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Bacterial-induced weathering of ultramafic rock: implications in phytoextraction

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ABSTRACT

Bioavailability of metals in soil is often cited as a limiting factor of phytoextraction (or phytomining). Bacterial metabolites, such as organic acids, siderophores, or biosurfactants, have been shown to mobilise metals, and their use to improve metal extraction has been proposed. In this study, the weathering capacity and Ni mobilisation by bacterial strains was evaluated. Minimal medium containing ground ultramafic rock was inoculated with either of two *Arthrobacter* strains: LA44 (IAA-producer) or SBA82 (siderophore-producer, PO_4-solubiliser and IAA-producer). Trace elements and organic compounds were determined in aliquots taken at different time intervals after inoculation. Trace metal fractionation was carried out on remaining rock at the end of the experiment. Results suggest that the strains act upon different mineral phases. LA44 is a more efficient Ni-mobiliser, apparently solubilising Ni associated with Mn oxides and this appeared to be related to oxalate production. SBA82 also leads to release of Ni and Mn, albeit to a much lower extent. In this case, the concurrent mobilisation of Fe and Si indicates preferential weathering of Fe oxides and serpentine minerals possibly related to the siderophore production capacity of this strain. The same bacterial strains were tested in a soil-plant system: the Ni-hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* was grown in ultramafic soil in a rhizobox system and inoculated with each bacterial strain. At harvest, biomass production and shoot Ni concentrations were higher in plants from inoculated pots than from non-inoculated pots. Ni yield was significantly enhanced in plants inoculated with LA44. These results suggest that Ni-mobilising inoculants could be useful for improving Ni uptake by hyperaccumulator plants.

Introduction

Over the last two decades, there has been increased interest in the development of plant-based remediation techniques (phytoremediation) for the clean-up of polluted soils. These techniques are intended to provide a viable alternative to traditional and civil-engineering methods. Phytoextraction uses plants that take up metals from the soil and accumulate them in their aboveground biomass. Recently, the incorporation of plant-associated bacteria into these systems has been suggested as means of enhancing phytoextraction efficiency (1, 2). Microorganisms play an essential role in the global biogeochemical cycling of metals and nutrients. Their activity can either increase or reduce the mobility of these elements in soils (3). Bacteria influencing the availability of plant nutrients (such as N, Fe or P) have been used as "biofertilizers" to enhance plant nutrient uptake and alleviate nutrient deficiencies (4). Inoculation of pine seedlings with *Burkholderia glathei* PML1(12) significantly improved plant growth and nutrition through the weathering of biotite and release of nutrients such as K and Mg (5). Similarly, the use of metal-mobilising bacteria to enhance plant metal uptake has been proposed by several authors as a promising method to increase metal bioavailability, which can be a limiting factor in phytoextraction processes (6-9). Such microbial inoculants offer an alternative to the controversial use of metal chelants which can increase metal bioavailability but at the same time lead to environmental problems because of their limited biodegradability or to an enhanced leaching of metals to groundwater. Several microbial metabolites have been shown to enhance rock weathering through chemical interaction or, oxidation-reduction reactions, leading to mineral dissolution and metal solubilisation. These metabolites include inorganic acids (HNO_3 and H_2SO_4), organic acids (such as citric, oxalic, gluconic acid) and metal-chelating ligands such as iron-complexing siderophores or biosurfactants (10).

Microbial activity was associated with an enhanced release of Co and Ni in ultramafic soils of New Caledonia (11). Quantin et al. (12) showed that bacterial reduction of oxides led to the solubilisation of Fe, Mn, Ni and Co and modified metal distribution in the soil. Li et al. (13) related the production of short-chain organic acids by rhizosphere bacteria with the mobilisation of Cd and Zn. Cell-free culture filtrates of rhizobacterial strains have been shown to mobilise soil Ni (8, 9), Zn and Cd (6, 14). This bacterial-promoted solubilisation of metals led, in some cases, to an increase in metal uptake by plants. For instance, inoculation with Ni-mobilising rhizobacteria enhanced Ni uptake by the Ni-hyperaccumulator *Alyssum murale* (9, 15) and by the non-hyperaccumulator *Brassica juncea* (16). Similarly, Zn/Cd-mobilising bacteria enhanced metal accumulation in *Salix caprea* (14) and Zn concentration in *Thlaspi caerulescens* (6).

In this study, two bacterial strains isolated from the rhizosphere of the Ni-hyperaccumulator *Alyssum serpyllifolium* (ssp. *lusitanicum* and ssp. *malacitanum*) were evaluated for their weathering capacity and ability to mobilise Ni from different mineral phases of ultramafic rock. The influence of these strains on metal availability and plant uptake was further evaluated in a pot experiment by growing plants in ultramafic soil and adding bacterial inoculants.

Materials and methods

Bacterial strains and preparation of inoculants

Two bacterial isolates identified as members of the *Arthrobacter* genus were selected for this study. These strains were isolated from the rhizosphere soil of two subspecies of the Ni-hyperaccumulator *Alyssum serpyllifolium* (8). These included the strains (identified by partial sequencing of 16S rDNA) *Arthrobacter nitroguajacolicus*

LA44 (an IAA-producer) isolated from the rhizosphere of *Alyssum serpyllifolium* subsp. *lusitanicum* (Melide, NW Spain) and *A. oxydans* SBA82 (a siderophore-producer, PO_4-solubiliser and IAA-producer) isolated from *Alyssum serpyllifolium* subsp. *malacitanum* (Sierra Bermeja, S Spain).

To prepare the bacterial inoculants, strains were cultivated in 869 medium (17) for 3 days, harvested by centrifugation (4000 g, 15 min, 4 ºC), washed and re-suspended in 10 mM $MgSO_4$ to an optical density of 1.0 at 600 nm (about 10^8 cells per ml).

Batch culture experiment design

To evaluate the ability of the bacterial strains to mobilise Ni they were cultivated in minimal medium containing sterile ultramafic rock. The ultramafic rock was collected from the serpentinitic area of Morais (NE Portugal) where *A. serpyllifolium* subsp. *lusitanicum* is found growing. The chemical composition of this rock is given in Table 1. As is expected for an ultramafic rock, the SiO_2 content is less than 45%, the Al_2O_3, K_2O and CaO contents are low, whereas the MgO content and total Ni, Co and Cr concentrations are elevated. Mineral associations and element distribution were characterised by scanning electron microscope (SEM EVO LS 15) equipped with EDX microprobe analysis (INCA X-act, Oxford Instruments, United Kingdom) (Fig. 1).

A modified 284 medium (18) was used with no trace of Ni, Mn or Co and a reduced concentration of K and Fe. This modified 284 medium contained (per liter medium): 6.06 g Tris-HCl, 4.68 g NaCl, 0.015 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.20 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.04 g $Na_2HPO_4.2H_2O$ plus oligoelements (0.3 mg H_3BO_4, 0.02 mg $CuSO_4.5H_2O$, 0.036 mg $Na_2MoO_4.2H_2O$) adjusted to pH 7. The medium was supplemented with glucose (0.5 g l^{-1}) and fructose (0.5 g l^{-1}). Flasks containing 2 g of ground rock (< 100

µm) were autoclaved three times with a 24h interval between cycles. To each flask, 100 ml of modified 284 medium was added. Each flask was then inoculated with 1 ml of one of the two bacterial inoculants, LA44 or SBA82, or with 1 ml of 10 mM $MgSO_4$ (control treatment). Flasks were incubated in the dark for two weeks at 28 ºC and agitated on a horizontal shaker 150 rpm. Five replicates were prepared for each treatment.

Aliquots (of 3 ml) were taken at different times (1, 2, 4, 7, 10 and 14 days). Samples were centrifuged, the supernatant was decanted and filtered (0.22 µm pore size) and immediately frozen until analysis. Samples were analysed for element concentrations, organic acids and phenolic compounds. At the same sampling times, bacterial densities were determined by plating out serial dilutions of the samples on 10-fold diluted 869 agar medium, for three replicates of each treatment. At the end of the experiment (14 days), the medium pH was determined. The sterility of the control flasks was checked by plating on 1:10 diluted 869 agar medium. Finally, the cultivated bacterial strains (LA44 and SBA82) were compared with the original inoculants using BOX-PCR and following the methods described in Becerra-Castro et al. (8).

Analysis of supernatant samples

The concentration of Al, Co, Cr, Fe, Mn, Ni and Si in the culture medium was determined by ICP-MS (Elan 9000 DRCe, Perkin Elmer) and K by emission spectrophotometry (AAS; Perkin Elmer 2380, Norwalk, CT).

Carboxylic acids were separated by reversed-phase liquid chromatography on a C_{18} column with 5 µm particle size (GraceSmart, RP18, and 2.1 x 150 mm column from Grace Davison Discovery Sciences, Deerfield, Il, United State) and analysed by liquid chromatography -electrospray ionization - time of flight mass spectrometry (LC-ESI-

144 TOFMS, Agilent Technologies, Palo Alto, CA, United States) as described by Jaitz et al.
145 (19). Quantification of selected phenolic compounds (caffeic acid, catechin, p-coumaric
146 acid, 2.5-dihydroxybenzoic acid, epicatechin, ferulic acid, gallic acid, 4-hydroxy-3-
147 methoxycinnamidaldeyhde, resveratrol (3,5,4'-trihydroxy-trans-stilbene), sinapic acid,
148 syringic acid and vanillic acid) was performed via LC–MS/MS in negative ionisation mode
149 on a 6410 triple quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA,
150 United States) equipped with an electrospray ionisation (ESI) interface (20).
151
152 *Analysis of rock residues*
153 The remaining ground rock at the end of the experiment was recovered by
154 centrifugation, washed 4 times with sterile deionized water and air-dried. A metal
155 fractionation scheme was carried out on the residual rock samples according to Zeien and
156 Brümmer (21):
157 - First, soil samples were shaken with 1 M NH_4NO_3 (unbuffered) for 24 h at room
158 temperature, the solution was separated by centrifugation (15 min, 938 g) and filtered
159 and stabilized with HNO_3 (14.4 N). This extracts the *water soluble and exchangeable*
160 metals (F1).
161 - Second, the resulting residue was shaken for 24 h at room temperature with 1 M
162 NH_4OAc (adjusted with 50% HOAc to pH 6), centrifuged, filtered and stabilized with
163 HNO_3 (14.4 N). 25 ml NH_4NO_3 was added to the remaining residue and shaken for 10
164 min. The solution was separated by centrifugation, filtered and combined with the
165 NH_4NOAc extract. This step extracts *easily mobilisable* metals (F2).
166 - Third, the residue was shaken with 0.1 M NH_2OH-HCl + 1 M NH_4OAc (adjusted to pH
167 6 with diluted HCl) for 30 min, separated by centrifugation, filtered and stabilized with

HCl (12 N). 25 ml 1 M NH_4OAc were added to the residue and shaken for 10 min, centrifuged, filtered (repeated once) and combined with the above solution. This extracts metals bound to *Mn oxides* (F3).

- Fourth, 50 ml 0.2 M NH_4-oxalate (= 0.2 M Di-ammonium oxalate-monohydrate + 0.2 M oxalic acid-dihydrate adjusted to pH 3.3 with diluted NH_4OH) were added to the residue, and after shaking (over-head) for 4 h in the dark, the solution was separated by centrifugation (15 min, 938 g) and filtered. A second volume of 25 ml 0.2 M NH_4-oxalate (pH 3.3) was added to the residue and shaken over-head in the dark for 10 min. The solution was separated by centrifugation (15 min, 938 g), filtered and the two filtrates were combined. This step targets those metals bound to *amorphous Fe oxides* (F4).
- Fifth, 50 ml 0.1 M ascorbic acid + 0.2 M NH_4-oxalate (= 0.1 M L(+)-ascorbic acid + 0.2 M NH_4-oxalate buffer: 0.2 M oxalic acid-dihydrate adjusted to pH 3.25 with diluted NH_4OH) were added, and after shaking horizontally in a water bath for 30 min at 96 ºC ± 3 ºC, the solution was separated by centrifugation (15 min, 938 g) and filtered. The remaining solution was extracted with 25 ml 0.2 M NH_4-oxalate (pH 3.3) by shaking over-head in the dark for 10 min. The solution was separated by centrifugation (15 min, 938 g), filtered and the filtrates were combined. This extracts metals bound to *crystalline Fe oxides* (F5).
- Finally, the remaining residue was digested with *aqua regia* to determine the *residual* (silicate-bound) fraction (F6).

The concentration of Al, Co, Fe, Mn, Ni and Si were analysed in the filtered supernatants of each extraction by ICP-OES (Vista Pro; Varian Inc., Australia). In the case of Si, the residual fraction was obtained by subtracting the sum of all fractions from the

192 total Si (obtained by Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF).

193

194 *Rhizobox experiment*

195 Ultramafic soil was collected from a serpentine site in Redschlag, Austria (22). The
196 soils at this site present a slightly acidic pH (pH_{CaCl2} 6.55) with an organic C content of 13
197 g kg^{-1}, a predominance of Mg in the exchange complex and high concentration of total Ni
198 (2580 mg kg^{-1}). The soils were air-dried and sieved to <2 mm before being filled into the
199 rhizoboxes (bulk density, 1.2 g cm^{-3}). The rhizobox used in this study was based on the
200 system of Fitz et al. (23). Root growth was restricted to a central compartment by a 30 µm
201 mesh size nylon net (Labor Becker, Vienna, Austria) to avoid growth of root hairs into the
202 adjacent 2-mm-thick root-free rhizosphere soil compartment. The root-free rhizosphere
203 compartment was separated from bulk soil by the same 30 µm mesh size nylon net. The
204 rhizoboxes were made of Perspex acrylic material, allowing observation of root growth.
205 Rhizoboxes were wrapped with aluminium foil during the experiment to avoid growth of
206 photosynthetic soil organisms and weed germination.

207 For this experiment, the subspecies *malacitanum* of the Ni-hyperaccumulating *A.*
208 *serpyllifolium* was selected. Surface-sterilized seeds were germinated on perlite and
209 transplanted into rhizoboxes (2 seedlings per rhizobox). After 5 weeks, 20 ml of LA44 or
210 SBA82 bacterial suspensions (prepared as described previously) were added to the
211 rhizoboxes at the base of the plants. The same amount of 10 mM $MgSO_4$ was added to non-
212 inoculated plants in control rhizoboxes. All treatments were replicated five times. Plants
213 were grown for a further 2 months. At harvest, the shoots and roots of plants were
214 separated, washed with pressurised tap water (and 0.05 M $CaCl_2$ in the case of roots)
215 followed by deionised water, oven-dried at 45 ºC and ground. Shoot tissues (0.1 g) were

digested in a 2:1 mixture of concentrated HNO_3:HCl on a hot plate at 160 ºC, and the Ca, Mg, K, P, Fe and Ni concentration was measured by ICP-OES (Vista Pro; Varian Inc., Australia). Data were expressed on the basis of dry weight plant material. The full recovery of plant roots was not possible and only shoot dry weight was taken into account when assessing the effects of inoculation on plant biomass production or Ni yield.

Soil analyses were carried out on the <2 mm fraction of rhizosphere and bulk soil samples. Soil pH was measured in H_2O using a 1:2.5 soil:solution ratio. Exchangeable cations were extracted with 0.1 M $BaCl_2$, and Al, Ca, K, Mg and Na determined by ICP-OES (Vista Pro; Varian Inc., Australia). Water-soluble Ni concentration was analysed by ICP-OES in soil extracts after 30 min shaking using a 1:2.5 soil:H_2O ratio. $Ca(NO_3)_2$- and $Sr(NO_3)_2$-extractable Ni concentrations were determined by ICP-OES in soil extracts after 2 h shaking using a 1:4 soil:extractant ratio for both extractants.

Statistical analysis

Differences in element solubilisation and organic acid production in the batch culture experiment were determined using a repeated measures analysis of variance (rANOVA). A multiple comparison of means was determined by the "post-hoc" Bonferroni. Data were log transformed where necessary to achieve homogeneity of variance. Mann–Whitney U-tests were used to detect significant differences between microbial densities.

Changes in the culture medium composition (element and organic acid anion concentrations) were also analysed by principal component analysis (PCA). Values below detection limits (DL) were recorded as the ½ DL for statistical analysis. A varimax rotation was applied to the PCAs in order to facilitate the interpretation of the extracted principal components.

Differences in plant biomass and plant metal/nutrient concentration in the rhizobox-grown plants were determined using analysis of variance (ANOVA). A multiple comparison of means was determined by the "post-hoc" Least Significance Difference test. Data were log transformed where necessary to achieve homogeneity of variance. Comparison of means between bulk and rhizosphere soil was achieved by the Student's t test for related means.

Results

Bacterial growth in medium with ground rock

During the experiment, no significant differences were found in the bacterial densities of the two strains (data not shown). They presented a similar growth rate, reaching a stable density of 1.1-6.4 x 10^8 CFUs ml^{-1} medium by day 2. The identity of the recovered strains at the end of the experiment was confirmed by BOX-PCR to be the same as the originally inoculated strains.

Element solubilisation from rock and release of organic compounds

Element concentrations in control flasks (non-inoculated) varied slightly over time. They were generally lower than in experimental flasks (inoculated) (Fig. 2). Iron and K were added in low concentrations to the initial liquid medium (1.6 and 200 µM, respectively), while Co, Mn and Ni were absent. Bacterial activity and growth either led to a depletion in some elements compared to the non-inoculated control (such as K), or alternatively, to the release of some elements from the rock (such as Fe, Co, Mn, Ni, or Si). At the end of the experiment there was no difference in the pH of the culture medium between treatments. The repeated measures ANOVA showed that the time, inoculant

264 treatment, and the time x inoculant interaction factors, significantly affected changes in the
265 medium composition ($p < 0.01$; Table 2).

266 The K concentration in control flasks was similar to the initial K concentration in
267 inoculated flasks and remained constant for the duration of the experiment, values varied
268 from 216 µM on day 1 to 252 µM on day 14 (Fig. 2e). In contrast, bacterial growth led to a
269 significant decrease in the original K concentration of the medium. The K concentrations
270 were lower in the inoculated flasks than in the controls throughout the experiment.
271 Minimum values of 21 µM and 116 µM were detected on day 2 in LA44 and SBA82
272 cultures, respectively. From that time onwards however, there was a steady release of K
273 into the medium in the presence of either strain, although the concentrations never reached
274 the values of controls. On day 14 the K concentration was 104 µM and 209 µM in the
275 LA44 and SBA82 cultures, respectively.

276 Concentrations of Ni and Mn in the LA44 cultures on day 2 were 8.9- and 7.1-fold
277 higher than in the controls, and 3.1- and 2.7-fold higher than in the SBA82 cultures (Fig. 2c
278 and d). The concentrations of both elements were significantly correlated ($R^2 = 0.79$, $p <$
279 0.001). Inoculation with LA44 induced a rapid release of both Ni and Mn. Their
280 concentrations in the medium peaked on day 2. This bacterial strain also led to a higher
281 dissolution of Co than either SBA82 cultures or the control (Fig. 2f), although in this case
282 concentrations decreased with time. Concentrations of Fe or Si in LA44 cultures were
283 similar to those in the non-inoculated control (Fig. 2a and b).

284 In contrast to what was observed with the LA44 cultures and in the controls, the
285 presence of strain SBA82 led to a significant release of Fe and Si from the ultramafic rock
286 into the medium solution. By day 7 concentrations of these two elements were up to 4.1-
287 and 3.8-fold higher than those detected in controls, respectively. Both elements followed a

similar pattern with time, a higher rate of solubilisation occuring during the initial days which then stabilised between days 4-7 (Fig. 2a and b). Concentrations of Fe and Si in SBA82 cultures were significantly correlated ($R^2 = 0.93$; $p < 0.001$). Values for Ni and Mn in SBA82 cultures were also significantly higher than in controls (although lower than in LA44 cultures): by day 4 concentrations of these two elements were up to 2.8- and 2.6-fold higher than in the control, respectively (Fig. 2c and d), and were also significantly correlated ($R^2 = 0.95$; $p < 0.001$). The presence of this strain also induced the release of Al into the medium; concentrations of between 0.3 µM and 0.5 µM were detected. In contrast, Al was consistently below the detection limit in both controls and LA44 cultures.

None of the phenolic compounds were detected in the culture media. In contrast, detectable levels of malate, malonate, oxalate and succinate were measured. Aconitate and citrate were below the detection limit. Malate concentrations in control and SBA82 cultures were detected in a similar range, and remained always below 0.70 µM. LA44 cultures showed somewhat higher values of this anion, between 0.20 – 1.15 µM. In the control treatments the malonate concentration did not exceed 0.30 µM, and in SBA82 cultures the values ranged from 0.20 – 0.55 µM. Higher concentrations of malonate, varying from 0.30 – 0.90 µM, were found in LA44 cultures (data not shown). Significant differences were observed in inoculated flasks with respect to oxalate and succinate concentrations ($p < 0.05$; repeated measures ANOVA; Table 2). In control cultures, succinate was only detected at some sampling times (2, 7, 11 and 14 days).With an exception of day 1, succinate concentration in SBA82 cultures was generally higher (1.9 – 3.8 µM) than in LA44 cultures (0.6 – 1.9 µM) (Fig. 3a). With respect to oxalate, the difference in concentration between the two strains was more marked (Fig. 3b). The concentrations in SBA82 cultures were similar to those in control treatments (4.1 – 5.4 µM), whereas those in

LA44 cultures increased from 18 µM on day 1 to close to 50 µM on day 14.

Element solubilisation and organic acid production were subjected to principal components analysis (PCA). The Kaiser-Meyer-Oklin value was 0.71 and the Barlett's Test of Sphericity reached statistical significance ($p < 0.05$), supporting the factorability of the correlation matrix. The PCA extracted two principal components that explained 70% of total variance (Fig. 4). The first component (PC1; 41%) was mainly represented by Co, Ni and Mn concentration and by malonate, oxalate and malate concentrations. The second principal component (PC2; 29%) was related to the concentration of Fe, Al, Si, and to a lesser extent to Cr. In the PCA plot, control samples are grouped together, with negative scores on both axes. SBA82 samples are mainly placed on the positive axis of the second component (PC2) represented by Al, Fe and Si concentrations in the medium, whereas LA44 samples are grouped on the positive axis of the first component (PC1) associated with Co, Mn and Ni concentrations and organic acids (Fig. 4). The Principal component analysis confirmed the main differences observed between the two inoculants in the release of trace elements and organic acids into the medium.

Metal fractionation in rock samples

Fig. 5 shows the element fractionation in the recovered rock from the control treatment, as well as the bacterial-induced depletion/increase of each fraction relative to the control treatment. The residual fraction was the most important geochemical phase for Al, Cr and Si (representing more than 85% of the total content). This phase was also dominant for Fe and Ni (representing more than 60%) but for these two elements an important fraction was also associated with either amorphous or crystalline Fe oxides (32-35% in total). Mn was principally associated with the Mn oxide fraction (41% of total Mn). EDX

336 microprobe analysis confirmed the presence of Ni associated with Mn oxides (Fig. 1), Co
337 was distributed among Mn oxide, amorphous Fe oxide and residual fractions.

338 Bacterial activity significantly influenced element fractionation in the rock, and these
339 changes were dependent on the bacterial strain (Fig. 5). After incubation with strain LA44,
340 all elements associated with the Mn oxide fraction were significantly reduced compared to
341 the control. In the case of Mn and Ni, concentrations were reduced from 492 to 393 mg kg^{-1}
342 and from 108 to 87 mg kg^{-1}, respectively (Fig. 5d and f). These metals were then re-
343 distributed among the more labile fractions (F1 and F2) and amorphous Fe oxides (F4). In
344 F1, Ni concentrations increased from 16.9 to 40.0 mg kg^{-1}, and Mn from 26.6 to 72.8 mg
345 kg^{-1}. Corresponding shifts in F2 were less pronounced: Ni increased from 31.6 to 35.5 mg
346 kg^{-1} and Mn from 21.9 to 37.0 mg kg^{-1} Although not always statistically significant, a
347 similar pattern was observed for Co (Fig. 5g): an increase in the first two fractions and a
348 decrease in Co associated with the Mn oxide fraction. This effect of bacterial activity on
349 Mn oxides was either not detectable, or far less pronounced, in rock samples which were
350 incubated with strain SBA82 (Fig. 5).

351 In the case of SBA82, the presence of this bacterial strain led to a significant increase
352 in soluble and exchangeable concentrations of the major elements Al (from 1.5 to 1.8 mg
353 kg^{-1}), Si (from 399 to 840 mg kg^{-1}) and Fe (from 17.8 to 48.2 mg kg^{-1}) (Fig. 5a-c). This
354 strain also induced a significant increase in all elements associated with amorphous Fe
355 oxide fractions compared to controls. Concentrations of the major elements (Al, Fe and Si)
356 associated with this phase were increased by 20-27%, while the trace metals, Ni and Cr,
357 increased from 797 to 971 mg kg^{-1} and from 13.9 to 18.0 mg kg^{-1}, respectively. In parallel,
358 a reduction in the residual Ni fraction was observed (from 2322 to 2115 mg Ni kg^{-1}). These
359 effects were far less pronounced in the LA44 cultures (Fig. 5).

Pot experiment

After three months growth, plants grown in inoculated pots presented a higher biomass than plants from non-inoculated pots, although the difference was not significant for either inoculant (Fig. 6a). Inoculation did not significantly influence nutrient contents in shoot tissues (Table 3). However, some trends could be observed, for instance, plants grown in inoculated pots tended to have a higher Fe and Ca shoot content than plants grown in non-inoculated pots. Furthermore, inoculation with SBA82 also tended to increase shoot P content (Table 3).

No differences were observed in root Ni concentrations due to inoculation: values were in the range of 806 to 849 mg Ni kg^{-1} for all three treatments. In contrast, strain LA44 tended to increase Ni accumulation in shoots (reaching up to 11873 mg kg^{-1}) compared to plants grown in non-inoculated pots (mean Ni concentration of 9700 mg kg^{-1}), although this increase was not significant (Fig. 6b). Moreover, bacterial inoculation with both LA44 and SBA82 increased the total Ni phytoextracted by 1.4- and 1.3-fold, respectively, compared to plants grown in non-inoculated pots. This increase was statistically significant in the case of plants grown in LA44-inoculated pots ($p < 0.05$; Fig. 6c).

Plant growth induced few changes in physico-chemical soil properties and no significant effects of bacterial inoculation were observed. In plants grown in non-inoculated pots, rhizosphere soil pH was slightly higher than bulk soil pH: 8.4 ± 0.1 compared to 8.3 ± 0.1. As expected, the cation exchange complex was dominated by Mg (8.1 – 9.4 $cmol_c$ kg^{-1}). The CEC was 10.3 and 10.9 $cmol_c$ kg^{-1} in bulk and rhizosphere soil, respectively, and Ca/Mg quotients were consistently less than 1 (around 0.2). Water-soluble Ni concentration

was significantly higher ($p < 0.01$) in the rhizosphere compared to bulk soil (0.12 and 0.06 mg kg^{-1}, respectively). In contrast, $Sr(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations tended to be depleted in the rhizosphere (falling from 0.49 to 0.44 mg kg^{-1} and 6.44 to 5.89 mg kg^{-1}, respectively). Bacterial inoculation did not lead to any significant changes in these general physicochemical properties.

Discussion

The influence of two bacterial strains on the weathering and solubilisation of elements from an ultramafic rock was assayed. Bacterial activity was found to have a significant influence on mineral weathering, as indicated by the release of structural elements (Al, Fe, Si) or adsorbed or interlayer cations (such as Mn, Ni, Co) into the medium solution and their re-distribution among the different geochemical compartments. The release of elements into the medium was most pronounced during the first two days of incubation, coinciding with the period of maximum bacterial growth. Furthermore, based on the differences observed between the two bacterial strains, and on the PCA analysis, our results suggest that the strains act preferentially upon different mineral phases.

In the case of SBA82, the co-release of Al, Fe and Si into the culture medium suggest a preferential weathering of Ni-rich ferromagnesium silicates. Serpentine and chlorite have been identified as the dominant primary minerals in these rocks (unpublished data). Most Fe was included in primary silicate minerals and crystalline Fe oxides, and appears to be rendered amorphous during microbial weathering. Other elements released during weathering were also incorporated into amorphous Fe oxide phases (Al, Si, Co, Cr, Mn, Ni) or released into soluble and exchangeable pools. Amorphous Fe oxides have a high specific surface area and can act as important sorbents of trace metals (24). Our results are in line

with previously described weathering sequences of serpentine minerals (25). Since strain SBA82 is a siderophore-producer, its mechanism of weathering probably involves involve siderophore-induced mineral dissolution. Siderophores are iron-chelating secondary metabolites which are known to be produced under Fe-limiting conditions. The reduced Fe concentration of the culture medium used in this study will presumably have induced siderophore production by strain SBA82. Previous studies have demonstrated siderophore-promoted dissolution of goethite and hornblende and consequent release of Fe, Si, and Al (26, 27). Although siderophores have a high affinity for Fe(III) they are known to form complexes with other trace elements (28, 29). Their presence in the culture medium would therefore maintain elements such as Al, Fe and Si in solution (which was the case in SBA82 cultures). Siderophore mediated Fe solubilisation from Fe-rich minerals could also lead to the solubilisation and re-distribution of their companion trace metals (Cr, Ni).

On the contrary, since LA44 is not a siderophore-producer the concentration of major elements (Al, Fe and Si) was no different from controls. However, it cannot be completely ruled out that strain LA44 is able to alter ferromagnesian minerals by other means. This strain could alter ferromagnesian minerals by attack with oxalic acid and/or other metabolites that it produces. Bacterial secretion of organic acids has been related to the weathering of silicates and Fe (hydr)oxides (30). Extracellular polymers produced by bacteria have also been shown to affect mineral solubility (31). Nonetheless, the strong mobilisation of Co, Mn and Ni into the culture medium combined with a general reduction of elements associated with Mn oxides suggests that the LA44 strain principally acts on this mineral phase. Although Ni associated with Mn oxides is not the principal Ni fraction in this rock, these oxides seem to be important in determining Ni availability. The analysis of rock samples by SEM confirmed an association between Mn and Ni in this rock. The role

of Mn-oxides in determining Ni availability has also been demonstrated by other authors in ultramafic areas. In a study evaluating Ni and Cr extractability in ultramafic soils, Quantin et al. (24) concluded that Ni behaviour appeared to be partly controlled by pH and partly by Mn-oxides. Antić-Mladenović et al. (32) suggested that the dissolution and precipitation of Fe/Mn oxides, organic matter transformations, and adsorption on solids were important processes controlling Ni solubility in ultramafic soils. Similarly, a study carried out in the ultramafic region of Morais, in the same serpentine outcrop where the rock sample used in this study was collected, revealed that the Ni bioavailability in these soils is linked to the Mn oxide fraction (33). The strong release of Ni and Mn, and to a lesser degree of Co, in LA44 cultures seems to be related to the release of organic acids by this bacterial strain. Low molecular weight organic acids, and in particular oxalic acid, are most often cited as the main agent in biogeochemical weathering of silicate minerals. These organic ligands can attack minerals directly by complexing with ions at the surface, weakening metal-oxygen bonds, or catalysing dissolution reactions. Indirectly they affect weathering rates by complexing ions in solution, thus lowering the solution saturation rate (34). The production of organic acid anions by bacterial strains was related to the weathering of hornblende (35) and the mobilisation of metals from carbonates and oxides (13). In the present study, oxalate production seems to have an important role in the solubilisation of Mn and Ni. Oxalate-promoted dissolution of Mn oxides by reduction of Mn(IV) to Mn(II) has been proposed by several authors (36-38). The higher concentrations of Ni released into the culture medium (or the re-distribution of Ni towards labile phases) may be related to the ability of strain LA44 to create reducing conditions. Although no differences in Eh were detected in culture mediums inoculated with either strain, LA44 may generate redox microgradients at the rock surface which were not detected. Solubilisation of Ni in the case

of SBA82 came mainly from serpentine group minerals, although a possible influence of this strain on Mn oxides cannot be discarded. Siderophores can also interact with Mn oxides (39) and could therefore explain, at least partially, the solubilisation of Mn induced by SBA82. In fact, Mn oxides were also reduced after incubation with this strain, although its influence on this phase was far less pronounced than for the LA44 strain.

Metal availability in the soil, or the replenishment of labile metal pools from solid soil phases, is a key element to successful phytoextraction. There has been considerable debate as to whether or not hyperaccumulating plants are able to access metal fractions not available to non-accumulator plants thus increasing metal uptake (1, 40, 41). This study demonstrates the capacity of rhizobacterial strains associated with hyperaccumulating species to mobilize metals such as Ni from rocks. By increasing soil labile metal fractions these bacterial inoculants could also potentially increase metal uptake by metal-(hyper)accumulating plants. In phytoextraction (or phytomining) this would lead to an overall improvement in the efficiency of the process. In the present study, we tested the effect of strains LA44 and SBA82 on metal uptake by the hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* in a rhizobox experiment.

In rhizospheric studies of hyperaccumulators some authors show a depletion of labile metal fractions in the rhizosphere (attributed to plant uptake), while others indicate an increase in such fractions in the rhizosphere. In either case, the differences in the concentration of labile metal fractions do not explain the extreme metal uptake by these plants. It therefore continues to be a point of controversy whether or not these plants are able to access metal fractions not available to non-accumulating plants (thus increasing metal uptake), or if their root activity leads to a faster replenishment of soluble metal pools. In this study, water-soluble Ni concentrations increased in the rhizosphere of *A.*

479 *serpyllifolium* subsp. *malacitanum* compared to bulk soil. The same effect was surprisingly
480 not seen in $Sr(NO_3)_2$- or $Ca(NO_3)_2$-extractable Ni concentrations. However, a similar
481 increase in water-soluble Ni and a decrease in labile Ni in the rhizosphere has been
482 observed with other Ni hyperaccumulators such as *Thlaspi goesingense* (22, 42) and *A.*
483 *serpyllifolium* subsp. *lusitanicum (A. pintodasilvae*) (43). Wenzel et al. (22) and
484 Puschenreiter et al. (42) suggested a more intense weathering of Ni-rich minerals in the
485 rhizosphere of the hyperaccumulator *Thlaspi goesingense*, and a concurrent release of labile
486 Ni.

487 After 14 weeks growth, plant biomass of plants grown in inoculated pots tended to be
488 higher than that grown in non-inoculated pots: shoot biomass was up to 1.3- or 1.4-fold
489 greater in plants grown in pots inoculated with LA44 or SBA82, respectively (although
490 differences were not statistically significant). The increase in shoot biomass could be
491 related to the ability of both of these strains to produce IAA (2). Nutrient shoot
492 concentrations, such as Ca, Fe, and P also tended to be higher in plants grown in inoculated
493 pots than in plants grown in non-inoculated pots, especially in the case of the inoculation
494 with strain SBA82. This could be due to bacterial-induced mineral weathering; SBA82 is a
495 phosphate-solubiliser and a siderophore-producer which could lead to an improvement in
496 the plant P and Fe status. Both characteristics have previously been related to an
497 improvement in plant nutrition (44). This improvement in plant nutrition could also be
498 associated with the observed increase in biomass production.

499 Shoot Ni concentrations were far above the criteria given for Ni hyperaccumulation
500 (>1000 mg Ni kg^{-1}) (45) and were similar to concentrations found in field collected plants
501 of hyperaccumulating subspecies of *Alyssum serpyllifolium* (46). Shoot Ni concentrations
502 tended to be higher in plants grown in LA44-inoculated pots. Although the activity of either

503 bacterial strain did not significantly influence, either biomass or Ni concentration, the
504 combined effect led to an increase in Ni phytoextracted, and this was significant in the case
505 of plants grown in LA44-inoculated pots. Similar results were obtained by Cabello-Conejo
506 et al. (47) when they inoculated *A. pintodasilvae* with the same *Arthrobacter* strain LA44
507 and grew the plants in ultramafic soil from Trás-os-Montes (NE Portugal). The positive
508 influence of specific bacterial strains on metal uptake by hyperaccumulator plants has been
509 shown by several authors. For instance, inoculation with Ni-mobilising rhizobacteria
510 enhanced Ni uptake by the Ni-hyperaccumulator *Alyssum murale* (9, 15) and by the non-
511 hyperaccumulator *Brassica juncea* (16). The increase in shoot Ni concentration observed
512 with the LA44 treatment could be due to the ability of this strain to act on Mn oxides
513 through the production of organic acids, and consequent release of associated Ni. This
514 could effectively help to replenish metals in the more labile fractions and enhance metal
515 uptake by the plant.

516 In conclusion, the activity of two bacterial strains promoted the weathering of
517 ultramafic rock in *in vitro* batch cultures. The two bacterial strains studied acted on distinct
518 mineral phases and the mechanisms involved in this process were isolate-specific. Further
519 studies should be carried out using pure mineral phases (e.g. olivine, Mn oxides) to study
520 the different mechanisms operating in more detail. Nonetheless, bacterial activity led to an
521 increase in the availability of metals such as Mn, Ni and Co. Inoculation with either
522 bacterial strain had a positive, although not significant, effect on plant growth and Ni shoot
523 concentration of *A. serpyllifolium* subsp. *malacitanum*. Moreover, a significant increase in
524 phytoextracted Ni was observed with the bacterial inoculum which was able to solubilise
525 Ni associated with Mn oxides, a fraction which has previously been associated with Ni
526 bioavailability in serpentine soils. This type of plant-associated bacteria could potentially

be applied in phytoextraction (phytomining) systems as a means of improving their efficiency.

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671 **Tables**

672 **Table 1**

673 Chemical composition of the rock used in batch culture experiment obtained by Energy-

674 Dispersive X-Ray Fluorescence spectrometry (EDXRF)

	Major elements (wt.% oxides)								Trace metals (mg kg^{-1})		
	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	Ni	Cr	Co
Rock	42.79	1.49	15.73	0.39	34.00	0.98	0.54	0.05	3530	2660	130

675

676 **Table 2**

677 Effects of inoculant treatment (strain) and time (days) on element solubilisation and organic

678 acid production (results of eight separate 2-way repeated measure ANOVAs) (* p<0.01).

		Si		Fe		Mn		Ni	
	df	MS	F	MS	F	MS	F	MS	F
days	5	39.9	20.7*	0.1	18.5*	0.3	13.7*	0.1	17.2*
strain	2	514.2	329.4*	2.9	298.5*	5.4	283.1*	1.8	336.0*
days x strain	10	23.2	12.1*	0.1	24.3*	0.1	4.6*	0.1	15.7*

		K		Co		Oxalate		Succinate	
	df	MS	F	MS	F	MS	F	MS	F
days	5	17.2	66.0*	1.4×10^{-4}	15.5*	422.4	44.9*	13.2	27.4*
strain	2	336.8	3710.5*	1.5×10^{-4}	63.1*	7169.1	585.9*	9.7	12.0*
days x strain	10	1.8	6.8*	8.8×10^{-4}	16.0*	135.5	14.4*	15.4	32.0*

df, degree of freedom; MS, mean square; F, F statistic

Table 3

Shoot nutrients concentrations (mean ± SE) of *Alyssum serpyllifolium* subsp. *malacinatum*. For the same element, different letters indicate significant differences ($p < 0.05$).

	Ca (g kg^{-1})	Mg (g kg^{-1})	K (g kg^{-1})	P (g kg^{-1})	Fe (mg kg^{-1})
NI	24.9 ± 2.1 *a*	10.4 ± 0.7 *a*	16.5 ± 1.3 *a*	1.8 ± 0.2 *a*	69 ± 13 *a*
LA44	27.1 ± 1.7 *a*	12.0 ± 1.0 *a*	16.0 ± 1.6 *a*	1.7 ± 0.3 *a*	75 ± 4 *a*
SBA82	33.0 ± 2.9 *a*	11.8 ± 0.9 *a*	16.0 ± 1.6 *a*	2.5 ± 0.7 *a*	79 ± 4 *a*

Figure legends

Figure 1

SEM photomicrographs taken from two areas of original ground ultramafic rock (a.1 and b.1), element mapping of these two areas (a.2 and b.2) and results of microprobe analysis from selected spots within each area (a.3 and b.3).

Figure 2

Concentrations of Si (a), Fe (b), Mn (c), Ni (d), K (e) and Co (f) in culture medium during incubation of 14 days (n=5).

Figure 3

Succinate (a) and oxalate (b) concentrations in culture medium during incubation of 14 days (n=5).

Figure 4

Score and loading (upper right-hand corner) plots of principal component analysis (PCA) of elements and organic acids concentrations in medium of LA44, SBA82 and non-inoculated

treatments.

Figure 5

Element fractionation in the recovered rock from the control treatment and mean relative (%) bacterial-induced depletion/increase of each fraction relative to the control treatment (n=5). Depletion/increase was calculated by subtraction of the amount of metals in each fraction at bacterial treatment (LA44 or SBA82) from the amount in non-inoculated treatment. A significant depletion/increase in metal concentration is denoted with an asterisk ($p<0.05$). Differences between bacterial treatments are denoted with "≠".

Figure 6

Plant biomass (a), shoot Ni concentration (b) and Ni phytoextracted (c) (mean ± SE) of *Alyssum serpyllifolium* subsp. *malacitanum* (n=5). Bars with different letter indicate significant differences ($p<0.05$).