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THE EFFECT OF GLUCONIC ACID SECRETION BY PHOSPHATE-SOLUBILIZING *PSEUDOMONAS PUTIDA* BACTERIA ON DISSOLUTION OF PYROMORPHITE $Pb_5(PO_4)_3Cl$ AND Pb REMOBILIZATION

Justyna TOPOLSKA[1], Pawel BOROWICZ[2], Maciej MANECKI[1], Tomasz BAJDA[1], Stefan KASCHABEK[3] & Broder J. MERKEL[4]

[1] *AGH University of Science and Technology, Faculty of Geology, Geophysics and Environmental Protection, Department of Mineralogy, Petrography and Geochemistry, al. Mickiewicza 30, 30-059 Kraków, Poland; e-mail: jm.topolska@gmail.com*

[2] *Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa Str. 7, 30-387 Kraków, Poland*

[3] *Institute of Biosciences, TU Bergakademie Freiberg, Leipziger Str. 29, D-09596 Freiberg, Germany*

[4] *Institute of Geology, TU Bergakademie Freiberg, Gustav Zeuner Str. 12, D-09596 Freiberg, Germany*

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Abstract: The purpose of this study was to investigate the effect of bacterially produced gluconic acid on the dissolution of pyromorphite and Pb remobilization. Pyromorphite $Pb_5(PO_4)_3Cl$ is formed as a product of the phosphate-induced treatment of Pb-contaminated sites. This very stable mineral greatly decreases the bioavailability of Pb. In this study, bacterial and abiotic batch experiments on the dissolution of pyromorphite were carried out. In the microbial experiments, the mineral was dissolved in the presence of the phosphate–solubilizing soil bacterium, *Pseudomonas putida*. The bacterial growth medium was supplemented with glucose, which under natural conditions can be supplied to microbes via symbiosis with plants. *P. putida* acquired P from pyromorphite and enhanced its dissolution. Elevated Pb concentrations were observed in the suspensions with bacteria. The bacterial secretion of 16.5 mM gluconic acid played a significant role in Pb remobilization; the pH of the solution dropped down from an initial 7.4 to 3.5. In the abiotic experiments, pyromorphite was dissolved at several concentrations of gluconic acid and at an acidic to neutral pH range. Both acidification and formation of stable Pb-gluconate ligands enhanced the dissolution of pyromorphite and caused Pb remobilization.

Key words: Pyromorphite, *Pseudomonas putida*, gluconic acid, Pb remobilization, P-induced method.

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INTRODUCTION

Pyromorphite is lead apatite that commonly occurs in nature as a secondary mineral phase in the oxidation zones of ore deposits (Nakamoto *et al.*, 1969). There are several methods of synthesizing pyromorphite and the properties of this mineral have been studied extensively (Baker, 1966; Dai and Hughes, 1989; Manecki, 2007; Xie and Giammar, 2007). Pyromorphite forms a continuous solid solution with vanadinite $[Pb_5(VO_4)_3Cl]$ and mimetite $[Pb_5(AsO_4)_3Cl]$ (Flis *et al.*, 2010). The high thermodynamic stability of these minerals is the reason, for which they are considered as metal sequestration agents in areas contaminated by lead and arsenic (e.g., Bajda, 2010; Flis *et al.*, 2011 and the literature therein). Pyromorphite ($\log K_{sp} = -79$, after Flis *et al.*, 2011) is one of the most stable minerals in the environment and the precipitation of Pb in this form greatly decreases the bioavailability of the metal (Manecki *et al.*, 2006). Hence, *in situ* immobilization with the use of phosphate amendments is nowadays one of the best studied and recommended treatments for sites contaminated by Pb (USEPA, 2005). In this treatment method, the dissolution of apatite results in phosphate release. The PO_4^{3-} ions combine with Pb and precipitate as nanoparticles of the mineral pyromorphite, $Pb_5(PO_4)_3Cl$ (e.g., Ma *et al.*, 1993, 1994a, b, 1995; Ruby *et al.*, 1994; Cotter-Howells, 1996; Cotter-Howells and Caporn, 1996).

Recently, a controversial optimization of the method was presented by Park *et al.* (2011a, b). They propose to inoculate the phosphate amendments with a live strain of phosphate-solubilizing bacteria (PSB). It is assumed that the microbes enhance the dissolution of mineral P, promot-

ing its transformation into pyromorphite. The controversy of the method stems from the need to introduce a living, extraneous strain of bacteria into an uncontrolled environment, although the long-term effect of such a treatment is unknown. Recently, the present authors showed that PSB can affect the stability of pyromorphite and that the effectiveness of the process depends on the availability of dissolved phosphates in solution (Topolska *et al.*, 2014). However, the interaction between microbes and minerals are complex and some aspects of the potential involvement of PSB in remediation treatments remain unclear.

There is some evidence that various organic compounds, microbial metabolites and plant activity may increase the dissolution of pyromorphite and cause a secondary Pb release (e.g., Sayer *et al.*, 1997; Formina *et al.*, 2004; Manecki, 2007; Manecki and Maurice, 2008; Debela *et al.*, 2010, 2013). The secretion of organic acids is often indicated as the active bacterial strategy for solubilizing mineral nutrients (e.g., Vyas and Gulati, 2009; Debela *et al.*, 2010). In these terms, efficiency depends, among other things, on the availability of a source of organic carbon for the microbes and glucose seems to be particularly effective (Nautiyal, 1999). If the bacteria utilize glucose, they excrete considerable amounts of gluconic acid, which greatly enhances the dissolution of mineral phosphates (Lin *et al.*, 2006; Buch *et al.*, 2008). Some bacteria, including various species of the PSB, can live in the rhizosphere in symbiosis with the roots of plants, which supply nutrients, such as glucose, to a symbiotic microbial strain (e.g., Espinosa-Urgel *et al.*, 2002; Rosas *et al.*, 2006; Buch *et al.*, 2008; Vyas and Gulati, 2009). Hence, the symbiotic bacteria exhibit particular capabilities of dissolving the mineral forms of phosphates via organic acid production, which makes the nutrient P more available for the plants.

The effect of glucose as a substrate for the bacteria on pyromorphite stability is unknown and this issue is interesting for at least two reasons. The first one is related to the fact that the gluconic acid is secreted by bacteria via symbiosis with plants' roots and this is likely to occur in the rhizosphere, if the PSB are introduced into the Pb-contaminated soil. The second reason is that glucose is a recommended carbon source for bacterial growth-media, as a substrate supporting microbial capabilities for solubilizing mineral phosphates (Nautiyal, 1999). Hence, there is a strong possibility that the PSB strain, prepared in the laboratory for remediation treatment with the use of phosphate amendments, will be supplied with glucose and that the gluconic acid will be secreted as soon as the bacteria are in the soil.

To fill this gap in knowledge and clarify the vague issues, related to bacterial impact on pyromorphite stability, the authors conducted microbial and abiotic batch experiments, in which they investigated (i) the growth of the PSB *Pseudomonas putida* in the presence of glucose and pyromorphite as a phosphate source; (ii) the effect of glucose as a carbon source for the PSB *P. putida* on the stability of pyromorphite and Pb remobilization; and (iii) the effect of gluconic acid on pyromorphite stability to understand the mechanisms behind point (ii). The bacterium *Pseudomonas putida* was selected for this project for being common, rather than for being unique or unusual. These microbes are ubiquitous aerobic, gram-negative bacteria, commonly occurring in the environment. *Pseudomonas* can be found in unpolluted soils as well as in sites contaminated with heavy metals (Roane, 1999; Leung *et al.*, 2001; Rugierro *et al.*, 2005; Matlakowska *et al.*, 2008). *P. putida* has been reported to exhibit the capability of solubilizing the mineral forms of phosphates via symbiosis with plants (e.g., Espinosa-Urgel *et al.*, 2002) and it often serves as a model organism for environmental, genetic and bioengineering experiments (Reva *et al.*, 2006).

EXPERIMENTAL METHODS

Materials

Synthesis of pyromorphite (C)

A synthesized pyromorphite $Pb_5(PO_4)_3Cl$ was used in this study. The synthesis follows a method previously described by Flis *et al.* (2010) and Topolska *et al.* (2014). A combination of 0.3M $Pb(NO_3)_2$, 0.14M K_2HPO_4 and 0.05M NaCl solutions was used. Equal volumes of the solutions (500mL) were simultaneously introduced, using a peristaltic pump (flow rate 1.5 mL min^{-1}) into a glass beaker filled partly with 1 liter of distilled deionized water and stirred with a magnetic stir bar. The precipitate was aged in the suspension for 24 hours, as described by Scheckel and Ryan (2002). Then, the precipitate was washed thoroughly on a paper filter (Whatman) with DDIW, air dried, and kept in a desiccator until use.

The pyromorphite was identified, using a HITACHI S-4700 field emission scanning electron microscope coupled with NORAN Vantage energy dispersive spectrometer (SEM/EDS), at the Institute of Geological Sciences, Jagiellonian University, Kraków.

The X-ray powder diffraction (XRD) patterns of the synthesized solids were collected with a Philips PW 3020 X'Pert-APD Diffractometer system (with a Cu anode and a graphite monochromator), using a step scan mode at a step size of 0.02 2Θ and a rate of 1s per step.

Prior to the bacterial batch dissolution experiments, 0.05 g portions of the mineral were sterilized in a heater at 180°C for 3 hours. Prior to the abiotic batch dissolution experiments, 0.05 g portions of pyromorphite were preweathered in distilled deionized water for 24 h and centrifuged. The applied sterilization and aging procedures did not alter the properties of the mineral.

Bacteria

The *Pseudomonas putida* strain (IBPRS KKP 1136), used in this study, was obtained from the commercially available collection of the Institute of Agricultural and Food Biotechnology in Warsaw, Poland. For the batch dissolution experiments, the bacteria were prepared, as in the previous study by Topolska *et al.* (2014): the microbes were grown in a phosphorus-rich medium (MP), as described below, until an optical density at 600 nm (OD_{600}) of 0.8 was reached (mid-logarithmic growth); then they were pelleted by centrifugation, resuspended in the experimental solutions and inoculated 1:100 into flasks.

Table 1

The experimental set – bacterial batch dissolution experiments

Experiment	Solution	Mineral	Bacteria	Time/Temp.	Analysis
Bacterial	MG	Pyromorphite (0.05 g)	*Pseudomonas putida*	160 h/22°C	OD_{600}, pH, Pb, gluconic acid
Control 1	MS				
Control 2	MS	–			
Control 3	MG	–			
Control 4	MG	Pyromorphite (0.05 g)	–		
Control 5	MS				

(MS – succinate supplemented solution; MG – glucose supplemented solution)

Table 2

The experimental set – abiotic batch dissolution experiments

Experiment	Solution with gluconic acid [mM]	Mineral	pH adjustment	Time/ Temp.	Analysis
E1	0 0.1 1.0 10 50	Pyromorphite (0.05 g)	–	1400 h/ 22°C	pH, Pb, P
E2	0 0.1 1.0 10 50	Pyromorphite (0.05 g)	3.5		
E3	0 10	Pyromorphite (0.05 g)	2.0 3.0 4.0 5.0 6.0		

Batch dissolution experiments (B)

Bacterial experiments (C)

Three types of solution were used in the bacterial experiments: a phosphorus-rich solution (MP), a phosphorus-deficient, succinate supplemented solution (MS), and a phosphorus-deficient, glucose-supplemented solution (MG). These were created with the constituents necessary for bacterial growth. The MP solution contained the following ingredients per liter: succinic acid disodium salt anhydrous, 5 g; K_2HPO_4, 0.5 g; NH_4Cl, 1 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; KCl, 0.5 g; FeEDTA, 30 mM; glycerol 6.5 g and 0.125 mL of trace elements ($MnSO_4{\cdot}H_2O$, 0.005 g; $CoSO_4{\cdot}7H_2O$, 0.0065 g; $CuSO_4$, 0.0023 g; $ZnSO_4$, 0.0033 g; MoO_3, 0.0024 g per 100 mL of water). The MS and MG solutions were identical to MP, except for the absence of K_2HPO_4 and glucose introduced, instead of succinate into the MG solution. The pH of the solutions was adjusted to ~7.3 prior to autoclaving. Reagent grade chemicals and ultra-pure, distilled 18 $M\Omega\,cm^{-1}$ water (Milli-Q, Millipore) were used throughout.

In the experiments, pyromorphite was dissolved in the presence of *Pseudomonas putida* bacteria in solutions, containing glucose (MG) or succinate salt (MS) as a carbon source for the microbes. The suspensions were devoid of aqueous phosphate ions and the mineral pyromorphite was the only source of P for the bacteria. The optical density of the bacterial suspensions (OD_{600} – to monitor the culture growth) and the time evolution of Pb concentration and pH were investigated. When the pH of the solutions stabilized (after 24 hours), the suspensions were analyzed for the gluconic acid concentration. To ensure that *P. putida* can acquire phosphorus from pyromorphite, the strain was also grown in MG and MS solutions without any P source. To observe the potential effect of glucose on the solubility of pyromorphite, dissolution of the mineral in sterile MG and MS solutions were compared.

A summary of the experimental conditions is presented in Table 1. The experiments were run in triplicate in 500 mL flasks, containing 100 mL of the solution. Preparation of the mineral particles and of the bacteria for the experiments is described in the "Materials" section. The flasks were incubated at room temperature (21°C ± 1°C) on a gyratory shaker (100 rpm) for 160 h, until a full bacterial growth cycle was observed in the flask with the MG solution. The aeration speed allowed the mineral particles to settle to the bottom of the flask, so that they did not interfere with absorbance readings; a sterile MP solution amended with pyromorphite precipitate was used as a blank. Liquid growth cultures were monitored for contamination by streaking agar plates and examining the morphology of the resulting colonies. Any sign of contamination resulted in termination of the experiment.

Abiotic experiments

Three abiotic experiments (marked as E1, E2 and E3) on the dissolution of pyromorphite were carried out in the presence of gluconic acid; a detailed summary of the experimental setup is presented in Table 2. In Experiment 1 (E1) – investigating the total effect of gluconic acid on pyromorphite solubility – the mineral was dissolved in solutions with several gluconic acid concentrations. The pH of the solutions equilibrated spontaneously. The Experiment 2 (E2) determined the role of metal-chelating capabilities of the acid in dissolution of the mineral. Hence, the pH of all gluconic acid solutions was adjusted to 3.5 with the use of HNO_3 and KOH. In the Experiment 3 (E3), the dissolution reaction was performed under acidic to neutral conditions, which are likely to occur at Pb-contaminated sites.

In all experiments, 0.05 g portions of preweathered synthetic pyromorphite were introduced to polypropylene bottles, containing 100 mL of the solvent; 0.05 M KNO_3 was used as a background electrolyte. The flasks were incubated on a gyratory shaker (100 rpm), at 21°C ± 1°C for 2 months. For this time, the solutions were sampled regularly for analysis of the concentration of dissolved Pb and P, and for pH measurements. To remove the suspended solids, the samples were filtered, using 0.2 μm polycarbonate filters. After the experiments, the residual particles were separated from the solutions by centrifugation. The experiments were performed in triplicate.

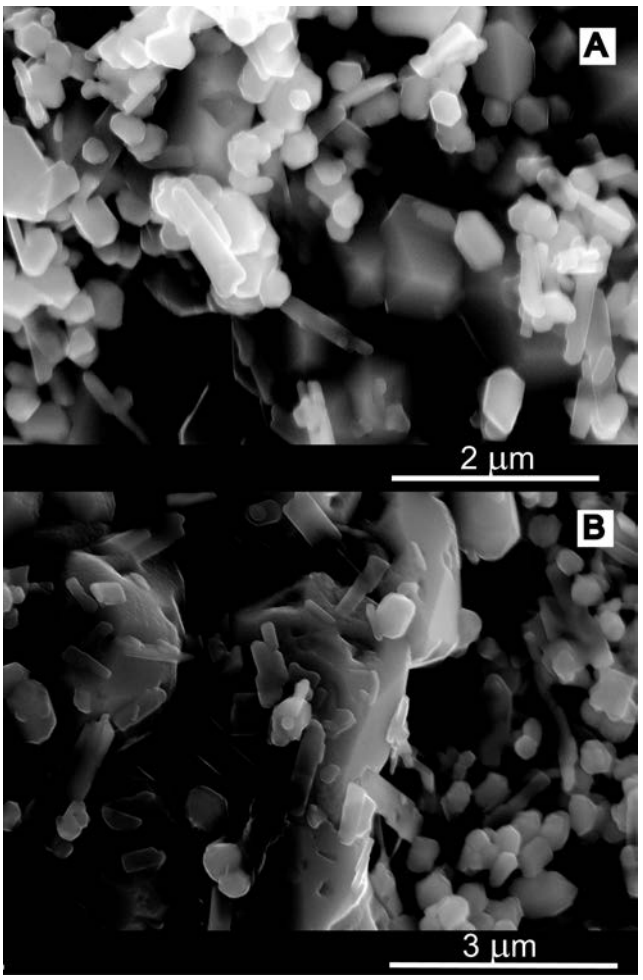

Fig. 1. Scanning electron microscope images of synthesized pyromorphite used in the experiments (upper image) and residuals from the abiotic experiments on dissolution of pyromorphite in 50 mM gluconic acid (lower image)

Analytical methods

Experimental residual particles were characterized, using a HITACHI S-4700 field emission scanning electron microscope coupled with NORAN Vantage energy dispersive spectrometer (SEM/EDS), at the Institute of Geological Sciences, Jagiellonian University, Kraków, and a FEI QUANTA 200 FEG at 15 kV at the Faculty of Geology Geophysics and Environmental Protection, AGH University of Science and Technology, Kraków.

The optical density of the bacterial suspensions was determined by absorbance measurements at 600 nm, with a use of a Cary 50 Bio UV-visible spectrophotometer. A sterile reaction solution with pyromorphite particles was used as a blank.

Total Pb concentration was analyzed with a Thermo Scientific X Series ICP-MS. Prior to the element analysis, the samples were centrifuged and filtered using 0.2 µm polycarbonate filters to remove the bacterial cells if needed. Then, the supernatants were diluted in 0.01M HCl and spiked with the internal standards of Ge and Re. Total P(V) was measured using colorimetry, the molybdene blue method of Dhar *et al.* (2004), using a Cary 50 Bio UV-visible spectrophotometer. The secreted gluconic acid was quantified, using HPLC RP-18 columns, according to the method described by Buch *et al.* (2008).

RESULTS AND DISCUSSION

Characterization of solids

The synthesis, carried out as a part of this study, resulted in a white homogeneous precipitate. It was identified as pure pyromorphite by means of XRD analysis. Examination by SEM/EDS yielded particles, ranging from 0.1 to 2 µm in size and containing Pb, P, O, Cl as major constituents (Fig. 1A). The results of the analysis were described by Topolska *et al.* (2014) with reference to the same material. The methodology of synthesis allowed for precipitation of small particles, similar to those formed in the rhizosphere at Pb-contaminated sites (Traina and Laperche, 1999).

Examination of experimental residuals did not yield significant changes in the morphology of the particles (Fig. 1B). Crystals partially lost their shape, becoming more rounded. The elemental composition of the residuals was unchanged, as indicated by EDS analysis, and no secondary phase was found.

Bacterial experiments

The bacterial experiment was terminated after 160 h, when the microbial extinction in the medium with glucose was observed – the OD_{600} of the suspension dropped by 25% (Fig. 2). In the flasks with the mineral, growth of *P. putida* was apparent, whereas both control experiments, totally devoid of phosphorus, did not yield bacterial culture evolution; after the initial growth, systematic and slow die off of the microbial population occurred. Phosphorus is an essential element necessary for life, thus, the apparent microbial growth in the medium with pyromorphite indicated that the mineral had effectively served as a phosphate source for the bacteria. The growth of the culture was significantly greater, when glucose instead of succinate served as a carbon source for the bacteria. During the 160 h of experimental time, a full growth cycle was observed in the flask with the MG and pyromorphite, whereas the number of the bacteria cultivated on succinate and the mineral was only slightly greater than in the flask without phosphorus. However, the difference between the last two was more apparent with time.

Acidification of the solution supplemented with glucose and inoculated with *P. putida* (Fig. 3A) was apparent. The decrease in pH from the initial 7.3 to the final 3.6 was relatively rapid; it started between the 3rd and the 5th h and lasted for a further 20 h (Fig. 3A). This was not observed for the other flasks. The pH of the abiotic controls, in which pyromorphite was dissolved in the sterile MG or MS solution, remained at the initial level (Fig. 3A). In the control with *P. putida*, MS and pyromorphite, the pH increased slightly from 7.3 to ~9 by the end of the experiment (Fig. 3A).

The significant acidification in the flask with the MG solution obviously inhibited the growth of the bacteria (Fig. 2). The optical density of the suspension with MS, kept increasing after the experimental 160 h (data not shown), reaching

the maximum absorbance of 3.5. However, it is worth mentioning here, that the culture extinction observed for the MG flask is unlikely to occur in nature. In the rhizosphere, the bacteria are exposed to different kinds of physicochemical stress (Reva *et al.*, 2006 and the literature therein). In response, the microbe controls the fluidity and permeability of its membrane by changing the length, degree of saturation, and the cis/trans ratio of fatty acids. In this way, it survives difficult conditions, including a low (~4) pH (Ramos *et al.*, 1997). In the work by Reva *et al.* (2006), it was reported, that in the acidic environment, the growth of *P. putida* is suppressed, when the bacteria are exposed to high oxygen tension and a homogeneous concentration of metabolites. In other words, it is likely that the agitation and laboratory conditions have hindered a natural protective mechanism of the bacteria and as a result the considerable rapid extinction of the culture was observed (Fig. 2). The production of organic acids via a glucose metabolic pathway is unlikely to inhibit the growth of *P. putida* in its natural habitat and the bacteria will grow as long as carbon, oxygen, and phosphorus sources are available.

The authors attribute the acidification of the MG solution to the bacterial secretion of gluconic acid. The measurements indicated that after stabilization of the pH, the concentration of the gluconic acid in the flask with pyromorphite, *P. putida* and MG was 16.5 ± 0.24 mM (HPLC). These findings are in agreement with observations reported by Lin *et al.* (2006) and Buch *et al.* (2008). They indicated that the bacterial metabolic pathway of glucose results in the secretion of significant amounts of gluconic acid and that the process is followed by relatively rapid and significant acidification of the microbial milieu. Lin *et al.* (2006) correlated a decrease in solution pH from an initial 8 to 3, with 16.3 mM of gluconic acid and 3.8 mM 2-keto-gluconic acid under laboratory conditions. The increase in the pH in the control solution with succinate salt from initial 7.3 to final ~9 was a typical result of microbial activity in this kind of medium and is due to sodium hydrolysis. This was observed and described e.g. by Dehner *et al.* (2010), when they were working with the same growth medium.

Dissolving pyromorphite released Pb ions into the solutions (Fig. 3B). In the flasks with *P. putida* and pyromorphite, the Pb concentration successively increased with time. At the end of the experiment, in both cases (MS and MG solutions), the Pb concentration was significantly higher than in the sterile controls. Thus, pyromorphite dissolution was enhanced by the bacterial activity. The rate of the reaction was significantly different for the solution, in which the bacteria were supplemented with glucose, as compared with the solution with succinate. At the end of the experiment, the amount of Pb in the solution with glucose was 20 times higher than in the abiotic controls, whereas in the solution with succinate, it was only 5 times higher. The final concentration of Pb dissolved in the suspensions with MG and MS was 20 ± 3 mM and 5 ± 0.4 mM, respectively. In the sterile solution, there was no effect of the presence of glucose on the solubility of pyromorphite and the concentration of dissolved Pb. In both abiotic controls with MG and MS, pyromorphite dissolved at statistically the same level.

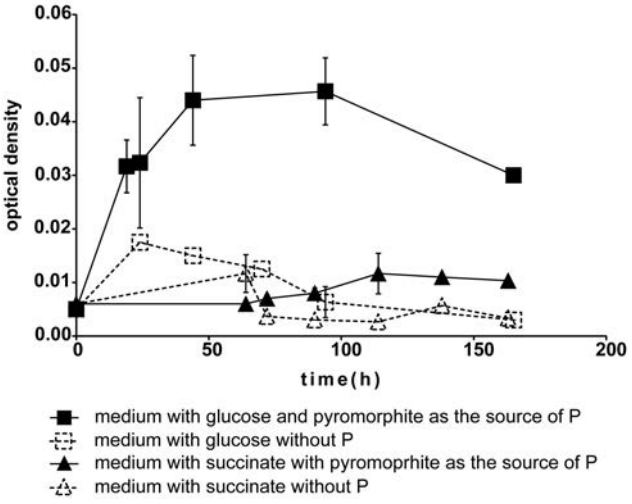

Fig. 2. Growth of *Pseudomonas putida*, expressed as the optical density of the bacterial suspensions. Error bars represent standard deviation of triplicates

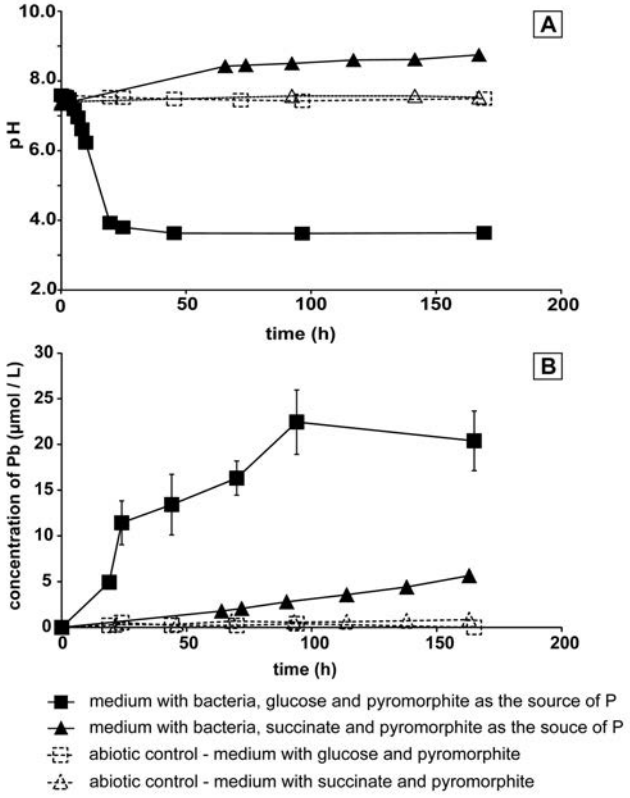

Fig. 3. Results of the bacterial experiments. **A.** Time change of pH in experimental flasks containing pyromorphite as sole source of phosphates in solution. Error bars are depicted within the marks. **B.** Concentration of Pb as a function of time in bacterial experiments and their abiotic controls. Pb was dissolved from pyromorphite. Error bars represent standard deviation of triplicates

The release of Pb correlated with the number of the bacterial cells in the suspensions; the more bacteria, the greater progress in the dissolution. However, regardless of the number of bacteria, the strain supplemented with glucose was particularly efficient in the solubilization process. The

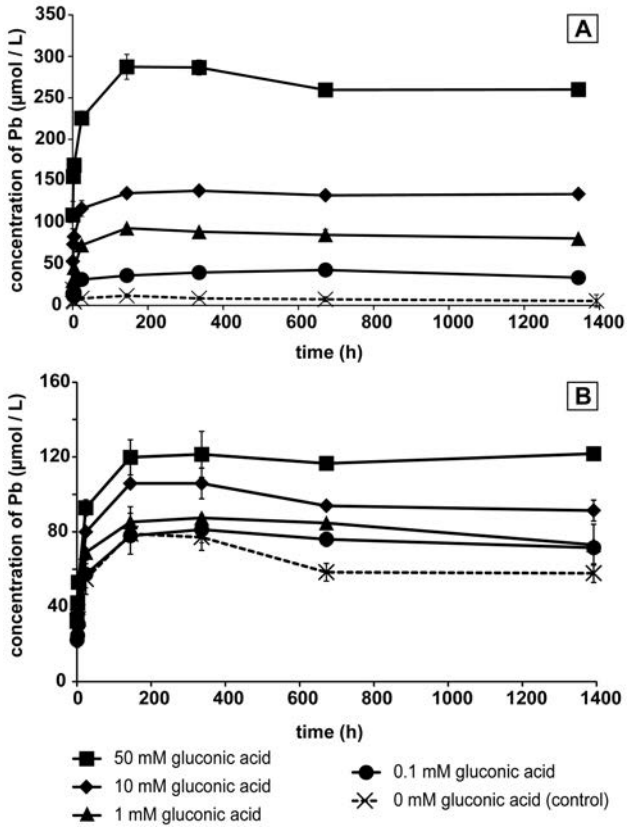

Fig. 4. Evolution of the Pb concentration in solutions of the abiotic experiment. **A.** Concentration of Pb as a function of time in the solutions from abiotic dissolution experiments of pyromorphite in presence of gluconic acid. The pH of the solutions was adjusted spontaneously during the reactions. **B.** The effect of formation of chelates between gluconate and Pb on concentration of Pb dissolved from pyromorphite. The pH of the solutions was adjusted to 3.5. The solution with KNO_3 was a control. Error bars represent standard deviation of triplicates

amount of Pb released during the bacterial experiments was normalized to the number of the microbes in the suspensions. The normalization was performed for the cultures in mid-logarithmic growth, when all of the energy of the organisms was converted into division of cells, and the microbes exhibit statistically identical features. For the flask with MG, the concentration of Pb was 0.55 ± 0.07 mmol per cell $*10^{-6}$, while for the flask with MS it was only 0.05 ± 0.005 mmol per cell $*10^{-6}$. Apparently, a *P. putida* bacterium can dissolve significantly more pyromorphite, when its metabolism is supported by glucose.

Abiotic experiments

In the abiotic experiment 1 (E1), pyromorphite was dissolved in the presence of several gluconic acid concentrations and in KNO_3 as a control. The pH of the solutions was allowed to equilibrate spontaneously and at the end of the experiment it was: 4.15 for the KNO_3 experiment, 3.78 for the 0.1 mM gluconic acid solution, 3.05 for 1mM, 2.38 for 10 mM and 2.00 for 50 mM gluconic acid. Even a small amount of the gluconic acid caused a significant decrease in the pH of the solution. Hence, the bacteria that base their metabolism on glucose are prone to acidify their milieu. In terms of dissolved Pb, in the control-KNO_3 experiment, steady-state conditions were reached within 24 h (Fig. 4A). The more gluconic acid in the solution, the later the plateau in the plot was observed. For example, in the 50 mM gluconic acid solution, the Pb concentration reached a maximum at 150 h of the experimental time, and stabilized after 350 hours. Pyromorphite solubility increased gradually with the gluconic acid concentration in the solution. At the end of the experiment, in the control-KNO_3 solution, the amount of dissolved Pb was almost 50 times lower than in the 50 mM gluconic acid solution. As stated by Drever and Stillings (1997), there are at least three mechanisms, in which organic acids can induce mineral weathering. Decrease of pH along with an ability to chelate metals are the most frequently reported (Shen *et al.*, 1996; Welch and Ullman, 1996; Jones, 1998; Banfield *et al.*, 1999; Strobel, 2001; Jarosz-Wilkolazka and Gadd, 2003; Jones *et al.*, 2003; Gadd 2004; Debela *et al.*, 2010; Bajda, 2011). The protonation of a surface and the formation of organic ligand-metal complexes are complementary and additive processes. The role of gluconic acid as a metal-chelating agent in the dissolution of pyromorphite can be discussed on the basis of Fig. 4B.

In the abiotic experiment 2 (E2), the pH of the solutions was adjusted to 3.5 using HNO_3 or KOH. The experimental pH was chosen on the basis of the results of the microbial experiments, in which bacterial activity acidified the suspensions to pH = 3.5. In all experiments with gluconic acid, the steady-state conditions were reached after 150 h, but in the control-KNO_3 solution, the plateau of the Pb evolution pattern appeared after 350 h (Fig. 4B). In general, the concentrations of Pb in the solutions from the Experiment 2 were lower than in the Experiment 1. However, the amount of dissolved Pb increased gradually with gluconic acid concentration in the reactors. The pH of the solutions in abiotic experiment 2 was constant. Thus the differences in Pb concentration between particular flasks were the effects of the formation of gluconate-metal complexes. Even the lowest concentration of gluconic acid in the solution (0.1 mM) resulted in enhanced pyromorphite dissolution. The amount of Pb dissolved through complexation, expressed as a percentage of the total Pb released from pyromorphite, was: 19% for 0.1 mM gluconic acid solution, 21% for 1 mM, 37% for 10 mM and 52% for 50 mM gluconic acid solution.

The stability constant ($lgK_{1:1}$) reported for 1:1 Pb-gluconate chelates varies between 2.49 (Escandar *et al.*, 1996) and 2.13 (Vicedomini, 1983). This puts gluconic acid among chelating agents of medium strength. However, in the context of pyromorphite dissolution, the Pb-gluconate complexes seem to be more stable than, for example, oxalate, acetate, citrate or malate. In their paper on pyromorphite dissolution in 0.1 mM low molecular weight organic acids (LMWOA), Debela *et al.* (2010) observed that the Pb-oxalate, -acetate, -citrate or -malate chelates had disintegrated within the first 100 hours of the experiment. This was not the case in the present study: the concentration of Pb increased with time and after steady state conditions had been reached, the amount of Pb in the solutions was stable till the end of the experiment (Fig. 4). An interesting issue is that

although in the bacterial experiments, the pH of the suspension with GM and pyromorphite was 3.5 and the concentration of gluconic acid was 16.5 mM, the total dissolved Pb concentration was for the entire time of the experiment much lower than in any of the solutions from abiotic experiment 2 (compare Figs 3B, 4B). The difference is probably due to the biosorption of Pb ions on the bacterial surface. Considering the fact that the experimental conditions (solid to solution ratio, volume of solvent, aeration speed, etc.) were the same for the bacterial and abiotic experiments and the SEM analysis of residual particles did not yield any phases other than pyromorphite, there is no other rational reason for such a difference. The problem of biosorption, in the context of pyromorphite dissolution in the presence of *P. putida*, was discussed by Topolska *et al.* (2014). Sometimes, the process of biosorption is mistakenly associated with non-metabolizing bacteria and for this reason neglected during interpretation of in-vivo experiments. Considering that the bacterially bound metal may go back into solution with time, the process should be appreciated to avoid misleading findings.

The capability of gluconic acid to bind Pb ions varies with the pH of the solution. The concentration of Pb, released by pyromorphite during its 6-weeks dissolution in 10 mM gluconic acid and a pH range from 2 to 6, is shown in Fig. 5A (abiotic experiment 3). Experiments in KNO_3 were carried out simultaneously as a control. In terms of dissolved Pb, the solubility of pyromorphite decreased with the pH of the solutions. As mentioned before, dissolution of pyromorphite strongly depends on the acidity of the environment (Manecki and Maurice, 2008). For the entire experimental pH range, the effect of formation of Pb-gluconate chelates on dissolution of pyromorphite was apparent: the Pb concentration was higher in the 10 mM gluconic acid solutions than in the KNO_3 controls (Fig. 5A). However, the difference was more significant at pH values above 3.0; such conditions are likely to occur in the rhizosphere.

In the solutions with an initial pH of 2.0, the molar Pb/P ratio was identical, within the limits of experimental error, with the stoichiometric ratio of these elements in the pyromorphite's structure (Fig. 5B). Furthermore, at this pH, there was no significant difference between the solution with gluconic acid and the KNO_3 control (Fig. 5B). Under these conditions, the dissolution of pyromorphite was congruent. This is in agreement with previously reported findings (Flis *et al.*, 2011). The final Pb/P ratio in the experimental solutions decreased with increasing pH. The authors attribute this mainly to readsorption of Pb cations on the mineral crystals. The process might occur preferentially in solutions that are weaker proton donors. A possible readsorption of Pb also was indicated by Bajda (2010), as a third step of dissolution of mimetite in organic acids. Mimetite, $Pb_5(AsO_4)_3Cl$, is an arsenic analogue of pyromorphite and exhibits similar thermodynamic properties. It is also possible, that at pH = 5.0 and 6.0, minor precipitation of some secondary Pb phases, such as $Pb(OH)_2$, occurred (Manecki and Maurice, 2008). However, the examination of the experimental residue, did not yield the presence of any phases other than pyromorphite. At a pH above 3.0, the Pb/P ratio was significantly higher in the gluconic acid solution than in

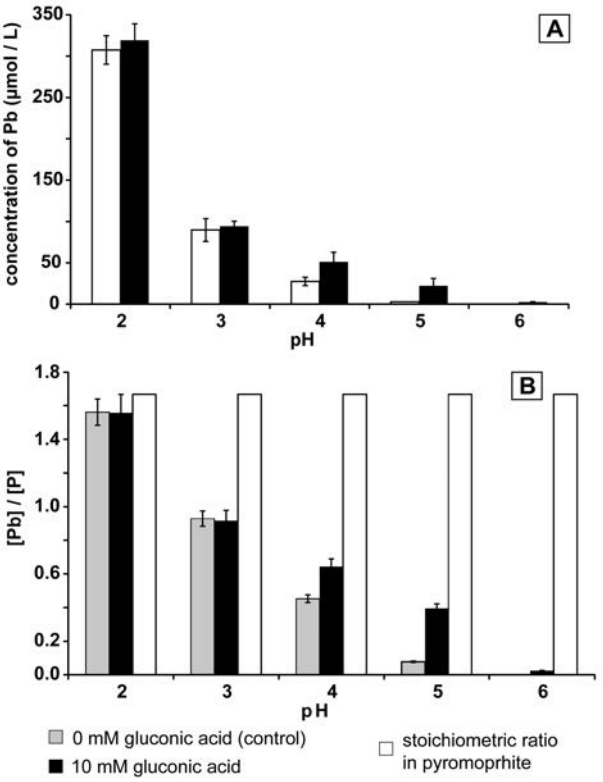

Fig. 5. Effect of pH on concentration of Pb dissolved from pyromorphite (A) and on stoichiometry of the solutions (B) in the experiments on dissolution of pyromorphite in 10 mM gluconic acid and KNO_3 (control). Error bars represent standard deviation of triplicates

the KNO_3 control. We attribute this to the formation of Pb-gluconate complexes, which as discussed above at pH > 3.0 play a significant role in the dissolution of pyromorphite. Seemingly, the formation of chelates inhibits to some extent the readsorbtion of Pb ions on the mineral surface. Lang and Kaupenjohann (2003) and Hashimoto *et al.* (2009) have shown that the formation of pyromorphite in soil can be inhibited by organo-metal complexes. The property of gluconic acid of forming stable chelates with Pb might also hinder the reprecipitation of the dissolved Pb.

The processes determining the bioavailability of dissolved Pb and P at sites remediated by the phosphate-induced method are complex. It is likely that the authors' findings do not address all aspects of the problem. However, the complementary sets of experiments, involving in-vivo and abiotic systems, yielded results, which shed new light on the use of a phosphate-solubilizing bacterium (PSB) on sites contaminated by Pb.

The negative impact of the soil bacteria on the stability of pyromorphite is apparent. Thus it cannot be ruled out that at sites contaminated by Pb, the autochthonous microorganisms might affect the P-induced remediation treatment. Introducing an extraneous strain of P-solubilizing bacteria, as proposed by Park *et al.* (2011a, b), to optimize this Pb-immobilization method might bring the opposite result. Nevertheless, if one decides on such a remediation treatment, the authors recommend against the use of bacteria, which enter

into symbiosis with native plants, as these might inhibit the formation of pyromorphite. The authors also suggest that glucose not be used as a substrate for growth and preparation of the potential PSB strain; otherwise the bacteria will produce gluconic acid, which is an effective solvent of pyromorphite.

CONCLUSIONS

Pyromorphite, which is a stable (log Ksp – 79; Flis *et al.*, 2011) form of Pb, considered not to be bioavailable, effectively served as a phosphate source for the soil bacterium, *Pseudomonas putida* IBPRS KKP 1136 strain, which is a common phosphate-solubilizing microorganism and enhanced the dissolution of pyromorphite. The process was particularly efficient, when the bacterial suspension was supplemented with glucose. As a result of the metabolism of glucose, *P. putida* secreted gluconic acid in the amount of 16.5 ± 0.24 mM. This caused a significant acidification of the bacterial milieu and increased the dissolved Pb concentration in solution. Furthermore, the capability of gluconic acid to form complexes with divalent cations, including Pb, played a significant role in the dissolution of pyromorphite, especially at pH > 3. The Pb-gluconate chelates are relatively stable and they seem to impede the readsorbtion of Pb ions on mineral surfaces. The processes described here might have considerable consequences in the environment at Pb-contaminated sites, remediated by the P-induced method.

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