution fluxes and concentrations. In order to assess changes in chromium behavior during the biodegradation process is necessary to consider both chemical speciation in soil solution and the kinetics of exchange between solution and solid phase. In this study, the effect of phenanthrene removal by the fungus *P. frequentans* and native soil microflora on Cr behavior in both sterilized and non-sterilized soil was investigated using the Diffusive Gradient in Thin Films (DGT) technique and filtration.

Material and methods

Soil characterization

A sandy clay loam soil from a tropical site in the state of Tabasco, Mexico was used in this study. Soil was homogenized (gentle blending) and characterized before treatment. The main soil characteristics are: pH 5.7, organic matter 6.83%, water holding capacity 15%, cationic exchange capacity 5.1 meq 100 g^{-1}. Concentrations of PAHs were below detection limits (0.002 mg g^{-1}) prior to spiking, and the total chromium concentration was 23 mg g^{-1} (Amezcua-Allieri *et al.* 2003).

Solid-state culture

To propagate *P. frequentans*, 0.02 g of mycelium was added to 0.4 g of bagasse with 0.2 ml of deionised sterilized water and incubated in 125 ml sealed vials at 28 °C for 15 days. Subsequently, 6 g of sterilized/non-sterilized soil was spiked with phenanthrene. The final soil concentration of phenanthrene was 200 mg g^{-1}. On day 15, the soil was brought to an optimal C:N ratio of 40 and moisture content of 44%. Those conditions were previously selected for maximum phenanthrene removal and as optimal for the operation of DGT (Amezcua-Allieri *et al* 2003).

Treatments

Different treatments with sterilized and non-sterilized soil were run following the same inoculation procedure: (A) Soil + fungus + phenanthrene, (B) Soil + fungus, (C) Soil + phenanthrene, (D) Soil. All analyses were performance in triplicate.

Sterility of soil and bagasse was checked by adding 2 ml of soil or bagasse suspension to agar plates and incubated for 7 days in darkness at room temperature. Three different culture media were used: nutrient agar, potato-dextrose agar, and Czapek agar. These stimulated bacteria, fungal and Actinomycetes growth, respectively. Sterilization efficiency was confirmed by the absence of CO_2 production in controls. Subsequent to treatment, the soil pH decreased slightly to pH 5.4. Speciation calculations and blanks showed this change had a minimal effect on Cr.

Carbon dioxide evolution

The CO_2 evolution was measured every three days as an indirect method of quantifying heterothrophic activity (Mitchell 1992). The CO_2 content of headspace samples was determined with a Gow–Mac 550 gas chromatograph equipped with a thermal conductivity detector and an Alltech CTRI stainless steel column.

Phenanthrene extraction and analysis

Phenanthrene removal was evaluated after 30 days of incubation. Soil samples were extracted according to the EPA-3540 method. Samples were analyzed by gas chromatography/mass spectrometry (GC/MS), using a GCQ Finnigan Mat under the following conditions: Hewlett Packard capillary column (0.32 × 50 m, 5% phenyl and 95 methyl silicon), initial column temperature 60 °C for 4 min, 250 °C at 5 °C m^{-1}, 42 min. Helium was used as carrier gas at a flow rate of 15 ml min^{-1}. The injector temperature was 50 °C, and the detector was an electromultiplier. Phenanthrene was identified based on its fragmentation peak in the GC/MS library. Identification was validated by comparison of the retention time using a phenanthrene standard (99% purity, Sigma–Aldrich) and quantified using the internal standard of naphthalene.

Diffusive Gradient in Thin-films (DGT)

DGT has been fully described elsewhere (Davison *et al.* 2000a). In brief, metal species freely diffuse through a layer of hydrogel and are then immobilized in an underlying layer of binding agent (Chelex resin). Because the diffusion layer

thickness during deployment is well defined by a known thickness of the gel, the mean concentration in solution can be calculated using Fick's laws (Davison & Zhang 1994). With DGT, separation of chemical species is based on both diffusive mass transport and on the lability. A steady-state situation is attained during deployment, allowing the results to be interpreted as a time-averaged flux from the solid soil as a concentration (Davison *et al.* 2000a).

The DGT device was based on a simple tight-fitting piston design (Davison *et al.* 2000a). 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.

DGT deployment and retrieval

DGT measurements were made directly on the soil which was the subject of bioremediation. The DGT units were placed on the surface of the soil slurry at an angle and pushed gently into the surface, making sure that there was no air bubble 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 (28 $\pm$ 0.1 °C) for every three days for 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 measure DGT chromium concentration, the chelex resin was retrieved, rinsed and was completely immersed in 1 ml of 1 M HNO_3 for 24 h to extract the metal into solution. Metal concentration in the resin layer and fluxes were calculated according to Davison & Zhang 1994 and Zhang *et al.* 1998.

Filtration

Soil solution samples were extracted by centrifugation at 24000 $\times$ *g* for 45 min. Centrifugation at low centrifugal forces is a standard separation method in microbiological studies and no cell lysis was expected. The resulting soil solutions were filtered through 0.45 μm Millipore cellulose nitrate membrane using on-line syringe filters. The filtered samples were acidified to pH 2 with Aristar HNO_3. Metal analysis was performed by GFAAS (Varian M493).

Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA), at 0.005 significance level, using three replicates ($n = 3$). Where more than two means were compared, significant differences between treatments were analyzed using a test for least significant difference (LSD). Statistical analyses were carried out using the software SAS 6.01 (Statistical Analysis System) and STATISTICA® 6.0.

Results and discussion

CO_2 evolution

Evolution of CO_2 has been used to approximate heterothrophic activity (Mitchell 1992). Evolution increased after mixing of soil and fungi, i.e. the start of the bioremediation process, in all treatments (data not shown). This increase observed is due to microbial action (either fungal or mixed fungal and native microflora) on the soil. Higher activities are observed in the non-sterilized soil due to the additional action of native microflora. Maximal heterothrophic activity (180 μg CO_2 IDM^{-1}) was found in non-sterilized soil spiked with phenanthrene (Treatment A). In the case of sterilized soil, maximal activity (90 μg CO_2 IDM^{-1}) was found due to the microbial activity in the soil in the absence of phenanthrene (Treatment B).

There was a statistically significant difference ($P < 0.005$) between treatment B and A in both types of soil. In Treatment A (in non-sterilized soil), phenanthrene appears to stimulate heterothrophic activity by providing an easily convertible carbon and energy source. The fungi and native microflora in combination also appear to mitigate any harmful effects of the phenanthrene. In sterilized soil, addition of phenanthrene appears to

inhibit heterothrophic activity, as the fungi alone are incapable of mitigating the harmful effects of phenanthrene. However, they are capable of utilizing phenanthrene as an energy source as fungi alone can still degrade phenanthrene.

Phenanthrene removal

The highest phenanthrene removal was observed at day 21, in non-sterilized soil (which include the autochthonous microorganisms from soil and bagasse), with a total phenanthrene removal of 73 ± 3.2%. In the system with sterilized soil (with the addition of the *P. frequentans* inoculum) total phenanthrene removal was 63 ± 2.8% and was maximal at day 27. Therefore, sterilized soil removed less phenanthrene and took longer to remove this lower amount. According to the results from an ANOVA statistical analysis, the factors that were statistically significant for phenanthrene removal were: time after the start of bioremediation ($P < 0.0001$), treatment type ($P < 0.0005$) and soil type, i.e. sterilized and non-sterilized soil ($P < 0.005$). Maximal removal is higher in the non-sterilized soil and occurs earlier on day 21, rather than day 24, again indicating a possible synergistic action of the combined fungi and microbes from bagasse. The data presented here confirm bioremediation has occurred, although it is acknowledged that contamination of soil from the environment, rather than by spiking in the laboratory, would have resulted in more strongly bound phenanthrene, which would have been harder to degrade. However, the importance of the presented results lies with the changes in metal behavior subsequent to bioremediation, rather than with bioremediation *per se*.

Cr fluxes

Figure 1 shows fluxes of chromium in both sterilized and non-sterilized soil as measured by DGT. Chromium fluxes increased after fungal inoculation (day 15) in all cases. 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 sterilized soil and by similar, but elevated levels (0.3–0.4 pg cm^{-2} s^{-1}) in non-sterilized soil. There was no significant difference ($P < 0.05$) between treatments A (Soil + fungus + phenanthrene) and B (Soil + fungus) in non-sterilized soil (Figure 1a), but there was a significant difference in the sterilized soil. The factors that were statistically significant in metal fluxes were: soil type and treatment type ($P < 0.005$) and time after the start of bioremediation ($P < 0.003$). In the LSD test (Critical value of $T = 2.31$, $\alpha = 0.05$, LSD = 0.0352), there were significant differences in all treatments over time in sterilized soil.

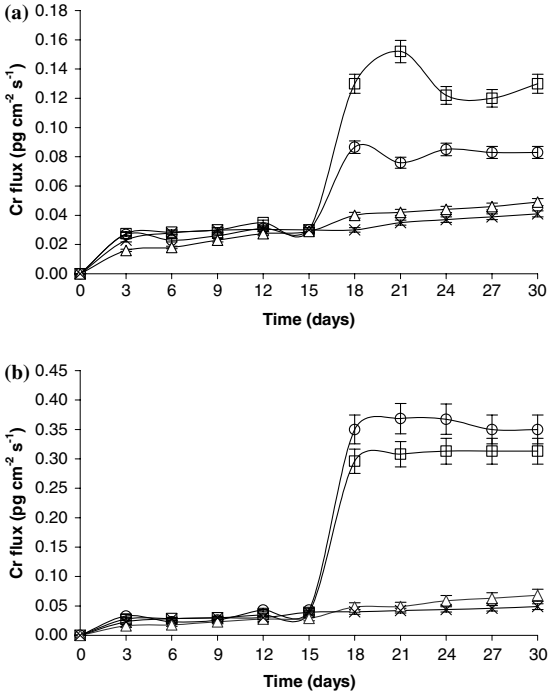

Figure 1. Chromium fluxes measured by DGT: (a) sterilized soil; (b) non-sterilized soil. Treatment A (O): soil + fungus + phenanthrene, Treatment B (□): soil + fungus, Treatment C (△): soil + phenanthrene, Treatment D (x): soil. Soil and fungi were mixed at day 15. Two different scales are used to clarify the trends of sterilized and non-sterilized soils.

Addition of phenanthrene results in higher Cr fluxes from solid phase to solution in the non-sterilized soil, compared to the sterilized soil (Figure 1). We postulate that Cr fluxes into solution are due to the action of microbial activity. This suggests that the phenanthrene may be less toxic and more readily utilized by the action of the native microflora and added fungi together compared to the action of the added fungi alone, suggesting a synergistic behavior. Moreover, consideration of CO_2 evolution suggests that heterothrophic activity in the non-sterilized soil is much greater than in the sterilized soil, which is reflected

in its higher metal fluxes and higher phenanthrene removal.

Maximal net fluxes due to the fungal activity in the presence of phenanthrene to a large extent could be explained in terms of phenanthrene toxicity on non-sterilized soil. In sterilized soil, phenanthrene must be reducing fungal activity (lower fluxes) due to its toxicity. In the presence of native soil microflora (non-sterilized soil), the synergistic behavior observed between *P. frequentans* and native soil microflora means that microorganisms can use phenanthrene as a carbon and energy source, while minimizing any toxic effects. However, the difference in total phenanthrene removal is only 10%, between sterilized and non-sterilized soil. Therefore, increased the ability to use phenanthrene as an energy source is likely to be insufficient to explain the increase in Cr fluxes due to microbial activity. Thus, we suggest that the presence of soil microflora helps to offset the toxic effects of phenanthrene.

Chromium concentration measured by DGT

Figure 2 shows the concentration of Cr measured by DGT. Chromium in pore waters significantly ($P < 0.05$) increased after adding soil with or without microflora and *P. frequentans* inoculum in a similar manner to the increased DGT fluxes. Prior to fungal addition, the concentration of Cr in pore waters is < 0.2 $\mu g\ l^{-1}$ in both types of soil and increases to 0.5 $\mu g\ l^{-1}$ in sterilized soil and to 10 $\mu g\ l^{-1}$ in non-sterilized soil after addition of fungus. This represents an crease of 50-fold in Cr concentration in non-sterilized soil.

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. However, DGT concentrations are not absolute measures of solution metal concentration, as there may be insufficient Cr to supply the DGT device. 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

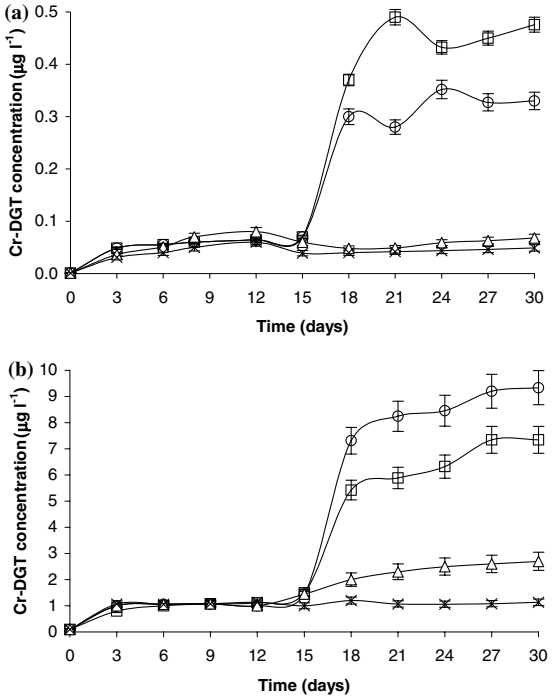

Figure 2. Chromium concentration in pore waters measured by DGT: (a) sterilized soil; (b) non-sterilized soil. Treatment A (O): soil + fungus + phenanthrene, Treatment B (□): soil + fungus, Treatment C (△): soil + phenanthrene, Treatment D (x): soil. Soil and fungi were mixed at day 15. Two different scales are used to clarify the trends of sterilized and non-sterilized soils.

(Lead *et al.* 1997; Davison *et al.* 2000a). Although the exact nature of the speciation measurement made by DGT has not been elucidated, it does measure the most potentially bioavailable fraction, e.g. most mobile and labile metal species. 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. Therefore, the bioremediation process has been shown to cause an increase in potential plant uptake of Cr.

Chromium concentration measured by filtration

Data showing soil solution concentration measured by filtration are shown in Figure 3 Prior to fungal inoculation, concentrations were around 0.5 $\mu g\ l^{-1}$ and increased by up to 8-fold after fungal inoculation in sterilized soil (4 $\mu g\ l^{-1}$) and

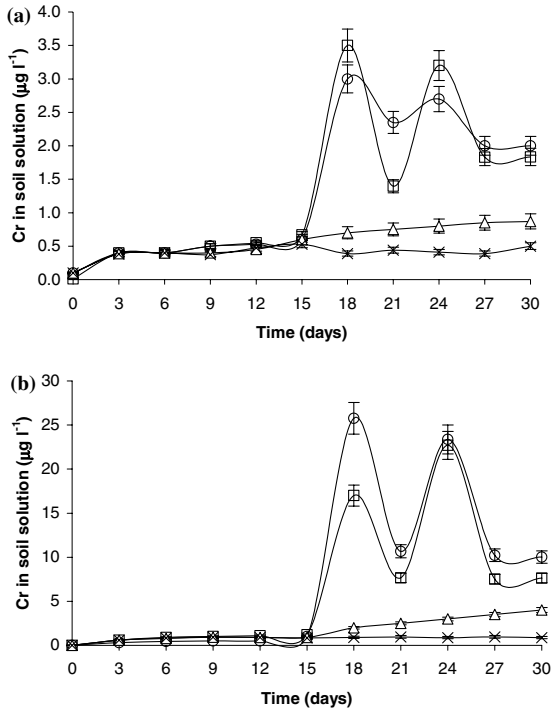

Figure 3. Chromium concentration in soil solution measured by filtration: (a) sterilized soil; (b) non-sterilized soil. Treatment A (O): soil + fungus + phenanthrene, Treatment B (□): soil + fungus, Treatment C (△): soil + phenanthrene, Treatment D (x): soil. Soil and fungi were mixed at day 15. Two different scales are used to clarify the trends of sterilized and non-sterilized soils.

by 50-fold times (25–30 μg l^{-1}) in non-sterilized soil. Fungal activity had a significant ($P < 0.005$) positive effect on metal concentration in soil solution fluxes after the inoculation of *P. frequentans* in both type of soils. Moreover, a bimodal distribution was found in Cr concentration after bioremediation. Two peaks in chromium concentration measured by filtration (< 0.45 μm) were found at days 18 and 24 in both sterilized and non-sterilized soils (Figure 3). During the phenanthrene bioremediation process, we postulate that the initial action of the fungi and soil microflora is to breakdown the solid soil to smaller, 'solution-phase' material, which contains Cr, leading to a peak in Cr concentration at day 18. Subsequently, this more bioavailable material will be used as carbon and energy source until depleted. Simultaneously, the Cr will be removed from solution (day 21). Subsequently, the fungi (in sterilized soil) and *P. frequentans* and soil microflora (in non-sterilized soil) will break down the solid soil again, leading to a Cr peak at day 24 and a trough at day 27. This behavior will not be identified by DGT, which gives a time-averaged metal concentration rather than a spot sample as with filtration. We acknowledge that further experiments including higher frequency sampling are required to unambiguously identify these peaks.

Chromium concentrations were much larger in soil solution measured by filtration than by DGT. There are two reasons for this. Firstly, because DGT underestimates concentrations due to reduced resupply from the solid phase of Cr to the DGT device. This study provides the first data on flux measurements of Cr in soil measured by DGT. However, there is a substantial literature data suggesting that fluxes from solid to solution are sufficient to resupply the DGT device for certain labile metals (e.g. Cd, Zn) and is insufficient for other less labile metals (e.g. Fe, Cu) (Davison *et al.* 2000a). Based on our knowledge of Cr chemistry, we expect that Cr fluxes will not fully meet the demand of DGT device, leading to an underestimate of concentration. Secondly, DGT measures more the mobile and labile fraction of metal species than filtration. Therefore, the two techniques perform different speciation measurements, with DGT measuring a smaller fraction of Cr. This difference has also been observed in freshwaters (Ozdak *et al.* 2002). However, the combination of both methods allows the consideration of a number of aspects of Cr behavior and provides extra information 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. These data are therefore useful in interpreting the chemical and physical behavior of chromium. In addition, metal bioavailability has been shown to be related to the concentration of labile metal species in solution (Hooda *et al.* 1999).

Although most soils contain significant amounts of chromium, its bioavailability is limited by being strong bounded to solid soil matrix, but it can be highly toxic in certain labile forms (ATSDR 2000). These results have indicated both the increase in the bioavailable fraction of metal and a possible mechanism by which the bioremediation process releases chromium from soil. Since the bioremediation of soil by *P. frequentans* increases the lability and mobility of trace metal species in

soil and the fluxes from solid to solution, it is likely to be significant in making Cr available to plants and other biota.

Biological activity was low in this study soil contaminated with PAHs and heavy metals. Therefore, bioaugmentation (the supplementation of microorganisms) and biostimulation (the stimulation of microbial degradation by the addition of nutrients) are ideal methods for bioremediation (Riha *et al*. 1993). In addition, it is well known that bioremediation of organic pollutants has advantages over other techniques as it is cheap, non-destructive and contamination remains localized (Wilson & Jones 1993; Riser-Roberts 1998). Despite these advantages, it is clear from these results that chromium flux, concentration and speciation are altered after phenanthrene bioremoval. This alteration is such that the resulting Cr is more labile and potentially bioavailable. These results therefore highlight the impact of bioremediation of organic contaminants on trace metal behavior. Although not directly quantified, it is likely that the increase in labile Cr is as the mobile Cr(VI) ion, as soils are under aerobic conditions. Readily soluble Cr(VI) in soils is highly toxic to plants and animals (Kabata-Pendias 2001), and more so than Cr(III). The bioremediation process therefore potentially 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.

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