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Phosphatase-mediated bioprecipitation of lead by soil fungi

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30 **Running title:** Phosphatase-mediated lead bioprecipitation by fungi

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Summary

Geoactive soil fungi were examined for their ability to release inorganic phosphate (P_i) and mediate lead bioprecipitation during growth on organic phosphate substrates. *Aspergillus niger* and *Paecilomyces javanicus* grew in 5 mM $Pb(NO_3)_2$ -containing media amended with glycerol 2-phosphate (G2P) or phytic acid (PyA) as sole P sources, and liberated P_i into the medium. This resulted in almost complete removal of Pb from solution and extensive precipitation of lead-containing minerals around the biomass, confirming the importance of the mycelium as a reactive network for biomineralization. The minerals were identified as pyromorphite ($Pb_5(PO_4)_3Cl$), only produced by *P. javanicus*, and lead oxalate (PbC_2O_4), produced by *A. niger* and *P. javanicus*. Geochemical modelling of lead and lead mineral speciation as a function of pH and oxalate closely correlated with experimental conditions and data. Two main lead biomineralization mechanisms were therefore distinguished: pyromorphite formation depending on organic phosphate hydrolysis and lead oxalate formation depending on oxalate excretion. This also indicated species specificity in biomineralization depending on nutrition and physiology. Our findings provide further understanding of lead geomycology and organic phosphates as a biomineralization substrate, and are also relevant to metal immobilization biotechnologies for bioremediation, metal and P biorecovery, and utilization of waste organic phosphates.

Keywords: lead, pyromorphite, lead oxalate, fungi, phosphatase, geochemical modelling, biomineralization

Introduction

Lead is one of the most widely found toxic metal pollutants in the environment, and arises from natural weathering of rocks and industrial activities, including mining and smelting of lead ores, manufacture of lead-acid batteries, ammunition waste and colouring elements in pigments (Flora *et al.*, 2012). In recent years, the application of metal and mineral transformation capabilities of microbes in bioremedial treatments for metal contamination has been widely accepted (Ruby *et al.*, 1994; Cotter-Howells, 1996; Sayer *et al.*, 1999; 2001; Fomina *et al.*, 2007, 2008; Debela *et al.*, 2010; Rhee *et al.*, 2012; 2014a,b; Wei *et al.*, 2013; Gupta and Lu, 2013; Li *et al.*, 2014; Gadd *et al.*, 2014). Pyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{X}$ (X = OH, Cl, or F)) is one of the most stable lead minerals and its formation can reduce the bioavailability of lead in the environment (Cotter-Howells and Giddens, 1990; Ruby *et al.*, 1994; Debela *et al.*, 2010; Rhee *et al.*, 2012; 2014a,b). It is a secondary lead mineral and can be formed by the weathering of galena (PbS) under oxidizing conditions although its formation may be limited by the availability of phosphorus (Cotter-Howells and Giddens, 1990). A commonly proposed strategy to immobilize Pb in the environment is by formation of pyromorphite by addition of an inorganic phosphate (P_i) source such as Ca-phosphate, orthophosphate, or polyphosphate (Cotter-Howells, 1996; Cotter-Howells and Caporn, 1996; Hashimoto *et al.*, 2009; Dick *et al.*, 2011). The elevation of P_i concentration and combination with Pb^{2+} and Cl^- results in precipitation as pyromorphite (Ruby *et al.*, 1994; Ma *et al.*, 1995; Cotter-Howells, 1996; Cotter-Howells and Caporn, 1996). Under general geochemical conditions, the pyromorphite family $\text{Pb}_5(\text{PO}_4)_3\text{X}$, where X = F, Cl, Br, OH, are the most stable environmental Pb compounds ($\text{Pb}_5(\text{PO}_4)_3\text{F}$, $K_{\text{sp}} = 10^{-71.6}$, $\text{Pb}_5(\text{PO}_4)_3\text{Cl}$, $K_{\text{sp}} = 10^{-84.4}$,

Pb₅(PO₄)₃Br, $K_{sp} = 10^{-78.1}$, and Pb₅(PO₄)₃OH, $K_{sp} = 10^{-76.8}$) over a wide range of conditions (Chen *et al.*, 2007; Chrysochoou *et al.*, 2007). However, it has been clearly demonstrated that certain microbial metabolites, e.g. mycogenic organic acids, can affect pyromorphite stability (Sayer *et al.*, 1999; Fomina *et al.*, 2004; Manecki and Maurice, 2008; Debela *et al.*, 2010; Topolska *et al.*, 2014). The formation of lead oxalate from pyromorphite by several free-living and mycorrhizal fungi has been demonstrated (Sayer *et al.*, 1999; Fomina *et al.*, 2004).

Phosphorus is abundant in the environment, but most is in an insoluble form, and soluble P_i readily reacts with a variety of divalent and trivalent cations in soil or aquatic systems to form inorganic compounds or large insoluble complexes (Van Ho *et al.*, 2002). Insoluble inorganic and organic-P compounds can be utilized by bacteria and fungi by, e.g. organic acid production and phosphatase enzyme activity respectively (Whitelaw, 2000; Chen *et al.*, 2007). Microbes can import phosphate from phosphate-containing organic compounds by first cleaving C-O-P ester bonds with phosphatases that release P_i (Hyšek and Šarapatka, 1997; Hayes *et al.*, 2000; Yadav and Tarafdar, 2001; Bull *et al.*, 2002). Phosphate-solubilizing bacteria and fungi can solubilize inorganic phosphate-containing minerals, e.g. apatites and other phosphates (Sayer *et al.*, 1995, 1997, 1999; Di Simine *et al.*, 1998; Sayer and Gadd, 1997, 2001; Whitelaw, 2000; Jacobs *et al.*, 2002a,b; Fomina *et al.*, 2004, 2005; Ahemad *et al.*, 2009; Topolska *et al.*, 2014) but can also release phosphate from organic phosphate sources through phosphatase activity (Aickin *et al.*, 1979; ; Macaskie *et al.*, 1992, 1994, 2000; Yong and Macaskie, 1995; Boswell *et al.*, 1999, 2001; Dick *et al.*, 2011). Released phosphate from organic sources can precipitate with metals forming insoluble metal phosphates, a phenomenon first characterized in bacteria (Aickin *et al.*, 1979; Macaskie *et al.*, 1992, 1994, 2000; Yong and Macaskie,

1995; Boswell *et al.*, 1999, 2001). Formation of metal phosphates has been observed in fungi, e.g. *Penicillium ochro-chloron* precipitated cupric oxalate and cupric phosphate (Crusberg, 2004), while *Penicillium cyclopium* removed up to 95% supplied lead in the presence of phosphate (Tsekova *et al.*, 2006). Rhee *et al.* (2012, 2014a,b) have shown fungal mediation of pyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$) formation from lead metal. Under conditions of Cu and Cd toxicity, the ectomycorrhizal fungus *Xerocomus chrysenteron* was exuded soluble protein and acid phosphatase that appeared to be involved in metal tolerance and precipitation (Zheng *et al.*, 2009). *Laccaria laccata* and *Suillus bovinus* have also been shown to produce more acid phosphatase in the presence of high concentrations of Cr (VI) (Raman *et al.*, 2002).

The objective of this work was to determine the possible role of fungal phosphatases in the bioprecipitation of lead phosphate when organic phosphate substrates are the only substrate source of phosphorus. The pH and P dependence of lead phosphate formation, and the significance of excreted oxalate in lead biomineralization was also investigated, coupled with geochemical modelling of lead and lead mineral speciation as a function of pH and oxalate.

Results

Growth and P_i release from different organic-P sources

All fungi were able to grow in organic phosphorus-amended MCD media at both pH 5 and pH 8. Negligible growth was observed in MCD medium without any phosphorus source. With equivalent amounts of P in the MCD medium, all fungi grew better and released more P_i into the medium when amended with 30 mM G2P than with 5 mM PyA. Among the test fungi, *A. niger* grew well with G2P and released up to 23 mM P_i from 30 mM G2P after 30 days incubation at 25°C. There were no significant differences in the amount of P_i released by *A. niger* and *P. javanicus* when grown with G2P at the different initial pH values. *P. javanicus* had a higher ability to hydrolyze PyA than the other test species, with 8.5 mM P_i released from 5 mM PyA after growth in MCD medium for 30 days at both initial pH values (Fig. 1). There were no significant differences in growth rate and P_i release by the organisms when grown at different initial pH values except for *B. caledonica* and *R. rubescens*. The latter released more P_i from the organic phosphorus sources in acidic rather than in alkaline conditions. *B. caledonica* and *R. rubescens* released up to 18 and 13 mM P_i, respectively, from 30 mM G2P in acidic conditions, compared with 11 and 7 mM P_i, respectively, in alkaline conditions. From analysis of the fungal growth rates and P_i release from organic-P, *A. niger* and *P. javanicus* were selected for detailed experiments.

Growth and P_i release from different organic-P sources in the presence of Pb by *A. niger* and *P. javanicus*

In the presence of $\text{Pb}(\text{NO}_3)_2$, growth of *A. niger* was reduced, while *P. javanicus* showed a relatively better tolerance in lead-amended MCD medium (Table 1). Tolerance indices (TI) were used to compare the biomass yields of *A. niger* and *P. javanicus* grown on MCD medium with or without 5 mM $\text{Pb}(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA (Table 1). A TI value lower than 100% indicates growth inhibition. Biomass yields of *A. niger* were markedly reduced in the presence of 5 mM $\text{Pb}(\text{NO}_3)_2$ when grown with 30 mM G2P, with growth inhibition resulting on 5 mM $\text{Pb}(\text{NO}_3)_2$ and 5 mM PyA. For *P. javanicus*, there was some inhibition of biomass yield over the first 10 days incubation, but after this time, biomass yields were generally larger than on control medium. The pH of all MCD media was adjusted to pH 6 before inoculation. The pH value of medium containing 30 mM G2P dropped to pH 3.1 after growth of *A. niger* for 30 days while the corresponding pH for *P. javanicus* in the same culture medium increased to pH 6.8. The pH values of medium containing 5 mM PyA showed similar trends after growth of *A. niger* and *P. javanicus* (Table 1). P_i concentrations in MCD medium containing 30 mM G2P or 5 mM PyA increased with time during growth of *A. niger* and *P. javanicus*. *A. niger* released around 25-26 mM P_i from 30 mM G2P and 5 mM PyA respectively after 30 days growth (Table 1). Similar concentrations of free P_i were released by *P. javanicus*. However, *P. javanicus* released more P_i at an earlier stage of growth than *A. niger*, with around 9-10 mM P_i being released from *A. niger* and around 15 mM P_i being released from *P. javanicus* after 20 days (Table 1).

Pb removal from solution and the formation of lead mineral phases

Both *A. niger* and *P. javanicus* were able to remove almost all of the lead from media amended with 5 mM Pb(NO₃)₂ and the organic-P sources (Table 1). Both *A. niger* and *P. javanicus* removed most of the Pb²⁺ from solution after 10 days and showed increasing Pb accumulation with time (Table 1).

Both *A. niger* and *P. javanicus* were able to grow in the presence of 5 mM Pb(NO₃)₂ and precipitated secondary minerals (Fig. 2). In contrast to *A. niger* from control Pb-free MCD medium (Fig. 2A), extensive secondary mineral formation occurred when *A. niger* was grown with 5 mM Pb(NO₃)₂ and 30 mM G2P (Fig. 2B). Some secondary minerals with a similar shape were also found on the biomass of *A. niger* grown with 5 mM Pb(NO₃)₂ and 5 mM PyA (Fig. 2E). For *P. javanicus*, the produced secondary minerals showed a completely different shape from those associated with *A. niger*, with arborescent and blade-like shapes above the hyphae (Fig. 2C,F) with some arborescent and globular (Inset Fig. 2C).

Energy-dispersive X-ray analysis (EDXA)

EDXA revealed the elemental composition of the secondary minerals found on the fungal hyphae. The minerals associated with *A. niger* after growth with Pb(NO₃)₂ and G2P showed peaks for carbon, oxygen, potassium and lead as the main elements detected (Fig. 3A). The minerals produced by *P. javanicus* grown with Pb(NO₃)₂ and G2P (Fig. 3B) showed peaks for carbon, oxygen, sodium, phosphorus and lead as the main elements. The minerals produced by *A. niger* grown with Pb(NO₃)₂ and PyA that showed a cylindrical form showed carbon, oxygen, sodium, potassium and lead (Fig. 3C). The minerals produced by *P. javanicus* grown with Pb(NO₃)₂ and PyA (Fig.

3D) showed peaks for carbon, oxygen, sodium, aluminium, phosphorus and lead as the main elements present.

Transmission electron microscopy (TEM) revealed the presence of electron-dense deposits around the biomass of both *A. niger* and *P. javanicus* after growth with 5 mM $\text{Pb}(\text{NO}_3)_2$ and 30 mM G2P (Fig. 4). Compared to the electron-dense granules found with *P. javanicus*, the amount of crystals found with *A. niger* seemed of lower abundance. These observations are consistent with the presence of the secondary minerals found with SEM.

X-ray powder diffraction (XRPD)

The secondary mineral precipitates associated with *A. niger* and *P. javanicus* grown with lead and G2P showed the presence of well-crystallized compounds with an excellent match to reference patterns for lead oxalate (PbC_2O_4) and pyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$) (Fig. 5). Lead oxalate was detected from both *A. niger* and *P. javanicus*, but pyromorphite was only detected with *P. javanicus* (Fig. 5).

Geochemical modelling

The predominance diagrams for chemical species of lead can be simulated by PHREEQC, and this software was used to generate a diagram for the bioprecipitation of lead oxalate and pyromorphite. For lead, the dominant minerals found in the simulated systems were simple oxides, carbonates and phosphates. Lead minerals differ widely in water solubility depending on the presence of H^+ , HCO_3^- , PO_4^{3-} , SO_4^{2-} , and the total ionic charge of the solution. The diagram shows

the most dominant mineral species of lead theoretically present in the MCD medium (Fig. 6). The data was calculated for a temperature of 25°C and with total dissolved lead in the presence of dissolved chloride, carbonate, phosphate, nitrate and sulfate. Fig. 6A indicates that pyromorphite dominates over the range pH 2-11 in the absence of oxalic acid, $\text{Pb}(\text{H}_2\text{PO}_4)_2$ dominates at pH values < 2, $\text{Pb}(\text{OH})_2$ dominates over pH 11-13, and $\text{Pb}(\text{OH})_4^{2-}$ occurs at pH values > 13. In the presence of oxalic acid, the situation is different and lead oxalate and lead hydroxide become the two main species in the system (Fig. 6B). Lead oxalate dominates over the pH-range 2-7, while lead hydroxide dominates over pH 7-13. Pb^{2+} and $\text{Pb}(\text{OH})_4^{2-}$ dominate at pH < 2 and pH > 13, respectively. In Fig. 6C, the mineral stability diagrams as a function of pH and oxalate activity show the dominance of lead oxalate at a pH < 5 and at an oxalate activity > 0.1 mM. These pH-ranges correspond with the pH values measured in the experimental conditions (Table 1). In contrast, anglesite can become dominant over the same acidic pH range when the oxalate concentration is lower than 0.1 mM. Pyromorphite is dominant over the approximate range of pH 4.8-8 even at high concentrations of oxalate. At pH > 8, lead hydroxide becomes dominant at any oxalate concentration. The presence of pyromorphite in the *P. javanicus*-organic-P-Pb system simulation and the presence of lead oxalate in the *A. niger*-organic-P-Pb system closely correlates with the XRPD data, confirming that the theoretical simulation closely agrees with experimental findings. It is important to note that with the function of pH and E_h , the concentration of lead in the aqueous system strongly affects the formation of the different lead species, while aqueous Pb complexes can also undergo several protonation or deprotonation reactions together with polymerization to form various aqueous species of lead.

Discussion

The availability of P is one of the most crucial determinants of microbial growth (Eide, 1998; Whitelaw, 2000). However, a large proportion of the phosphorus pool in nature is insoluble and this raises the question of how organisms obtain P from organic and insoluble phosphate sources (Wenzel *et al.*, 1994; Whitelaw, 2000; Fomina *et al.*, 2004). The dissolution of inorganic phosphates by fungi is well known and this can result in release of the associated metals (Lang and Kaupenjohann, 2003; Fomina *et al.*, 2004; Hashimoto *et al.*, 2009; Gadd, 2007, 2010). The possibility of utilizing organic phosphate sources in the environment holds some promise for toxic metal bioremediation or biorecovery through metal phosphate precipitation, and a large number of microorganisms, including bacteria and fungi, can produce various extracellular phosphatases (Schurr and Yagi, 1971; Sano *et al.*, 1999; Millán, 2006). Fungal phosphatases are able to hydrolyse different organic-P sources releasing inorganic phosphate (P_i) (Field and Schekman, 1980; Dick *et al.*, 2011). The enzymes can be classed as alkaline phosphatases with pH optima above 7, and acid phosphatases with pH optima below 7. Acid phosphatases can cleave the C-O-P ester bonds, which do not penetrate the plasma membrane, and release P_i (Yadav and Tarafdar, 2001; Hayes *et al.*, 2000). These enzymes are therefore usually associated with the cell walls of fungi (Field and Schekman, 1980; Novick *et al.*, 1980; González *et al.*, 1993; Ito *et al.*, 2007). Furthermore, phytase enzymes, which are meso-inositol hexaphosphate phosphohydrolases, catalyse the step-wise phosphate splitting of phytic acid (inositol phosphate: IP6) or phytate to lower inositol phosphate esters (IP5-IP1) and P_i (Hayes *et al.*, 2000). *Aspergillus* and *Penicillium* spp. have been shown to produce extracellular acid phosphatases that hydrolysed

extracellular organic phosphates in the presence of copper which enhanced the removal of copper from solution (Haas *et al.*, 1991; Tsekova *et al.*, 2000; 2002). The P_i released can be accumulated within cells via specific transport systems, but can also react with and precipitate toxic metals as insoluble metal phosphates (Crusberg, *et al.*, 2003).

In this work, organic phosphates were supplied as the sole P source. After growth in MCD medium amended with organic phosphorus and $Pb(NO_3)_2$, both *A. niger* and *P. javanicus* removed nearly all the soluble Pb^{2+} in the medium. This appeared to be mainly by two different mechanisms, (i) phosphatase-mediated hydrolysis of the phosphate source leading to phosphate precipitation and (ii) oxalate excretion leading to oxalate formation. The precipitation of metal oxalates has previously been considered as a means of metal detoxification in fungi (Manley and Evans, 1986; Müller *et al.*, 1995; Banfield, 1999; Gadd, 1999; 2004; Fomina *et al.*, 2004; Wei *et al.*, 2013; Gadd, *et al.*, 2014). Previous research has demonstrated the effect of mycogenic organic acids on the solubility of pyromorphite and other minerals (Sayer *et al.*, 1999; Fomina *et al.*, 2004). Pyromorphite can be dissolved by organic acid-producing fungi, such as *A. niger*, and this can release free P_i and Pb from pyromorphite when it is the sole source of P (Sayer *et al.* 1999). In contrast, other fungi are capable of mediating pyromorphite formation from sources of metallic lead (Rhee *et al.*, 2012; 2014a,b). In this work, formation of pyromorphite only occurred with *P. javanicus* grown with organic-P in the presence of lead while lead oxalate was found with both *A. niger* and *P. javanicus*.

Oxalate can be secreted by many fungi and this can mediate efficient ligand-promoted Pb mobilization and immobilization (Sayer *et al.*, 1999; Fomina *et al.*, 2004). The dominance of lead oxalate rather than pyromorphite is the result of ion

competition with the soluble P (either from the organic-P or the P_i released from organic-P), biosorption to fungal biomass, the pH of the system and Pb complexation with dissolved organic carbon or organic acids (Lang and Kaupenjohann, 2003; Gadd, 2004; Ryan *et al.*, 2004; Hashimoto *et al.*, 2009). When additional phosphate was added to Pb-contaminated soil without any microorganisms present, about 70% of the Pb species present were not transformed into pyromorphite or indeed any lead phosphate (Hashimoto *et al.*, 2009), which may emphasize the importance of fungal and other microbial activities in the formation of pyromorphite and other lead secondary minerals (Rhee *et al.*, 2012; 2014a,b).

Previous research has shown that pyromorphite formation in soil can be inhibited by the presence of dissolved organic carbon (Lang and Kaupenjohann, 2003) and organic matter (Hashimoto *et al.*, 2009) through organic-metal complex formation. Commonly occurring low molecular weight organic acids (LMWOA) secreted by fungi in the rhizosphere were examined for dissolution of pyromorphite at a 100 μ M concentration. The means of the highest amounts of Pb (μ M Pb) in solution followed the order: oxalic acid (17.6) > citric acid (6.2) > malic acid (5.6) > acetic acid (3.0) (Debela *et al.*, 2010). Solution pH, concentration of the carbon, phosphorus and nitrogen sources, the buffering capacity of the medium, the presence or absence of certain metals and some trace elements can influence organic acid secretion (Sayer and Gadd, 1997, 2001; Gharieb and Gadd, 1999; Gadd, 1999; Gadd *et al.*, 2012; 2014). Solution pH strongly influences the activities of metals and the de/protonation of LMWOA (Debela *et al.*, 2010). However, solution pH alone cannot explain the lead secondary mineral formation observed in this study but may be a contributing factor in the formation of lead oxalate. The high affinity of oxalate for Pb seems to be a major factor in lead oxalate formation. Oxalic acid excretion can be strongly

influenced by the presence of toxic metals (Fomina *et al.*, 2004). For instance, during growth of *A. niger* in the presence of $\text{Co}_3(\text{PO}_4)_2$ and $\text{Zn}_3(\text{PO}_4)_2$, there was increased production of gluconic acid and citric acid respectively (Sayer and Gadd, 2001). Generally, fungi acidify the medium during growth, but such acidification can depend on the nitrogen source, tending to result with NH_4^+ as nitrogen source rather than NO_3^- (Lapeyrie, 1988; Wenzel *et al.*, 1994; Whitelaw, 2000; Gharieb and Gadd, 1999; Fomina *et al.*, 2004). The mechanisms for lead secondary mineral formation therefore appear to include: (1) organic acid secretion by fungi decreasing the solution pH and forming surface lead complexes through adsorption to fungal surfaces, (2) the saturation state of the solution being altered by the generation of organic acids with respect to mineral formation, (3) metal speciation being greatly influenced by the presence of organic acids and P_i released by the fungal phosphatases, and (4) pH control of metal hydrolysis and complexation (McBride, 1994). Fungal cell walls have an important role in metal bioprecipitation and biotransformation providing many binding sites for toxic metals (Van Tichelen, *et al.*, 2001; Adriaensen *et al.*, 2004; Fomina *et al.*, 2004; Gadd, 1993, 2007, 2009; Gadd *et al.*, 2014; Li *et al.*, 2014). The range in Pb:P molar ratios suggests that the formation of pyromorphite was stoichiometric.

Lead oxalate is stable and dominant in the medium as long as the activity of oxalate remains high (>0.1 mM) and the pH remains low ($\text{pH} < 5$), which are the conditions close to fungal growth in our experiments. Lead oxalate tends to be stable in acidic environments with a low sulfur concentration which avoids the formation of anglesite (PbSO_4). Anglesite, and pyromorphite formation, has been shown to occur with several yeast strains incubated in Pb^{2+} - and SO_4^{2-} -containing media (Liang *et al.* 2015a). As oxalate activity decreases or the medium become more alkaline, other

minerals can become more stable. Because sulfate concentration is generally higher in soils than oxalate, lead oxalate in soil is not so stable. Over a wide pH range, there is a possibility that lead oxalate could be transformed into pyromorphite in the presence of phosphate. The conversion of lead oxalate to pyromorphite suggests that a dynamic equilibrium exists between chemical and biological processes and even compared to lead oxalate ($\log K_{sp} = -8.07$), chloropyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$) ($\log K_{sp} = -84.4$) and hydroxypyromorphite ($\text{Pb}_5(\text{PO}_4)_3(\text{OH})$) ($\log K_{sp} = -76.8$) are far less soluble than many other lead minerals (Shevade *et al.*, 2001; Scheckel and Ryan, 2002; Fomina *et al.*, 2004).

In conclusion, this work has shown that filamentous fungi are capable of phosphatase-mediated bioprecipitation of lead when utilizing an organic-P-containing substrate. This complements first discoveries of this phenomenon in bacteria (Aickin *et al.* 1979) and yeasts (Liang *et al.* 2015a). Fungal biomineralization was, however, species specific and therefore must depend on the physiology of the biomineralizing organism, and the changes it can effect in its environment. Pyromorphite was only produced by *P. javanicus*, this organism previously shown to be capable of pyromorphite formation from lead metal (Rhee *et al.* 2012, 2014a,b) while only lead oxalate was produced by *A. niger*, a result of significant oxalate secretion properties of this organism (Gadd, 1999). Lead speciation modelling closely correlated with experimental results and the physico-chemical conditions of the media. It is likely therefore, that fungal species specificity of biomineralization depends on nutritional characteristics and metabolism, such as the substrate and the nature and amount of organic acids excreted, the inorganic nitrogen source, as well as phosphatase activity in the case of organic-P sources. Filamentous fungi have received lower attention than bacteria regarding phosphate precipitation but it has been shown that

368 fungi can precipitate U-containing phosphate minerals during growth in the presence
369 or inorganic or organic sources of P (Fomina *et al.*, 2007, 2008; Liang *et al.*, 2015b).
370 The ability of fungi to precipitate metal phosphates from organic P resources
371 provides further understanding of fungal interactions with toxic metals and minerals
372 in polluted sites and their roles in the biogeochemical cycling of metals as well as P.
373 Metal bioprecipitation is also relevant to new approaches in bioremediation of
374 polluted environments, as well as metal biorecovery and bioprocessing, and the
375 manufacture and use of new biomaterials.

376

Experimental procedures

Organisms and media

Aspergillus niger van Tieghem (ATCC 201373), *Beauveria caledonica* Bisset and Widden 4 (originally supplied by D. Genney (CEH Merlewood collection)), *Rhizopogon rubescens* Tulasne (kindly provided by Dr. H. Wallander) and *Paecilomyces javanicus*(Friedrichs & Bally) A.H.S. Brown & G. Smith were used. These fungi have significant abilities in mineral and toxic metal biotransformations (Sayer *et al.*, 1999; Fomina *et al.*, 2007; 2008; Rhee *et al.*, 2012; 2014a,b; Wei *et al.*, 2013; Gadd *et al.*, 2014). Test fungi were grown in modified Czapek-Dox liquid medium (MCD) amended with 30 mM glycerol 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P \cdot xH_2O$) (G2P) (Sigma-Aldrich, St. Louis, MO, USA) or 5 mM phytic acid sodium salt hydrate ($C_6H_{18}O_{24}P_6 \cdot xNa^+ \cdot yH_2O$) (PyA) (Sigma-Aldrich, St. Louis, MO, USA) as sole phosphorus sources in 250-ml Erlenmeyer conical flasks containing 100 ml nutrient medium on an orbital shaking incubator (Infors Multitron Standard, Rittergasse, Switzerland) at 125 rpm at 25°C in the dark. 10 x 5 mm diameter inoculum plugs, cut from the margins of actively growing fungal colonies using a sterile cork borer (autoclaved at 121°C for 15 min) were used as inoculum. *A. niger* was grown for 4 d, *B. caledonica* was grown for 5 d, while *P. javanicus* and *R. rubescens* were grown for 10 d prior to experimental subculture. MCD medium consists of (L^{-1} Milli-Q water (Merck Millipore, Billerica, Massachusetts, USA)): D-glucose 30 g (Merck, Readington Township, NJ, USA), $NaNO_3$ 3 g (Sigma-Aldrich), $MgSO_4 \cdot 7H_2O$ 0.5 g (VWR, Radnor, PA, USA), KCl 0.5 g (Sigma-Aldrich), $Fe(NO_3)_3 \cdot 9H_2O$ 0.01 g (Sigma-Aldrich). G2P and PyA were dissolved

separately in Milli-Q water and sterilized by membrane filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved MCD medium (121°C, 15 min) at room temperature to give final concentrations of 30 mM G2P and 5 mM PyA. After autoclaving, the pH of liquid medium was adjusted to pH 5 using sterile 1 M HCl or to pH 8 using sterile 1 M NaOH.

A. niger and *P. javanicus* were chosen as test organisms for detailed experiments. These were grown in MCD amended with 5 mM $\text{Pb}(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA as sole phosphorus sources in 250-ml conical flasks containing 100 ml nutrient medium on an orbital shaking incubator at 125 rpm at 25°C in the dark. $\text{Pb}(\text{NO}_3)_2$, G2P and PyA were dissolved separately in Milli-Q water and sterilized by membrane filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved MCD medium (121°C, 15 min) at room temperature, to give 5 mM $\text{Pb}(\text{NO}_3)_2$, 30 mM G2P and 5 mM PyA final concentrations. All experiments were conducted at least in triplicate.

Tolerance indices (TI), P_i release, lead measurement and pH analysis

Test fungi were grown in MCD with or without 30 mM G2P or 5 mM PyA at pH 5 and pH 8. 10 mL aliquots of supernatant were collected at time intervals for up to 30d for analysis of P_i and pH measurements.

Inorganic P_i released during growth was quantified spectrophotometrically after reaction in the malachite green assay (Irving and McLaughlin, 1990). For these phosphate measurements, 15 μL aliquots of supernatant were sterilized by membrane filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone,

424 Kent, UK), and added to wells in a 96-well plate with 185 μ L Milli-Q water followed by
425 addition of 100 μ L malachite green reagent. After mixing for 15 min, the absorbance
426 at 620 nm was read using a RosysAnthos 2001 microplate reader, and calculations
427 were carried out using the manufacturer's Windows-based control and evaluation
428 software (AnthosLabtec Instruments, Wals-Siezenheim, Austria). For the malachite
429 green background comparison, 200 μ L Milli-Q water was mixed with 100 μ L
430 malachite green reagent standard in individual wells in the 96-well plate giving a final
431 volume of 300 μ L. After incubating the plate for 15 min, the absorbance at 620 nm
432 was determined as described previously. The pH of supernatants was measured
433 using a pH 210 Microprocessor pH Meter (Hanna Instruments, Woonsocket, RI,
434 USA). All experiments were conducted at least in triplicate.

435 For examining the role of fungal phosphatase in lead bioprecipitation, test fungi were
436 grown in MCD with 5 mM $\text{Pb}(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA in 250-ml conical
437 flasks containing 100 ml nutrient medium on an orbital shaking incubator at 125 rpm
438 at 25°C in the dark. Fungal biomass was harvested by centrifugation at 4000 rpm
439 (4880g) for 30 min and supernatants were obtained by filtering through membrane
440 filters (0.2 μ m pore diameter, Whatman, Maidstone, Kent, UK) at appropriate time
441 intervals and analysed for lead concentration, and pH. Metal tolerance was
442 evaluated using a tolerance index (TI) as follows: (dry weight of lead-exposed
443 mycelium/dry weight of control mycelium x 100%) (Sayer *et al.*, 1995; Wei *et al.*,
444 2013). Fungal biomass was oven-dried at 105°C to constant weight and then ground
445 to a powder using a pestle and mortar (Milton Brook, Dorset, UK). Samples for lead
446 analysis were prepared by digestion of 50 mg dried biomass powder which was
447 placed in a 50 mL glass test tube to which 3 mL concentrated nitric acid (15.8 M)
448 was added with heating at 100°C until the solution was clear (~ 4 h). The resulting

digest was then diluted to a suitable concentration with Milli-Q water and passed through a 0.2 μm syringe filter (Whatman, Maidstone, Kent, UK) prior to analysis. Samples were stored at 4°C before analysis of metal content by atomic absorption spectrophotometry (AAS, Perkin Elmer, AAnalyst 400) with reference to appropriate standards. Calibration standards were prepared from 1000 mgL^{-1} AAS standard solutions for lead (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). Any difference in means between treatments was assessed using one way analysis of variance (ANOVA) to a 0.05 significance level.

Examination of fungal biominerals produced in the presence of lead

Secondary mineral formation in association with fungal biomass grown with $\text{Pb}(\text{NO}_3)_2$ and G2P or PyA was examined using scanning electron microscopy (SEM). Fungal pellets grown for 30 d were cut in half using a sterile scalpel (Swann-Morton, Sheffield, UK) and fixed in 2.5 % (v/v) triple-distilled glutaraldehyde in 5 mM 1,4-piperazine N,N' bis (2-ethane sulphonic acid) (PIPES) buffer, pH 7.2, for at least 24 h at room temperature. The pH of 5 mM PIPES was adjusted using 1 M NaOH using a Corning pH meter 120 (Corning Incorporated, Corning, NY 14831, USA). After fixation, samples were rinsed twice in 5 mM PIPES buffer, pH 7.2 (15 min per rinse) and then dehydrated through a graded ethanol series (50-100 % (v/v), 15 min per step). Samples were then critical point dried using a liquid CO_2 BAL-TEC CPD 0.30 critical point dryer (BAL-TEC company, Canonsburg, USA) and subsequently mounted on aluminium stubs using carbon adhesive tape and stored in a desiccator at room temperature. Prior to electron microscopy, samples were coated with 25nm Au/Pd using a Cressington 208HR sputter coater (Ted Pella, Inc., Redding, CA, USA)

473 and examined using a Philips XL30 environmental scanning electron microscope
474 (ESEM) (Philips XL 30 ESEM FEG) operating at an accelerating voltage of 15kV.
475 Secondary minerals formed on the fungal hyphae were examined for elemental
476 composition using energy-dispersive X-ray analysis (EDXA) before Au/Pd coating
477 the samples in order to exclude the Au/Pd peak which overlaps P/Cl peaks. Spectra
478 were acquired using a Phoenix EDXA analysis system embedded within the
479 environmental scanning electron microscope (Philips XL30 ESEM FEG) operating at
480 an accelerating voltage of 20kV. X-ray powder diffraction (XRPD) was used to
481 identify the secondary minerals produced. Diffraction patterns were recorded from 3
482 to 60° 2-θ using Ni-filtered Cu K-alpha radiation, and scanning from 3-60° 2-theta
483 counting for 300 seconds per step on a Panalytical X-pert Pro diffractometer using a
484 X-celerator position sensitive detector. Mineral phases were identified by reference
485 to patterns in the International Centre for Diffraction Data Powder Diffraction File
486 (PDF).

487 For transmission electron microscopy (TEM), small cubes (~ 1 mm³) from fungal
488 pellets grown in liquid media were cut using a clean scalpel. These were fixed in
489 2.5% (v/v) triple distilled glutaraldehyde in 5 mM PIPES buffer, pH 6.5, overnight at
490 room temperature, and then washed four times (15 min/wash) with pH 6.5, 5 mM
491 PIPES buffer. Dehydration was then performed through a 25-100% (v/v) ascending
492 series of ethanol in sterile distilled water, samples being left overnight at each stage.
493 L. R. Whyte resin (Agar Scientific Ltd, Stansted, UK) in absolute ethanol was used to
494 infiltrate samples on a rotary mixer overnight after which time 100% resin was
495 substituted twice, samples again being incubated overnight. After final infiltration, the
496 small cubes containing fungal colonies were placed in gelatin capsules with fresh
497 resin and polymerized at 60°C for 1 d. Ultrathin sections were cut on a Reichert

OMU-3 microtome (C. Reichert, Vienna, Austria) and mounted on formvar-coated copper grids. Unstained and stained sections (uranyl acetate and lead citrate) were examined on a Jeol-1200 EX transmission electron microscope (Jeol Ltd, Welwyn Garden City, UK).

Modelling of lead speciation by PHREEQC

Geochemical software (PHREEQC, Ver. 3 (Parkhurst and Appelo, 2013) was used to calculate the chemical equilibria of chemical and mineral species of lead, phosphorus and oxalate to produce a geochemical model for the biotransformation of pyromorphite and lead oxalate. The results are shown as predominance diagrams calculated by Phreeplot, the graphic supporting software for PHREEQC (Andres *et al.*, 1993; 1994; Fowle *et al.*, 2000; Kinniburgh and Cooper, 2004; 2014). The available Minteq v4 database was integrated with critically selected stability constants of metal-oxalate complexes taken from the database of the National Institute of Standards and Technology (NIST), Ver. 8 (Martell *et al.*, 2004). There are some previous studies that characterized thermodynamic data for pyromorphite from which some data can be used for construction of the predominance diagrams for lead species under specific conditions (Sayer *et al.*, 1999; Shevade *et al.*, 2001). Total concentrations for Pb and P in the system were assumed to be 5 and 30 mM respectively, and 30 mM for oxalate, and the concentrations of other elements were taken to be the same values as present in MCD medium. The oxalate value took into account previous measurements of oxalic acid excreted by *A. niger*, with 15.4 mM being recorded after 10 d incubation at 25°C in Czapek-Dox liquid medium containing nitrate as a nitrogen source (Gharieb and Gadd, 1999).

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523 **Statistical analysis**

524 All data presented in the paper are the means of at least three replicates and error
525 bars represent one standard error either side of the mean. SigmaPlot, version 12.5,
526 was used to perform statistical analyses. One-way ANOVA tests on means were
527 performed for dry weight, the malachite green P_i assay, pH and lead concentration
528 measurements.

529

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777 Figure Legends

778 **Fig. 1.** P_i release from different P sources by *A. niger*, *B. caledonica*, *P. javanicus*
779 and *R. rubescens* grown in MCD liquid media for 30 d at initial pH 5 and 8 at 25°C.
780 The figure shows P_i release in MCD medium without any added phosphorus source
781 at pH 8 (▨) and pH 5 (▩), MCD medium amended with 30 mM G2P at pH 8 (▧) and pH 5 (▦) and MCD medium amended with 5 mM PyA at pH 8 (■) and pH 5 (□).
782 All organisms were grown in the dark on an orbital shaking incubator at 125 rpm.
783 Values shown are means of at least three measurements and the bars indicate
784 typical standard deviations of about 5%.
785

786 **Fig. 2.** Scanning electron microscopy of biominerals formed by *A. niger* and *P.*
787 *javanicus* grown in 5 mM $Pb(NO_3)_2$ and 30 mM G2P or 5 mM PyA-amended MCD
788 medium. (A) Control *A. niger* hyphae grown in Pb-free 30 mM G2P-amended MCD
789 medium, scale bar = 50 μm . (B,C) Biominerals formed in 5 mM $Pb(NO_3)_2$ and 30 mM
790 G2P-amended MCD medium after growth of (B) *A. niger* and (C) *P. javanicus* for 30
791 days, scale bars: B,C = 50 μm . Inset C is a higher magnification image of the area
792 indicated by the square (scale bar = 5 μm). (D) Control *P. javanicus* hyphae grown in
793 Pb-free 5 mM PyA-amended MCD medium for 30 days, scale bar = 20 μm . (E,F)
794 Biominerals formed in 5 mM $Pb(NO_3)_2$ and 5 mM PyA-amended MCD medium after
795 growth of (E) *A. niger* and (F) *P. javanicus* for 30 days, scale bars: E,F = 20 μm . All
796 organisms were grown for 30 days at 25°C in the dark on an orbital shaking
797 incubator at 125 rpm. Typical images are shown from several examinations.
798

Fig. 3. Energy-dispersive X-ray analysis (EDXA) of crystals produced by *A. niger* and *P. javanicus* during growth in 5 mM Pb(NO₃)₂ and 30 mM G2P or 5 mM PyA-amended MCD medium. (A,C) Lead-containing biomineral produced by *A. niger* (shown in Fig. 3B,E). (B,D) Lead-containing biomineral produced by *P. javanicus* (shown in Fig. 3C,F). Typical spectra are shown from one of at least three determinations.

Fig. 4. Transmission electron microscopy (TEM) of biominerals formed by *A. niger* and *P. javanicus* after growth in 5 mM Pb(NO₃)₂ and 30 mM G2P-amended MCD liquid medium. (A,B,C) Biominerals formed in 5 mM Pb(NO₃)₂ and 30 mM G2P-amended MCD liquid medium after growth of *A. niger* for 30 d. Scale bars: A = 3 µm, B,C = 6 µm. (D,E,F) Biominerals formed in 5 mM Pb(NO₃)₂ and 30 mM G2P-amended MCD liquid medium after growth of *P. javanicus* for 30 d. Scale bars: D = 7 µm, E = 4 µm, F = 2 µm. All organisms were grown for 30 d at 25°C in the dark on an orbital shaking incubator at 125 rpm. Typical images are shown from several examinations.

Fig. 5. X-ray powder diffraction (XRPD) patterns of biominerals extracted from 5 mM Pb(NO₃)₂ and 30 mM G2P or 5 mM PyA-amended MCD medium after growth of (A) *A. niger* and (B) *P. javanicus*. Patterns for dominant mineralogical components are shown, as well as the new biominerals produced as a result of fungal activity, lead oxalate (PbC₂O₄) and pyromorphite, syn (Pb₅(PO₄)₃Cl). Mineral particulates were harvested after growth for 30 days at 25°C in the dark on an orbital shaking incubator at 125 rpm. Typical patterns are shown from one of several determinations.

822 **Fig. 6.** Predominance diagrams of chemical and mineralogical species of lead. (A)
823 The stability field of pyromorphite in the system Pb, Mg, S, K, Cl, N, C, Na, Fe, P, H
824 and O. (B) The stability field of lead oxalate in the system Pb, Mg, S, K, Cl, N, C,
825 Na, Fe, P, H and O. (C) Lead mineral stability diagrams pH vs log oxalate
826 concentration. Total concentrations for Pb and P in the system were assumed to be
827 5 and 30 mM respectively, and 30 mM for oxalate, and the concentrations of other
828 elements were taken to be the same values as present in MCD medium.

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