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1 **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.

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 ($Pb_5(PO_4)_3X$ (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 $Pb_5(PO_4)_3X$, where X = F, Cl, Br, OH, are the most stable environmental Pb compounds ($Pb_5(PO_4)_3F$, $K_{sp} = 10^{-71.6}$, $Pb_5(PO_4)_3Cl$, $K_{sp} = 10^{-84.4}$,

83   Pb$_5$(PO$_4$)$_3$Br, K$_{sp}$ = 10$^{-78.1}$, and Pb$_5$(PO$_4$)$_3$OH, K$_{sp}$ = 10$^{-76.8}$) over a wide range of

84   conditions (Chen *et al.*, 2007; Chrysochoou *et al.*, 2007). However, it has been

85   clearly demonstrated that certain microbial metabolites, e.g. mycogenic organic

86   acids, can affect pyromorphite stability (Sayer *et al.*, 1999; Fomina *et al.*, 2004;

87   Manecki and Maurice, 2008; Debela *et al.*, 2010; Topolska *et al.*, 2014). The

88   formation of lead oxalate from pyromorphite by several free-living and mycorrhizal

89   fungi has been demonstrated (Sayer *et al.*, 1999; Fomina *et al.*, 2004).

90   Phosphorus is abundant in the environment, but most is in an insoluble form, and

91   soluble P$_i$ readily reacts with a variety of divalent and trivalent cations in soil or

92   aquatic systems to form inorganic compounds or large insoluble complexes (Van Ho

93   *et al.*, 2002). Insoluble inorganic and organic-P compounds can be utilized by

94   bacteria and fungi by, e.g. organic acid production and phosphatase enzyme activity

95   respectively (Whitelaw, 2000; Chen *et al.*, 2007). Microbes can import phosphate

96   from phosphate-containing organic compounds by first cleaving C-O-P ester bonds

97   with phosphatases that release P$_i$ (Hyśek and Sărapatka, 1997; Hayes *et al.*, 2000;

98   Yadav and Tarafdar, 2001; Bull *et al.*, 2002). Phosphate-solubilizing bacteria and

99   fungi can solubilize inorganic phosphate-containing minerals, e.g. apatites and other

100   phosphates (Sayer *et al.*, 1995, 1997, 1999; Di Simine *et al.*, 1998; Sayer and Gadd,

101   1997, 2001; Whitelaw, 2000; Jacobs *et al.*, 2002a,b; Fomina *et al.*, 2004, 2005;

102   Ahemad *et al.*, 2009; Topolska *et al.*, 2014) but can also release phosphate from

103   organic phosphate sources through phosphatase activity (Aickin *et al.*, 1979; ;

104   Macaskie *et al.*, 1992, 1994, 2000; Yong and Macaskie, 1995; Boswell *et al.*, 1999,

105   2001; Dick *et al.*, 2011). Released phosphate from organic sources can precipitate

106   with metals forming insoluble metal phosphates, a phenomenon first characterized in

107   bacteria (Aickin *et al.*, 1979; Macaskie *et al.*, 1992, 1994, 2000; Yong and Macaskie,

108   1995; Boswell *et al.*, 1999, 2001). Formation of metal phosphates has been

109   observed in fungi, e.g. *Penicillium ochro-chloron* precipitated cupric oxalate and

110   cupric phosphate (Crusberg, 2004), while *Penicillium cyclopium* removed up to 95%

111   supplied lead in the presence of phosphate (Tsekova *et al.*, 2006). Rhee *et al.* (2012,

112   2014a,b) have shown fungal mediation of pyromorphite ($Pb_5(PO_4)_3Cl$) formation from

113   lead metal. Under conditions of Cu and Cd toxicity, the ectomycorrhizal fungus

114   *Xerocomus chrysenteron*was exuded soluble protein and acid phosphatase that

115   appeared to be involved in metal tolerance and precipitation (Zheng *et al.*, 2009).

116   *Laccaria laccata* and *Suillus bovinus* have also been shown to produce more acid

117   phosphatase in the presence of high concentrations of Cr (VI) (Raman *et al.*, 2002).

118   The objective of this work was to determine the possible role of fungal phosphatases

119   in the bioprecipitation of lead phosphate when organic phosphate substrates are the

120   only substrate source of phosphorus. The pH and P dependence of lead phosphate

121   formation, and the significance of excreted oxalate in lead biomineralization was also

122   investigated, coupled with geochemical modelling of lead and lead mineral

123   speciation as a function of pH and oxalate.

124

125 **Results**

126

127 **Growth and P$_i$ release from different organic-P sources**

128 All fungi were able to grow in organic phosphorus-amended MCD media at both pH

129 5 and pH 8. Negligible growth was observed in MCD medium without any

130 phosphorus source. With equivalent amounts of P in the MCD medium, all fungi

131 grew better and released more P$_i$ into the medium when amended with 30 mM G2P

132 than with 5 mM PyA. Among the test fungi, *A. niger* grew well with G2P and released

133 up to 23 mM P$_i$ from 30 mMG2P after 30 days incubation at 25°C. There were no

134 significant differences in the amount of P$_i$ released by *A. niger* and *P. javanicus*

135 when grown with G2P at the different initial pH values. *P. javanicus* had a higher

136 ability to hydrolyze PyA than the other test species, with 8.5 mMP$_i$ released from 5

137 mM PyA after growth in MCD medium for 30 days at both initial pH values (Fig. 1).

138 There were no significant differences in growth rate and P$_i$ release by the organisms

139 when grown at different initial pH values except for *B. caledonica* and *R. rubescens*.

140 The latter released more P$_i$ from the organic phosphorus sources in acidic rather

141 than in alkaline conditions. *B. caledonica* and *R. rubescens* released up to 18 and 13

142 mM P$_i$, respectively, from 30 mM G2P in acidic conditions, compared with 11 and 7

143 mM P$_i$, respectively, in alkaline conditions. From analysis of the fungal growth rates

144 and P$_i$ release from organic-P, *A. niger* and *P. javanicus* were selected for detailed

145 experiments.

146

147 **Growth and P$_i$ release from different organic-P sources in the presence of Pb**

148 **by *A. niger* and *P. javanicus***

149   In the presence of $Pb(NO_3)_2$, growth of *A. niger* was reduced, while *P. javanicus*

150   showed a relatively better tolerance in lead-amended MCD medium (Table 1).

151   Tolerance indices (TI) were used to compare the biomass yields of *A. niger* and *P.*

152   *javanicus* grown on MCD medium with or without 5 mM $Pb(NO_3)_2$ and 30 mM G2P

153   or 5 mM PyA (Table 1). A TI value lower than 100% indicates growth inhibition.

154   Biomass yields of *A. niger* were markedly reduced in the presence of 5 mM $Pb(NO_3)_2$

155   when grown with 30 mM G2P, with growth inhibition resulting on 5 mM$Pb(NO_3)_2$ and

156   5 mM PyA. For *P. javanicus*, there was some inhibition of biomass yield over the first

157   10 days incubation, but after this time, biomass yields were generally larger than on

158   control medium. The pH of all MCD media was adjusted to pH 6 before inoculation.

159   The pH value of medium containing 30 mM G2P dropped to pH 3.1 after growth of *A.*

160   *niger* for 30 days while the corresponding pH for *P. javanicus* in the same culture

161   medium increased to pH 6.8.The pH values of medium containing 5 mM PyA

162   showed similar trends after growth of *A. niger* and *P. javanicus* (Table 1). P_i

163   concentrations in MCD medium containing 30 mM G2P or 5 mM PyA increased with

164   time during growth of *A. niger* and *P. javanicus*. *A. niger* released around 25-26

165   mMP_i from 30 mM G2P and 5 mM PyA respectively after 30 days growth (Table 1).

166   Similar concentrations of free P_i were released by *P. javanicus.* However, *P.*

167   *javanicus* released more P_i at an earlier stage of growth than *A. niger*, with around 9-

168   10mM P_i being released from *A. niger* and around 15 mM P_i being released from *P.*

169   *javanicus* after 20 days (Table 1)*.*

170

171   **Pb removal from solution and the formation of lead mineral phases**

172   Both *A. niger* and *P. javanicus* were able to remove almost all of the lead from media

173   amended with 5 mM $Pb(NO_3)_2$ and the organic-P sources (Table 1). Both *A. niger*

174   and *P. javanicus* removed most of the Pb^{2+} from solution after 10 days and showed

175   increasing Pb accumulation with time (Table 1).

176   Both *A. niger* and *P. javanicus* were able to grow in the presence of 5 mM $Pb(NO_3)_2$

177   and precipitated secondary minerals (Fig. 2). In contrast to *A. niger* from control Pb-

178   free MCD medium (Fig. 2A), extensive secondary mineral formation occurred when

179   *A. niger* was grown with 5 mM $Pb(NO_3)_2$ and 30 mM G2P (Fig. 2B). Some secondary

180   minerals with a similar shape were also found on the biomass of *A. niger* grown with

181   5 mM $Pb(NO_3)_2$ and 5 mM PyA (Fig. 2E). For *P. javanicus*, the produced secondary

182   minerals showed a completely different shape from those associated with *A. niger*,

183   with arborescent and blade-like shapes above the hyphae (Fig. 2C,F) with some

184   arborescent and globular (Inset Fig. 2C).

185

186   **Energy-dispersive X-ray analysis (EDXA)**

187   EDXA revealed the elemental composition of the secondary minerals found on the

188   fungal hyphae. The minerals associated with *A. niger* after growth with $Pb(NO_3)_2$ and

189   G2P showed peaks for carbon, oxygen, potassium and lead as the main elements

190   detected (Fig. 3A).The minerals produced by *P. javanicus* grown with $Pb(NO_3)_2$ and

191   G2P (Fig. 3B) showed peaks for carbon, oxygen, sodium, phosphorus and lead as

192   the main elements. The minerals produced by *A. niger* grown with $Pb(NO_3)_2$ and PyA

193   that showed a cylindrical form showed carbon, oxygen, sodium, potassium and lead

194   (Fig. 3C). The minerals produced by *P. javanicus* grown with $Pb(NO_3)_2$ and PyA (Fig.

195 3D) showed peaks for carbon, oxygen, sodium, aluminium, phosphorus and lead as

196 the main elements present.

197 Transmission electron microscopy (TEM) revealed the presence of electron-dense

198 deposits around the biomass of both *A. niger* and *P. javanicus* after growth with 5

199 mM $Pb(NO_3)_2$ and 30 mM G2P (Fig. 4). Compared to the electron-dense granules

200 found with *P. javanicus*, the amount of crystals found with *A. niger* seemed of lower

201 abundance. These observations are consistent with the presence of the secondary

202 minerals found with SEM.

203

204 **X-ray powder diffraction (XRPD)**

205 The secondary mineral precipitates associated with *A. niger* and *P. javanicus* grown

206 with lead and G2P showed the presence of well-crystallized compounds with an

207 excellent match to reference patterns for lead oxalate (PbC_2O_4) and pyromorphite

208 ($Pb_5(PO_4)_3Cl$) (Fig. 5). Lead oxalate was detected from both *A. niger* and *P.*

209 *javanicus*, but pyromorphite was only detected with *P. javanicus* (Fig. 5).

210

211 **Geochemical modelling**

212 The predominance diagrams for chemical species of lead can be simulated by

213 PHREEQC, and this software was used to generate a diagram for the

214 bioprecipitation of lead oxalate and pyromorphite. For lead, the dominant minerals

215 found in the simulated systems were simple oxides, carbonates and phosphates.

216 Lead minerals differ widely in water solubility depending on the presence of H^+,

217 HCO_3^-, PO_4^{3-}, SO_4^{2-}, and the total ionic charge of the solution. The diagram shows

218   the most dominant mineral species of lead theoretically present in the MCD medium

219   (Fig. 6). The data was calculated for a temperature of 25°C and with total dissolved

220   lead in the presence of dissolved chloride, carbonate, phosphate, nitrate and sulfate.

221   Fig. 6A indicates that pyromorphite dominates over the range pH 2-11 in the

222   absence of oxalic acid, $Pb(H_2PO_4)_2$ dominates at pH values < 2, $Pb(OH)_2$ dominates

223   over pH 11-13, and $Pb(OH)_4^{2-}$ occurs at pH values > 13. In the presence of oxalic

224   acid, the situation is different and lead oxalate and lead hydroxide become the two

225   main species in the system (Fig. 6B). Lead oxalate dominates over the pH-range 2-7,

226   while lead hydroxide dominates over pH 7-13. Pb^{2+} and $Pb(OH)_4^{2-}$ dominate at pH< 2

227   and pH >13, respectively. In Fig. 6C, the mineral stability diagrams as a function of

228   pH and oxalate activity show the dominance of lead oxalate at a pH < 5 and at an

229   oxalate activity >0.1 mM. These pH-ranges correspond with the pH values measured

230   in the experimental conditions (Table 1). In contrast, anglesite can become dominant

231   over the same acidic pH range when the oxalate concentration is lower than 0.1 mM.

232   Pyromorphite is dominant over the approximate range of pH 4.8-8 even at high

233   concentrations of oxalate. At pH > 8, lead hydroxide becomes dominant at any

234   oxalate concentration. The presence of pyromorphite in the *P. javanicus*-organic-P-

235   Pb system simulation and the presence of lead oxalate in the *A. niger*-organic-P-Pb

236   system closely correlates with the XRPD data, confirming that the theoretical

237   simulation closely agrees with experimental findings. It is important to note that with

238   the function of pH and E_h, the concentration of lead in the aqueous system strongly

239   affects the formation of the different lead species, while aqueous Pb complexes can

240   also undergo several protonation or deprotonation reactions together with

241   polymerization to form various aqueous species of lead.

242

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

268 extracellular organic phosphates in the presence of copper which enhanced the

269 removal of copper from solution (Haas *et al.*, 1991; Tsekova *et al.*, 2000; 2002). The

270 P_i released can be accumulated within cells via specific transport systems, but can

271 also react with and precipitate toxic metals as insoluble metal phosphates (Crusberg,

272 *et al.*, 2003).

273 In this work, organic phosphates were supplied as the sole P source. After growth in

274 MCD medium amended with organic phosphorus and $Pb(NO_3)_2$, both *A. niger* and *P.*

275 *javanicus* removed nearly all the soluble Pb^{2+} in the medium. This appeared to be

276 mainly by two different mechanisms, (i) phosphatase-mediated hydrolysis of the

277 phosphate source leading to phosphate precipitation and (ii) oxalate excretion

278 leading to oxalate formation. The precipitation of metal oxalates has previously been

279 considered as a means of metal detoxification in fungi (Manley and Evans, 1986;

280 Müller *et al.*, 1995; Banfield, 1999; Gadd, 1999; 2004; Fomina *et al.*, 2004; Wei *et al.*,

281 2013; Gadd, *et al.*, 2014). Previous research has demonstrated the effect of

282 mycogenic organic acids on the solubility of pyromorphite and other minerals (Sayer

283 *et al.*, 1999; Fomina *et al.*, 2004). Pyromorphite can be dissolved by organic acid-

284 producing fungi, such as *A. niger*, and this can release free P_i and Pb from

285 pyromorphite when it is the sole source of P (Sayer *et al.* 1999). In contrast, other

286 fungi are capable of mediating pyromorphite formation from sources of metallic lead

287 (Rhee *et al.*, 2012; 2014a,b). In this work, formation of pyromorphite only occurred

288 with *P. javanicus* grown with organic-P in the presence of lead while lead oxalate

289 was found with both *A. niger* and *P. javanicus*.

290 Oxalate can be secreted by many fungi and this can mediate efficient ligand-

291 promoted Pb mobilization and immobilization (Sayer *et al.*, 1999; Fomina *et al.*,

292 2004). The dominance of lead oxalate rather than pyromorphite is the result of ion

293   competition with the soluble P (either from the organic-P or the P_i released from

294   organic-P), biosorption to fungal biomass, the pH of the system and Pb complexation

295   with dissolved organic carbon or organic acids (Lang and Kaupenjohann, 2003;

296   Gadd, 2004; Ryan *et al.*, 2004; Hashimoto *et al.*, 2009). When additional phosphate

297   was added to Pb-contaminated soil without any microorganisms present, about 70%

298   of the Pb species present were not transformed into pyromorphite or indeed any lead

299   phosphate (Hashimoto *et al.*, 2009), which may emphasize the importance of fungal

300   and other microbial activities in the formation of pyromorphite and other lead

301   secondary minerals (Rhee *et al.*, 2012; 2014a,b).

302   Previous research has shown that pyromorphite formation in soil can be inhibited by

303   the presence of dissolved organic carbon (Lang and Kaupenjohann, 2003) and

304   organic matter (Hashimoto *et al.*, 2009) through organic-metal complex formation.

305   Commonly occurring low molecular weight organic acids (LMWOA) secreted by fungi

306   in the rhizosphere were examined for dissolution of pyromorphite at a 100 µM

307   concentration. The means of the highest amounts of Pb (µM Pb) in solution followed

308   the order: oxalic acid (17.6) > citric acid (6.2) > malic acid (5.6) > acetic acid (3.0)

309   (Debela *et al.*, 2010). Solution pH, concentration of the carbon, phosphorus and

310   nitrogen sources, the buffering capacity of the medium, the presence or absence of

311   certain metals and some trace elements can influence organic acid secretion (Sayer

312   and Gadd, 1997, 2001; Gharieb and Gadd, 1999; Gadd, 1999; Gadd *et al.*, 2012;

313   2014). Solution pH strongly influences the activities of metals and the de/protonation

314   of LMWOA (Debela *et al.*, 2010). However, solution pH alone cannot explain the lead

315   secondary mineral formation observed in this study but may be a contributing factor

316   in the formation of lead oxalate. The high affinity of oxalate for Pb seems to be a

317   major factor in lead oxalate formation. Oxalic acid excretion can be strongly

318   influenced by the presence of toxic metals (Fomina *et al.*, 2004). For instance, during

319   growth of *A. niger* in the presence of $Co_3(PO_4)_2$ and $Zn_3(PO_4)_2$, there was increased

320   production of gluconic acid and citric acid respectively (Sayer and Gadd, 2001).

321   Generally, fungi acidify the medium during growth, but such acidification can depend

322   on the nitrogen source, tending to result with NH_4^+ as nitrogen source rather than

323   NO_3^- (Lapeyrie, 1988; Wenzel *et al.*, 1994; Whitelaw, 2000; Gharieb and Gadd, 1999;

324   Fomina *et al.*, 2004). The mechanisms for lead secondary mineral formation

325   therefore appear to include: (1) organic acid secretion by fungi decreasing the

326   solution pH and forming surface lead complexes through adsorption to fungal

327   surfaces, (2) the saturation state of the solution being altered by the generation of

328   organic acids with respect to mineral formation, (3) metal speciation being greatly

329   influenced by the presence of organic acids and P_i released by the fungal

330   phosphatases, and (4) pH control of metal hydrolysis and complexation (McBride,

331   1994). Fungal cell walls have an important role in metal bioprecipitation and

332   biotransformation providing many binding sites for toxic metals (Van Tichelen, *et al.*,

333   2001; Adriaensen *et al.*, 2004; Fomina *et al.*, 2004; Gadd, 1993, 2007, 2009; Gadd

334   *et al.*, 2014; Li *et al.*, 2014). The range in Pb:P molar ratios suggests that the

335   formation of pyromorphite was stoichiometric.

336   Lead oxalate is stable and dominant in the medium as long as the activity of oxalate

337   remains high (>0.1 mM) and the pH remains low (pH<5), which are the conditions

338   close to fungal growth in our experiments. Lead oxalate tends to be stable in acidic

339   environments with a low sulfur concentration which avoids the formation of anglesite

340   ($PbSO_4$). Anglesite, and pyromorphite formation, has been shown to occur with

341   several yeast strains incubated in Pb^{2+} - and SO_4^{2-} -containing media (Liang *et al.*

342   2015a). As oxalate activity decreases or the medium become more alkaline, other

343 minerals can become more stable. Because sulfate concentration is generally higher

344 in soils than oxalate, lead oxalate in soil is not so stable. Over a wide pH range,

345 there is a possibility that lead oxalate could be transformed into pyromorphite in the

346 presence of phosphate. The conversion of lead oxalate to pyromorphite suggests

347 that a dynamic equilibrium exists between chemical and biological processes and

348 even compared to lead oxalate ($logK_{sp}$ = -8.07), chloropyromorphite ($Pb_5(PO_4)_3Cl$)

349 ($logK_{sp}$ = -84.4) and hydroxypyromorphite ($Pb_5(PO_4)_3(OH)$) ($logK_{sp}$ = -76.8) are far

350 less soluble than many other lead minerals (Shevade *et al.*, 2001; Scheckel and

351 Ryan, 2002; Fomina *et al.*, 2004).

352 In conclusion, this work has shown that filamentous fungi are capable of

353 phosphatase-mediated bioprecipitation of lead when utilizing an organic-P-containing

354 substrate. This complements first discoveries of this phenomenon in bacteria (Aickin

355 *et al.* 1979) and yeasts (Liang *et al.* 2015a). Fungal biomineralization was, however,

356 species specific and therefore must depend on the physiology of the biomineralizing

357 organism, and the changes it can effect in its environment. Pyromorphite was only

358 produced by *P. javanicus*, this organism previously shown to be capable of

359 pyromorphite formation from lead metal (Rhee *et al.* 2012, 2014a,b) while only lead

360 oxalate was produced by *A. niger*, a result of significant oxalate secretion properties

361 of this organism (Gadd, 1999). Lead speciation modelling closely correlated with

362 experimental results and the physico-chemical conditions of the media. It is likely

363 therefore, that fungal species specificity of biomineralization depends on nutritional

364 characteristics and metabolism, such as the substrate and the nature and amount of

365 organic acids excreted, the inorganic nitrogen source, as well as phosphatase

366 activity in the case of organic-P sources. Filamentous fungi have received lower

367 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). G2Pand PyA were dissolved

401   separately in Milli-Q water and sterilized by membrane filtration (cellulose nitrate, 0.2

402   μm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved MCD

403   medium (121^oC, 15 min) at room temperature to give final concentrations of 30 mM

404   G2P and 5 mM PyA. After autoclaving, the pH of liquid medium was adjusted to pH 5

405   using sterile 1 M HCl or to pH 8 using sterile1 M NaOH.

406   *A. niger* and *P. javanicus* were chosen as test organisms for detailed experiments.

407   These were grown in MCD amended with 5 mM $Pb(NO_3)_2$ and 30 mM G2P or 5 mM

408   PyA as sole phosphorus sources in 250-ml conical flasks containing 100 ml nutrient

409   medium on an orbital shaking incubator at 125 rpm at 25°C in the dark. $Pb(NO_3)_2$,

410   G2P and PyA were dissolved separately in Milli-Q water and sterilized by membrane

411   filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone, Kent, UK)

412   and added to autoclaved MCD medium (121^oC, 15 min) at room temperature, to give

413   5 mM $Pb(NO_3)_2$, 30 mM G2P and 5 mM PyA final concentrations. All experiments

414   were conducted at least in triplicate.

415

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

417   Test fungi were grown in MCD with or without 30 mM G2P or 5 mM PyA at pH 5 and

418   pH 8. 10 mL aliquots of supernatant were collected at time intervals for up to 30d for

419   analysis of P_i and pH measurements.

420   Inorganic P_i released during growth was quantified spectrophotometrically after

421   reaction in the malachite green assay (Irving and McLaughlin, 1990). For these

422   phosphate measurements, 15 μL aliquots of supernatant were sterilized by

423   membrane filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone,

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

For examining the role of fungal phosphatase in lead bioprecipitation, test fungi were grown in MCD with 5 mM $Pb(NO_3)_2$ and 30 mM G2P or 5 mM PyA in 250-ml conical flasks containing 100 ml nutrient medium on an orbital shaking incubator at 125 rpm at 25°C in the dark. Fungal biomass was harvested by centrifugation at 4000 rpm (4880g) for 30 min and supernatants were obtained by filtering through membrane filters (0.2 µm pore diameter, Whatman, Maidstone, Kent, UK) at appropriate time intervals and analysed for lead concentration, and pH. Metal tolerance was evaluated using a tolerance index (TI) as follows: (dry weight of lead-exposed mycelium/dry weight of control mycelium x 100%) (Sayer *et al.*, 1995; Wei *et al.*, 2013). Fungal biomass was oven-dried at 105°C to constant weight and then ground to a powder using a pestle and mortar (Milton Brook, Dorset, UK). Samples for lead analysis were prepared by digestion of 50 mg dried biomass powder which was placed in a 50 mL glass test tube to which 3 mL concentrated nitric acid (15.8 M) was added with heating at 100°C until the solution was clear (~ 4 h). The resulting

449   digest was then diluted to a suitable concentration with Milli-Q water and passed

450   through a 0.2 μm syringe filter (Whatman, Maidstone, Kent, UK) prior to analysis.

451   Samples were stored at 4°C before analysis of metal content by atomic absorption

452   spectrophotometry (AAS, Perkin Elmer, AAnalyst 400) with reference to appropriate

453   standards. Calibration standards were prepared from 1000 mgL^{-1} AAS standard

454   solutions for lead (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). Any

455   difference in means between treatments was assessed using one way analysis of

456   variance (ANOVA) to a 0.05 significance level.

457

458   Examination of fungal biominerals produced in the presence of lead

459   Secondary mineral formation in association with fungal biomass grown with

460    $Pb(NO_3)_2$ and G2P or PyA was examined using scanning electron microscopy (SEM).

461   Fungal pellets grown for 30 d were cut in half using a sterile scalpel (Swann-Morton,

462   Sheffield, UK) and fixed in 2.5 %(v/v) triple-distilled glutaraldehyde in 5 mM 1,4-

463   piperazine N,N' bis (2-ethane sulphonic acid) (PIPES) buffer, pH 7.2, for at least 24 h

464   at room temperature. The pH of 5 mM PIPES was adjusted using 1 M NaOH using a

465   Corning pH meter 120 (Corning Incorporated, Corning, NY 14831, USA). After

466   fixation, samples were rinsed twice in 5 mM PIPES buffer, pH 7.2 (15 min per rinse)

467   and then dehydrated through a graded ethanol series (50-100 %(v/v), 15 min per

468   step). Samples were then critical point dried using a liquid CO_2 BAL-TEC CPD 0.30

469   critical point dryer (BAL-TEC company, Canonsburg, USA) and subsequently

470   mounted on aluminium stubs using carbon adhesive tape and stored in a desiccator

471   at room temperature. Prior to electron microscopy, samples were coated with 25nm

472   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 mm^3) 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

498 OMU-3 microtome (C. Reichert, Vienna, Austria) and mounted on formvar-coated

499 copper grids. Unstained and stained sections (uranyl acetate and lead citrate) were

500 examined on a Jeol-1200 EX transmission electron microscope (Jeol Ltd, Welwyn

501 Garden City, UK).

502

Modelling of lead speciation by PHREEQC

504 Geochemical software (PHREEQC, Ver. 3 (Parkhurst and Appelo, 2013) was used

505 to calculate the chemical equilibria of chemical and mineral species of lead,

506 phosphorus and oxalate to produce a geochemical model for the biotransformation

507 of pyromorphite and lead oxalate. The results are shown as predominance diagrams

508 calculated by Phreeplot, the graphic supporting software for PHREEQC (Andres *et*

509 *al.*, 1993; 1994; Fowle *et al.*, 2000; Kinniburgh and Cooper, 2004; 2014). The

510 available Minteq v4 database was integrated with critically selected stability

511 constants of metal-oxalate complexes taken from the database of the National

512 Institute of Standards and Technology (NIST), Ver. 8 (Martell *et al.*, 2004). There are

513 some previous studies that characterized thermodynamic data for pyromorphite from

514 which some data can be used for construction of the predominance diagrams for

515 lead species under specific conditions (Sayer *et al.*, 1999; Shevade *et al.*, 2001).

516 Total concentrations for Pb and P in the system were assumed to be 5 and 30 mM

517 respectively, and 30 mM for oxalate, and the concentrations of other elements were

518 taken to bethe same valuesas present in MCD medium. The oxalate value took into

519 account previous measurements of oxalic acid excreted by *A. niger*, with 15.4mM

520 being recorded after 10 d incubation at 25°C in Czapek-Dox liquid medium

521 containing nitrate as a nitrogen source (Gharieb and Gadd, 1999).

522

Statistical analysis

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

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Figure Legends

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

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

Fig. 3. Energy-dispersive X-ray analysis (EDXA) of crystals produced by *A. niger* and *P. javanicus* during growth in 5 mM $Pb(NO_3)_2$ 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 $Pb(NO_3)_2$ and 30 mM G2P-amended MCD liquid medium. (A,B,C) Biominerals formed in 5 mM $Pb(NO_3)_2$ 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 $Pb(NO_3)_2$ 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 $Pb(NO_3)_2$ 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 (PbC_2O_4) and pyromorphite, syn ($Pb_5(PO_4)_3Cl$). 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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