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Lead mineral transformation by fungi

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Pyromorphite ($Pb_5(PO_4)_3Cl$), the most stable lead mineral under a wide range of geochemical conditions [1], can form in urban and industrially contaminated soils [2–5]. It has been suggested that the low solubility of this mineral could reduce the bioavailability of lead, and several studies have advocated pyromorphite formation as a remediation technique for lead-contaminated land [3,5,6], if necessary using addition of phosphate [6]. Many microorganisms can, however, make insoluble soil phosphate bioavailable [7–10], and the solubilisation of insoluble metal phosphates by free-living and symbiotic fungi has been reported [11–15]. If pyromorphite can be solubilised by microbial phosphate-solubilising mechanisms, the question arises of what would happen to the released lead. We have now clearly demonstrated that pyromorphite can be solubilised by organic-acid-producing fungi, for example *Aspergillus niger*, and that plants grown with pyromorphite as sole phosphorus source take up both phosphorus and lead. We have also discovered the production of lead oxalate dihydrate by *A. niger* during pyromorphite transformation, which is the first recorded biogenic formation of this mineral. These mechanisms of lead solubilisation, or its immobilisation as a novel lead oxalate, have significant implications for metal mobility and transfer to other environmental compartments and organisms. The importance of considering microbial processes when developing remediation techniques for toxic metals in soils is therefore emphasised.

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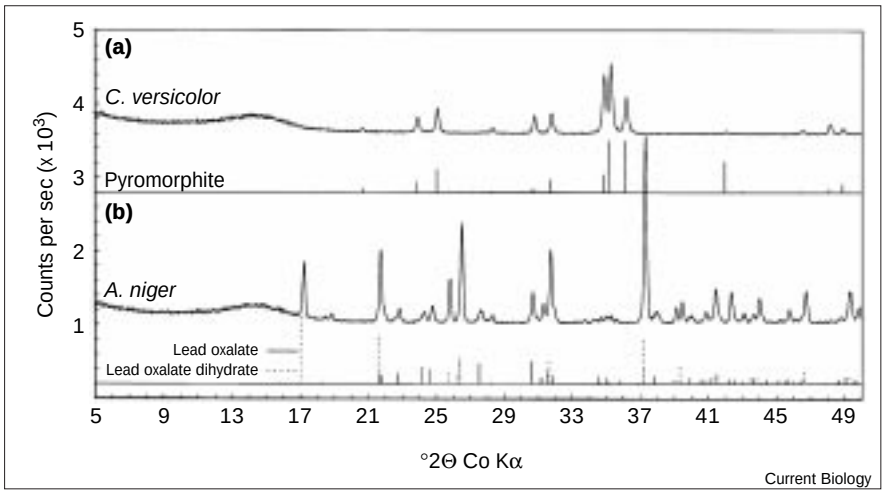
Results and discussion

Incorporation of 0.2% (w/v) pyromorphite in agar had no effect on the growth of the fungi *Coriolus versicolor*, *Penicillium bilaii*, *Rhizopus arrhizus* and *A. niger*, although the biomass of all species contained lead (Pb). After 10 days growth, *A. niger* contained 36.0 ± 9.3 nmol Pb per mg dry weight and the other fungi contained lower amounts: *C. versicolor* 1.14 ± 0.21, *P. bilaii* 3.35 ± 0.45 and *R. arrhizus* 5.23 ± 0.09 nmol Pb per mg dry weight (means ± standard error of the mean; four replicates), indicating that there was more pronounced transfer of lead from the pyromorphite to *A. niger*. Comparison of X-ray diffraction patterns of the 'pyromorphite' extracted from the agar with data from the ICDD (International Centre for Diffraction Data) Powder Diffraction File [16] revealed that samples from the fungal-free control, *C. versicolor*, *P. bilaii* and *R. arrhizus* were pure pyromorphite (Figure 1a), whereas this phase was absent from the sample taken from *A. niger*. This suggested that *C. versicolor*, *P. bilaii* and *R. arrhizus* had little ability to solubilise pyromorphite.

The *A. niger* sample contained lead oxalate and lead oxalate dihydrate, the latter identified by comparison of the X-ray diffraction pattern with that calculated from the crystal structure data for synthetic lead oxalate dihydrate (Figure 1b) [17]. Re-examination after heating revealed that the peaks from the lead oxalate dihydrate had disappeared and the intensity of peaks for anhydrous lead oxalate had increased, suggesting loss of water of crystallisation. Scanning electron microscopy of the pyromorphite extracted from the agar of fungal-free controls (Figure 2a) or from under colonies of *C. versicolor*, *P. bilaii* and *R. arrhizus* after 10 days growth (Figure 2b) revealed identical porous structures. The sample from the *A. niger* culture (Figure 2c), however, showed two distinct morphologies: fans of prismatic needles (Figure 2d) and pseudohexagonal platy crystals (Figure 2e). The pseudohexagonal symmetry expressed by the platy morphology (Figure 2e) is indicative of anhydrous lead oxalate (Figure 2f). The presence of dehydration cracks in the needles obtained from *A. niger* after heating also suggests that they are lead oxalate dihydrate [17]. Subsequent examination of the samples from *A. niger* by X-ray diffraction also revealed small amounts of lead carbonate, possibly an oxalate degradation product. It is apparent that pyromorphite is solubilised to produce lead oxalate dihydrate, which forms fans of needles radiating out from the original pyromorphite grains, as shown in Figure 3. It is therefore probable that *A. niger* produces only the dihydrate, which subsequently becomes anhydrous. *A. niger* was therefore able to completely solubilise pyromorphite, presumably extracting the phosphate and immobilising the majority of the lead as oxalate compounds in the process.

Figure 1

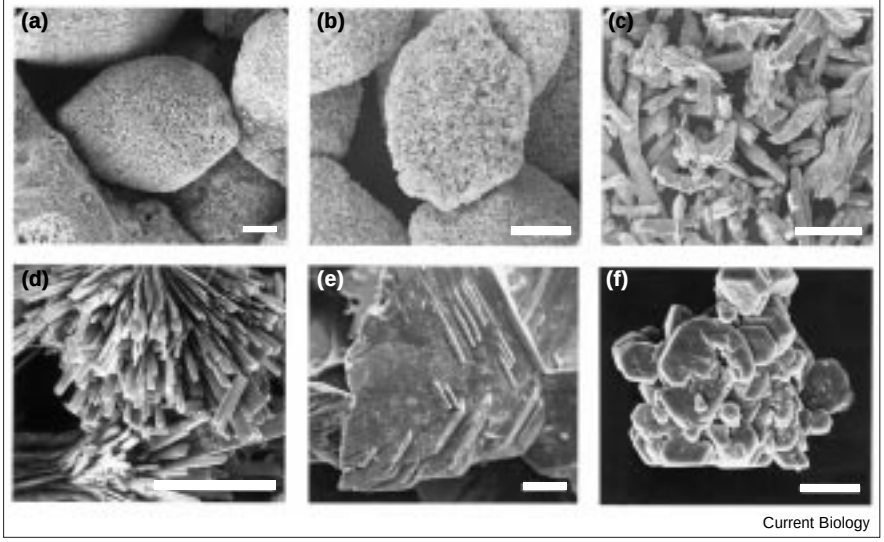


Typical X-ray diffraction patterns of pyromorphite phases extracted from agar. (a) The pattern from *C. versicolor* is compared to the pattern for pyromorphite taken from the ICDD Powder Diffraction File (PDF 19-0701) [16]. Patterns for the fungal-free controls, *P. bilaii* and *R. arrhizus*, are not shown but were identical to that for *C. versicolor*. (b) The pattern from *A. niger* is identified as a mixture of lead oxalate and lead oxalate dihydrate by comparison to the ICDD pattern for lead oxalate (PDF 14-803) [16] and an X-ray diffraction pattern for lead oxalate dihydrate calculated from crystal structure data [17].

Whereas most crystalline metal oxalates are highly insoluble [18,19], it is unlikely that lead oxalate would be thermodynamically stable in many soils. Figure 4 shows that the stability field for lead oxalate (that is, the geochemical conditions under which lead oxalate is stable) is at its minimum oxalate activity of $10^{-5.4}$ generally between pH 4 and 5.5 with respect to other lead minerals. Determinations of oxalate concentration are of the order of 10^{-6} M for forest soil solutions [20] which, if oxalate is controlled at this activity, is too low for lead oxalate to be stable. Lead oxalate is most likely to be stable in acidic environments with low sulphur conditions that suppress the formation of anglesite. Note that the conversion of lead oxalate back to pyromorphite is possible over a wide pH range (Figure 4), which encompasses most of the normal range for soils even at a low phosphate concentration (10^{-9} M). Preliminary laboratory experiments suggest that this backwards conversion can be achieved in days in the presence of dilute phosphate and chloride solutions (data not shown).

Given the efficacy of fungi like *A. niger* in extracting phosphate from minerals, there would be competition for phosphate between such fungi and lead in the soil. The observation of pyromorphite in lead-contaminated soil at normal soil-phosphorus levels [4,5] suggests that a dynamic equilibrium at least exists between chemical and biological processes because, if pyromorphite were solubilised without re-forming, it would not be present. Furthermore, pyromorphite (solubility product, $\log K_{sp} = -84.4$ [1]) is far less soluble than many common phosphate minerals. Therefore,

Figure 2

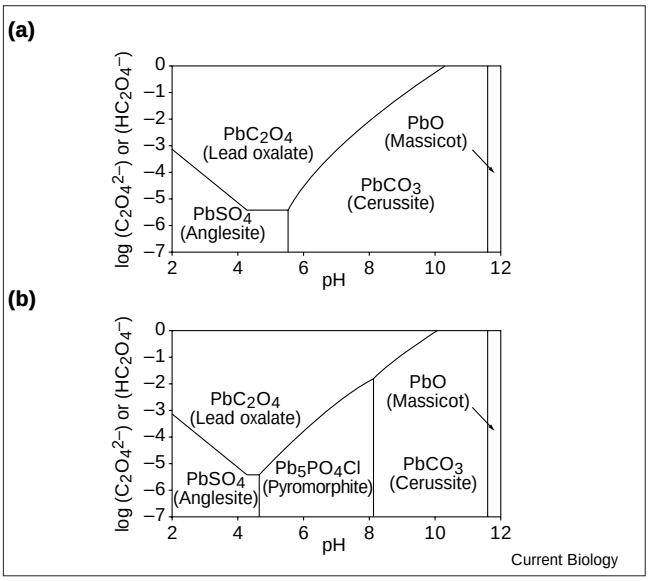


Typical scanning electron micrographs of samples of pyromorphite extracted from agar on which the various fungi had grown. (a) Control agar containing pyromorphite incubated for 10 days in the absence of test fungi. (b) Sample from under colonies of *C. versicolor* that were 10 days old. (c) Sample from under colonies of *A. niger* that were 10 days old. (d) Needle-type crystals from under colonies of *A. niger*, indicative of lead oxalate dihydrate. (e) Platy-type crystals from under colonies of *A. niger*, indicative of anhydrous lead oxalate. (f) Chemically synthesised anhydrous lead oxalate illustrating platy morphology. Scale bars represent 10 μm in (a,b,d–f) and 100 μm in (c).

Figure 3

Scanning electron micrograph showing a partially solubilised grain of pyromorphite from under a colony of *A. niger*. Needles of lead oxalate dihydrate can be seen emerging from the grain. Scale bar represents 10 μm.

Figure 4



The stability fields of lead minerals in the system (a) Pb, C, O, S and H, or (b) Pb, P, Cl, C, O, S and H as a function of pH and oxalate activity. Only oxidised species are considered and hydrocerussite ($Pb_3(CO_3)_2(OH)_2$) is omitted. Pertinent thermodynamic data are from Lindsay [29] except $\Delta G°_f(PbC_2O_4)$ = –747.88 kJ/mol, which is from Kapustinskii *et al.* [30]. Oxalate is considered as $HC_2O_4^-$ below pH 4.27 and as $C_2O_4^{2-}$ above. Activities of phosphate and carbonate species are calculated from their dissociation equilibria, assuming total dissolved (Σ_{diss}) P = 10^{-9} M, $\Sigma_{diss}CO_3 = 10^{-3}$ M and activity coefficients to be unity. The activities of Cl^-_{diss} and $SO_4^{2-}{}_{diss}$ are assumed to be 10^{-3}.

in the natural environment, fungi may preferentially solubilise other phosphate minerals, for example apatite ($Ca_5(PO_4)_3OH$). Apatite is the least soluble calcium phosphate in aerated soil and is therefore less readily available as a phosphorus source than other, more soluble calcium phosphates, for example, brushite ($CaHPO_4.2H_2O$), commonly present in phosphate fertilisers. In addition, organic phosphorus may be more readily available to fungi via enzymatic release. Therefore, pyromorphite may not be subject to such extreme attack in the soil.

Nevertheless, the solubilisation of pyromorphite by *A. niger* demonstrates that pyromorphite may not be as effective at immobilising lead as previous studies have suggested [3,5,6]. Indeed, we have demonstrated uptake of both phosphorus and lead by *Lolium perenne* L. (rye grass), when grown on pyromorphite as sole phosphorus source. Values of shoot phosphorus (± 1 standard deviation, $n = 5$) in Hoaglands' solution alone, in phosphate-free Hoaglands' solution plus apatite and in phosphate-free Hoaglands' solution plus pyromorphite were 42.5 ± 4.6, 24.8 ± 2.4 and 13.3 ± 2.1 μmol/g dry weight, respectively; corresponding values for shoot lead were 29.0 ± 17.4, 48.7 ± 6.3 and 782.0 ± 124.5 nmol/g dry weight, respectively. Shoot phosphorus concentrations from the Hoaglands' alone and apatite treatments were significantly greater ($p < 0.0005$ and $p < 0.05$, respectively) than those of plants grown on pyromorphite, demonstrating the relative difficulty in extracting phosphorus from pyromorphite. Shoot lead concentrations in the pyromorphite treatment were nearly 30-fold greater than background lead concentrations (using Hoaglands' solution as a phosphorus source), however. Although our data for lead uptake in the pyromorphite treatment are within the range of lead uptake by plants grown on lead-amended soils reported elsewhere [21–23], the amount of lead taken up by plants grown on apatite is rather high, possibly because the apatite itself contained some lead. In conclusion, this study has demonstrated the importance of considering microbial processes when developing remediation techniques for toxic metals in soils, because mechanisms of solubilisation or immobilisation can have significant consequences for metal mobility and transfer to other environmental compartments and organisms [24,25].

Materials and methods

Experimental organisms and lead analysis

A. niger (ATCC 201373), *C. versicolor* (Fr.) Quélet (C7B 863A), *P. bilaii* Chalabuda and *R. arrhizus* Fischer (IMI 57412) were used in solubilisation experiments according to [8]. The fungi were inoculated onto 10 cm^3 malt extract agar (MEA), either without (control) or with 0.2% (w/v) pyromorphite, using 7 mm diameter discs of mycelium cut from the edges of colonies which had been grown on MEA at 25°C for at least 24 h [26]. After the agar had set and before inoculation, discs of autoclaved sterile dialysis membrane were placed under aseptic conditions onto the surface of the agar [8,26]. For lead analysis, mycelia were removed and washed for 30 min in 0.1 M HCl before digestion in 2 ml concentrated HNO_3 for 18 h at room temperature. Digests were analysed for lead by atomic absorption spectrophotometry (AAS; Pye Unicam SP9). Pyromorphite was synthesised by mixing solutions of

Pb, P and Cl in stoichiometric proportions (5:3:1). $PbNO_3$ (200 ml of 0.5 M) was mixed with 200 ml of a solution containing 0.3 M Na_2HPO_4 and 0.1 M NaCl at approximately 90°C. When cool, the precipitate was isolated by filtration, washed in deionised water (DIW) and dried at 60°C.

Analysis of solubilisation products

Mycelia were removed from the agar and the pyromorphite was extracted by gently homogenising with warm deionised distilled water (ddH_2O) [8,26]. Scanning electron microscopy used a JEOL JSM-35. Morphological analysis of the sample from *A. niger* was repeated after heating in an oven at 100°C for 2 h. X-ray diffraction was used to confirm the purity of the pyromorphite and the identity of the compounds produced by the fungi. X-ray diffraction analysis of the sample from *A. niger* was repeated after heating at 100°C for 2 h.

Uptake of phosphorus and lead by L. perenne *L.*

Seeds of *L. perenne* were sterilised in H_2O_2 for 1 h, rinsed in DIW and germinated on acid-washed sand (pH 6.8). After 20 days they were transplanted into pots containing either sand or sand plus phosphate minerals (apatite or pyromorphite), giving 500 mg phosphorus per kg dry weight. Plants were supplied with phosphate-free 1/10 strength Hoaglands' solution (0.5 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.5 mM FeEDTA, 0.10 μM H_3BO_3, 2.9 μM $MnCl_2$, 0.16 μM $ZnCl_2$, 0.074 μM $CuCl_2$, 0.022 μM $Na_2MoO_4.2H_2O$) every second day for 30 days except for the control, which was given Hoaglands' solution containing 0.1 mM KH_2PO_4 [27]. All tissues were soaked in 1 mM NaEDTA for 3 h [28] before analysis by atomic absorption spectrophotometry.

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