Short Communication

Pentachlorophenol transformation in soil: a toxicological assessment

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Abstract

Bioremediation is recognised as an economically viable method to treat contaminated soil and the use of microbial inoculants, in particular, white-rot fungi, has been proposed to enhance the remediation process. During bioremediation, a variety of pollutant transformation products will be created which may have toxic synergistic interactions and may not at all be detected by chemical analysis. This work examined the potential for formation of toxic breakdown products during pentachlorophenol (PCP) transformation in soil after inoculation with Phanerochaete chrysosporium; a frequently used fungal inoculant. To monitor toxicity during bioremediation, changes in soil dehydrogenase activity and effects of soil methanol extracts on the growth of a common soil bacterium Bacillus megaterium in liquid culture were determined. After 6 weeks of remediation, soil PCP levels had dropped from an initial 250 mg kg$^{-1}$ to 2 mg kg$^{-1}$, and inoculation with P. chrysosporium did not improve PCP remediation over uninoculated PCP-contaminated soil. Soil dehydrogenase activity remained very low in all soils containing PCP and did not recover throughout the experiment (6 weeks) despite the decrease in PCP levels. Soil methanol extracts varied in their toxicity towards growth of B. megaterium and were most toxic after 6 weeks incubation when extracts obtained from PCP-contaminated soil inoculated with P. chrysosporium completely inhibited B. megaterium. A longer incubation time would probably result in removal of toxic products and as soil methanol extracts were used in growth inhibition studies, the bioavailability of these toxic compounds remains in question. However, this work indicates that toxic transformation products may be formed during bioremediation and that ecotoxicological assays are useful to complement chemical analysis during bioremediation of contaminated soil.

Keywords: Bioremediation; Soil; White-rot fungi; Toxicity; Pentachlorophenol; Phanerochaete chrysosporium

Pentachlorophenol is a general biocide and has been used for a variety of purposes such as agriculture and timber preservation. Worldwide use of the chemical has led to severe contamination problems particularly around former timber treatment plants (Ceroici and Beresford, 1993; Yu and Shephard, 1997). The known toxicity (Megharaj et al., 1998) and persistence of PCP necessitates the clean-up of contaminated sites, especially if they are to be redeveloped for housing or if there are other human exposure risks involved. A variety of treatment options are available and bioremediation has been used as a commercial remediation method (Valo and Salkinoja-Salonen, 1986). The success of any commercial remediation operation is generally determined by chemical analysis only and is based upon the successful removal of the parent compound, in this case, PCP. However, during the microbial transformation of PCP, a variety of breakdown products may be formed some of which may be as toxic, or even more toxic than the parent product. It has been estimated that there are over 30 PCP transformation products (Middaugh et al., 1993) and toxicity of
biodegraded PCP samples towards fish embryos (Menidia beryllina) has been observed (Middaugh et al., 1993). Due to the heterogeneous (physical, chemical and biological) nature of soil it is likely that a variety of microbial (aerobic and anaerobic) and chemical transformations will occur during bioremediation, leading to the possible formation of many breakdown products. Chemical analysis of such mixtures is time consuming and also gives no indication of the possible synergistic toxic effects that may occur. Therefore, it appears that methods which give an indication of residual soil toxicity after bioremediation will supplement more traditional chemical analysis and give a good estimate of the success of the reclamation process. However, it is first necessary to demonstrate that toxic products are formed during bioremediation of contaminated soil. To examine this possibility it was decided to spike soil with a known amount of PCP and examine the toxicity of the soil using dehydrogenase activity as a measure of microbial activity over time. In addition, toxicity of soil methanol extracts was determined by addition to a culture of Bacillus megaterium previously found to be sensitive towards PCP (R. McGrath, unpub. Ph.D. thesis, University College Dublin, 1995). Bacillus megaterium was chosen as representative of common soil bacteria since certain Bacillus species have been shown to be sensitive to PCP transformation products (Ruckdeschel and Renner, 1986). In addition, B. megaterium grew well in the soil methanol extracts used for the toxicity assay.

Sieved soil (2 mm, 40 g) was amended to 250 mg PCP kg\(^{-1}\) with PCP dissolved in 25 mM NaOH. Samples were then inoculated with Phanerochaete chrysosporium CMI 174727 (a model fungal inoculant) grown on peat (2.5 g peat per 40 g soil) or left uninoculated. Uncontaminated soil samples (with or without peat addition) were also included as controls. Soil was collected from the campus at University College Dublin and was a clay loam (pH 6.4). Samples were incubated at 25°C for 6 weeks and maintained at a constant moisture content (30% w/w) by regular addition of sterile distilled water. Sterile soils (autoclaved on three consecutive days for 1 h at 121°C) were also included to determine the amount of PCP transformation in the absence of microbial activity. Four replicates of each treatment were used. Analysis of test samples was carried out after 1 week of incubation and all subsequent analyses carried out on a fortnightly basis.

PCP concentrations in soils were determined after soxhlet extraction and subsequent HPLC analysis (McGrath and Singleton, 1997). Anhydrous sodium sulphate (5 or 10 g) was added to a cellulose extraction thimble, overlaid with soil (5 or 10 g) at 25% (w/w) moisture content and extracted for 16 h with hexane. Extracts were concentrated to approximately 5 ml in a rotary film evaporator and transferred to volumetric flasks (10 ml). Evaporating flasks were rinsed with hexane and washings added to volumetric flasks. After equilibration at 20°C volumes were made up to 10 ml. Samples were filtered through PTFE filters (Gelman, USA) and stored in glass Universal bottles at −18°C prior to analysis. HPLC analysis was carried out on an Envirosep-PP column (125 × 3.2 mm, Phenomenex, England) maintained at 45°C by a temperature control unit (Model 3, Waters Associates, USA). Flow rate was maintained at 1 ml min\(^{-1}\) by a gradient controller (Model 680, Waters Associates, USA) and solvent delivery system (M45, Waters Associates, USA). Mobile phase was methanol:water 70:30. Samples (20 µl) were applied to the column by a Marathon autosampler (Spark Holland BV, The Netherlands). PCP was detected at 254 nm and readings integrated by a data module (Model 740, Waters Associates, USA). PCP concentrations were calculated by reference to appropriate standard PCP solutions.

Dehydrogenase activity in soil was assessed by the method of Bauer et al. (1991). In this, soil (5 g) was mixed with 5 ml of 2,3,5-triphenyl tetrazolium chloride reagent and incubated at 25°C for 24 h. After this, 25 ml acetone was added and samples incubated for a further 2 h with periodic mixing. Samples were then filtered through Whatman No. 2 filter paper and diluted with acetone to bring readings within range of the standards. The absorbances of standards and samples were read at 546 nm on a LKB Biochrom Ultraspec 2 (LKB, England). Relevant controls allowing for background colour development in soils were included (Bauer et al., 1991).

The toxicity of soil towards B. megaterium was tested by the following method. Soil samples (10 g) from all test flasks were soxhlet extracted with methanol for 4 h. Extracts were concentrated till a final volume of 10 ml was obtained. Samples (1 ml) of extracts were sterilised by filtration (0.2 µm, non-organic binding filter, Gelman, USA) and added to 10% (w/v) Tryptone Soy Broth (TSB) (23 ml). To this was added B. megaterium inoculum (1 ml, Optical Density (OD) at 650 nm of 0.3) from a culture grown in 10% TSB for 18 h at 37°C and 160 rpm\(^{-1}\). An OD of 0.3 at 650 nm was obtained by addition of sterile distilled water to the culture as required. Final volume of TSB, soil extract and inoculum was 25 ml. Cultures were subsequently incubated at 37°C, 160 rpm in the dark for 8 h. OD at 650 nm was measured periodically on a Coleman spectrophotometer (Perkin Elmer, USA).

When required comparison between means was carried out using ANOVA followed by Tukey Multiple Comparisons test or Student t-test. All statistical analysis was carried out using Instat statistical package (Graph Pad, Intuitive Software for Science, USA).
Analysis of raw data showed normal distribution allowing the above statistical tests to be carried out.

PCP concentrations in all non-sterile soils declined to approximately 2 mg PCP kg\(^{-1}\) after 6 weeks incubation at 25\(\circ\)C (Fig. 1). Inoculation with \(P.\) \textit{chrysosporium} did not alter the final amount of PCP degradation obtained. No PCP transformation was observed in sterile soils and PCP extraction efficiencies of at least 90\% were obtained. This demonstrates that despite the lack of previous exposure of this soil to PCP, an active soil microbial population capable of PCP transformation was present. We did not attempt to examine for the presence of PCP degradation products. Clearly given the right environmental conditions, PCP is not persistent. However, higher PCP concentrations and repeated PCP addition coupled with sorption of the chemical to soils high in organic matter (Lee et al., 1990) will alter persistence levels obtained.

Dehydrogenase activity decreased considerably in all soils contaminated with PCP (Fig. 2) and did not recover even though PCP concentrations had dropped to 2 mg kg\(^{-1}\) in soil after 6 weeks incubation. Dehydrogenase activity reflects soil microbial activity (Gerber et al., 1991) and this result shows that there is some residual soil toxicity after 6 weeks bioremediation, even though PCP concentrations were apparently very low. This suggests either that toxic PCP transformation products were formed or that the soil microbes had not fully recovered from initial toxic responses towards PCP. Such a large decrease in dehydrogenase activity was unexpected.

Methanol soil extracts were prepared from incubated control and test soil samples every 2 weeks and subsequently added to \(B.\) \textit{megaterium} cultures to determine the presence of methanol extractable toxic compounds. It was assumed that a decrease in growth of \(B.\) \textit{megaterium} would indicate that toxic compounds (potential PCP transformation products) were present. The most apparent toxic effect was observed with extracts prepared from soils which had been incubated for 6 weeks. Methanol extracts from PCP-contaminated soils with added fungal inocula completely inhibited the growth of \(B.\) \textit{megaterium} (Fig. 3). Extracts from PCP-contaminated soils without fungal inocula gave approximately 40\% inhibition of \(B.\) \textit{megaterium}. Growth was significantly lower (\(P < 0.05\)) than methanol extracts prepared from control flasks containing soil only (Fig. 3). The dilutions involved in preparing soil toxicity tests would have produced PCP concentrations of less than 0.1 mg l\(^{-1}\) in growth media. Pre-
vious work (results not shown) demonstrating that 1 mg PCP l−1 was toxic but not completely inhibitory to *B. megaterium*, suggests that other toxic compounds extracted from soil were present in the growth media. These compounds may have been exerting synergistic toxic effects on *B. megaterium*. Overall it appears that during PCP transformation in soil, some toxic compounds were produced. This result is supported by evidence of the production and persistence of 3,4,5-trichlorophenol and 2,3,4,5 tetrachlorophenol from PCP in soil environments (Kuwatsuka and Igarashi, 1975; Baker and Mayfield, 1980). These metabolites are considerably more toxic to fungi and bacteria than PCP (Ruckdeschel and Renner, 1986; Ruckdeschel et al., 1987), and mean that, even if present in small amounts, no reduction in soil toxicity may be achieved by bioremediation. Extreme care must be taken to avoid their production during bioremediation. The bioavailability of toxic metabolites must remain an issue, as methanol extracts were used to assess residual soil toxicity and compounds extracted by methanol may have been tightly bound to soil particles. However, there is evidence suggesting that sorbed organic compounds are accessed by microbes (Calvillo and Alexander, 1996) and as soil dehydrogenase activity had decreased, it would appear as if toxic compounds were available to microorganisms. It is very likely that given more time the eventual toxicity of the soil would decrease to acceptable amounts, however, we consider that the evidence presented here suggests that toxicological measures of assessing the residual toxicity of soil after bioremediation are required. This is particularly so in the case of large-scale bioremediations where large volumes of soil are treated and it is difficult to maintain optimal environmental conditions.

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**References**


