

1 **Bacterial-induced weathering of ultramafic rock: implications in phytoextraction**

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21 **Running title:** *Bacterial weathering of ultramafic rock*

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24 **ABSTRACT**

25 Bioavailability of metals in soil is often cited as a limiting factor of phytoextraction (or
26 phytomining). Bacterial metabolites, such as organic acids, siderophores, or biosurfactants,
27 have been shown to mobilise metals, and their use to improve metal extraction has been
28 proposed. In this study, the weathering capacity and Ni mobilisation by bacterial strains
29 was evaluated. Minimal medium containing ground ultramafic rock was inoculated with
30 either of two *Arthrobacter* strains: LA44 (IAA-producer) or SBA82 (siderophore-producer,
31 PO₄-solubiliser and IAA-producer). Trace elements and organic compounds were
32 determined in aliquots taken at different time intervals after inoculation. Trace metal
33 fractionation was carried out on remaining rock at the end of the experiment. Results
34 suggest that the strains act upon different mineral phases. LA44 is a more efficient Ni-
35 mobiliser, apparently solubilising Ni associated with Mn oxides and this appeared to be
36 related to oxalate production. SBA82 also leads to release of Ni and Mn, albeit to a much
37 lower extent. In this case, the concurrent mobilisation of Fe and Si indicates preferential
38 weathering of Fe oxides and serpentine minerals possibly related to the siderophore
39 production capacity of this strain. The same bacterial strains were tested in a soil-plant
40 system: the Ni-hyperaccumulator *A. serpyllifolium* subsp. *malacitanum* was grown in
41 ultramafic soil in a rhizobox system and inoculated with each bacterial strain. At harvest,
42 biomass production and shoot Ni concentrations were higher in plants from inoculated pots
43 than from non-inoculated pots. Ni yield was significantly enhanced in plants inoculated
44 with LA44. These results suggest that Ni-mobilising inoculants could be useful for
45 improving Ni uptake by hyperaccumulator plants.

46

47

48 Introduction

49 Over the last two decades, there has been increased interest in the development of
50 plant-based remediation techniques (phytoremediation) for the clean-up of polluted soils.
51 These techniques are intended to provide a viable alternative to traditional and civil-
52 engineering methods. Phytoextraction uses plants that take up metals from the soil and
53 accumulate them in their aboveground biomass. Recently, the incorporation of plant-
54 associated bacteria into these systems has been suggested as means of enhancing
55 phytoextraction efficiency (1, 2). Microorganisms play an essential role in the global
56 biogeochemical cycling of metals and nutrients. Their activity can either increase or reduce
57 the mobility of these elements in soils (3). Bacteria influencing the availability of plant
58 nutrients (such as N, Fe or P) have been used as “biofertilizers” to enhance plant nutrient
59 uptake and alleviate nutrient deficiencies (4). Inoculation of pine seedlings with
60 *Burkholderia glathei* PML1(12) significantly improved plant growth and nutrition through
61 the weathering of biotite and release of nutrients such as K and Mg (5). Similarly, the use
62 of metal-mobilising bacteria to enhance plant metal uptake has been proposed by several
63 authors as a promising method to increase metal bioavailability, which can be a limiting
64 factor in phytoextraction processes (6-9). Such microbial inoculants offer an alternative to
65 the controversial use of metal chelants which can increase metal bioavailability but at the
66 same time lead to environmental problems because of their limited biodegradability or to an
67 enhanced leaching of metals to groundwater. Several microbial metabolites have been
68 shown to enhance rock weathering through chemical interaction or, oxidation-reduction
69 reactions, leading to mineral dissolution and metal solubilisation. These metabolites include
70 inorganic acids (HNO₃ and H₂SO₄), organic acids (such as citric, oxalic, gluconic acid) and
71 metal-chelating ligands such as iron-complexing siderophores or biosurfactants (10).

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

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

89

90 **Materials and methods**

91 *Bacterial strains and preparation of inoculants*

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

96 LA44 (an IAA-producer) isolated from the rhizosphere of *Alyssum serpyllifolium* subsp.
97 *lusitanicum* (Melide, NW Spain) and *A. oxydans* SBA82 (a siderophore-producer, PO₄-
98 solubiliser and IAA-producer) isolated from *Alyssum serpyllifolium* subsp. *malacitanum*
99 (Sierra Bermeja, S Spain).

100 To prepare the bacterial inoculants, strains were cultivated in 869 medium (17) for 3
101 days, harvested by centrifugation (4000 g, 15 min, 4 °C), washed and re-suspended in 10
102 mM MgSO₄ to an optical density of 1.0 at 600 nm (about 10⁸ cells per ml).

103

104 *Batch culture experiment design*

105 To evaluate the ability of the bacterial strains to mobilise Ni they were cultivated in
106 minimal medium containing sterile ultramafic rock. The ultramafic rock was collected from
107 the serpentinitic area of Morais (NE Portugal) where *A. serpyllifolium* subsp. *lusitanicum* is
108 found growing. The chemical composition of this rock is given in Table 1. As is expected
109 for an ultramafic rock, the SiO₂ content is less than 45%, the Al₂O₃, K₂O and CaO contents
110 are low, whereas the MgO content and total Ni, Co and Cr concentrations are elevated.
111 Mineral associations and element distribution were characterised by scanning electron
112 microscope (SEM EVO LS 15) equipped with EDX microprobe analysis (INCA X-act,
113 Oxford Instruments, United Kingdom) (Fig. 1).

114 A modified 284 medium (18) was used with no trace of Ni, Mn or Co and a reduced
115 concentration of K and Fe. This modified 284 medium contained (per liter medium): 6.06 g
116 Tris-HCl, 4.68 g NaCl, 0.015 g KCl, 1.07 g NH₄Cl, 0.43 g Na₂SO₄, 0.20 g MgCl₂.6H₂O,
117 0.03 g CaCl₂.2H₂O, 0.04 g Na₂HPO₄.2H₂O plus oligoelements (0.3 mg H₃BO₄, 0.02 mg
118 CuSO₄.5H₂O, 0.036 mg Na₂MoO₄.2H₂O) adjusted to pH 7. The medium was supplemented
119 with glucose (0.5 g l⁻¹) and fructose (0.5 g l⁻¹). Flasks containing 2 g of ground rock (< 100

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

125 Aliquots (of 3 ml) were taken at different times (1, 2, 4, 7, 10 and 14 days). Samples
126 were centrifuged, the supernatant was decanted and filtered (0.22 μm pore size) and
127 immediately frozen until analysis. Samples were analysed for element concentrations,
128 organic acids and phenolic compounds. At the same sampling times, bacterial densities
129 were determined by plating out serial dilutions of the samples on 10-fold diluted 869 agar
130 medium, for three replicates of each treatment. At the end of the experiment (14 days), the
131 medium pH was determined. The sterility of the control flasks was checked by plating on
132 1:10 diluted 869 agar medium. Finally, the cultivated bacterial strains (LA44 and SBA82)
133 were compared with the original inoculants using BOX-PCR and following the methods
134 described in Becerra-Castro et al. (8).

135

136 *Analysis of supernatant samples*

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

140 Carboxylic acids were separated by reversed-phase liquid chromatography on a C_{18}
141 column with 5 μm particle size (GraceSmart, RP18, and 2.1 x 150 mm column from Grace
142 Davison Discovery Sciences, Deerfield, IL, United State) and analysed by liquid
143 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 methoxycinnamaldehyde, 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 $\text{NH}_2\text{OH-HCl}$ + 1 M NH_4OAc (adjusted to pH
167 6 with diluted HCl) for 30 min, separated by centrifugation, filtered and stabilized with

168 HCl (12 N). 25 ml 1 M NH₄OAc were added to the residue and shaken for 10 min,
169 centrifuged, filtered (repeated once) and combined with the above solution. This extracts
170 metals bound to *Mn oxides* (F3).

171 - Fourth, 50 ml 0.2 M NH₄-oxalate (= 0.2 M Di-ammonium oxalate-monohydrate + 0.2 M
172 oxalic acid-dihydrate adjusted to pH 3.3 with diluted NH₄OH) were added to the residue,
173 and after shaking (over-head) for 4 h in the dark, the solution was separated by
174 centrifugation (15 min, 938 g) and filtered. A second volume of 25 ml 0.2 M NH₄-
175 oxalate (pH 3.3) was added to the residue and shaken over-head in the dark for 10 min.
176 The solution was separated by centrifugation (15 min, 938 g), filtered and the two
177 filtrates were combined. This step targets those metals bound to *amorphous Fe oxides*
178 (F4).

179 - Fifth, 50 ml 0.1 M ascorbic acid + 0.2 M NH₄-oxalate (= 0.1 M L(+)-ascorbic acid + 0.2
180 M NH₄-oxalate buffer: 0.2 M oxalic acid-dihydrate adjusted to pH 3.25 with diluted
181 NH₄OH) were added, and after shaking horizontally in a water bath for 30 min at 96 °C
182 ± 3 °C, the solution was separated by centrifugation (15 min, 938 g) and filtered. The
183 remaining solution was extracted with 25 ml 0.2 M NH₄-oxalate (pH 3.3) by shaking
184 over-head in the dark for 10 min. The solution was separated by centrifugation (15 min,
185 938 g), filtered and the filtrates were combined. This extracts metals bound to *crystalline*
186 *Fe oxides* (F5).

187 - Finally, the remaining residue was digested with *aqua regia* to determine the *residual*
188 (silicate-bound) fraction (F6).

189 The concentration of Al, Co, Fe, Mn, Ni and Si were analysed in the filtered
190 supernatants of each extraction by ICP-OES (Vista Pro; Varian Inc., Australia). In the case
191 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 ($\text{pH}_{\text{CaCl}_2}$ 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 $<2 \text{ mm}$ 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 $30 \mu\text{m}$
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 $30 \mu\text{m}$ 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 $45 \text{ }^\circ\text{C}$ and ground. Shoot tissues (0.1 g) were

216 digested in a 2:1 mixture of concentrated HNO₃:HCl on a hot plate at 160 °C, and the Ca,
217 Mg, K, P, Fe and Ni concentration was measured by ICP-OES (Vista Pro; Varian Inc.,
218 Australia). Data were expressed on the basis of dry weight plant material. The full recovery
219 of plant roots was not possible and only shoot dry weight was taken into account when
220 assessing the effects of inoculation on plant biomass production or Ni yield.

221 Soil analyses were carried out on the <2 mm fraction of rhizosphere and bulk soil
222 samples. Soil pH was measured in H₂O using a 1:2.5 soil:solution ratio. Exchangeable
223 cations were extracted with 0.1 M BaCl₂, and Al, Ca, K, Mg and Na determined by ICP-
224 OES (Vista Pro; Varian Inc., Australia). Water-soluble Ni concentration was analysed by
225 ICP-OES in soil extracts after 30 min shaking using a 1:2.5 soil:H₂O ratio. Ca(NO₃)₂- and
226 Sr(NO₃)₂-extractable Ni concentrations were determined by ICP-OES in soil extracts after
227 2 h shaking using a 1:4 soil:extractant ratio for both extractants.

228

229 *Statistical analysis*

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

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

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

246

247 **Results**

248 *Bacterial growth in medium with ground rock*

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

254

255 *Element solubilisation from rock and release of organic compounds*

256 Element concentrations in control flasks (non-inoculated) varied slightly over time.
257 They were generally lower than in experimental flasks (inoculated) (Fig. 2). Iron and K
258 were added in low concentrations to the initial liquid medium (1.6 and 200 μM ,
259 respectively), while Co, Mn and Ni were absent. Bacterial activity and growth either led to
260 a depletion in some elements compared to the non-inoculated control (such as K), or
261 alternatively, to the release of some elements from the rock (such as Fe, Co, Mn, Ni, or Si).
262 At the end of the experiment there was no difference in the pH of the culture medium
263 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

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

297 None of the phenolic compounds were detected in the culture media. In contrast,
298 detectable levels of malate, malonate, oxalate and succinate were measured. Aconitate and
299 citrate were below the detection limit. Malate concentrations in control and SBA82 cultures
300 were detected in a similar range, and remained always below 0.70 μM . LA44 cultures
301 showed somewhat higher values of this anion, between 0.20 – 1.15 μM . In the control
302 treatments the malonate concentration did not exceed 0.30 μM , and in SBA82 cultures the
303 values ranged from 0.20 – 0.55 μM . Higher concentrations of malonate, varying from 0.30
304 – 0.90 μM , were found in LA44 cultures (data not shown). Significant differences were
305 observed in inoculated flasks with respect to oxalate and succinate concentrations ($p <$
306 0.05 ; repeated measures ANOVA; Table 2). In control cultures, succinate was only
307 detected at some sampling times (2, 7, 11 and 14 days). With an exception of day 1,
308 succinate concentration in SBA82 cultures was generally higher (1.9 – 3.8 μM) than in
309 LA44 cultures (0.6 – 1.9 μM) (Fig. 3a). With respect to oxalate, the difference in
310 concentration between the two strains was more marked (Fig. 3b). The concentrations in
311 SBA82 cultures were similar to those in control treatments (4.1 – 5.4 μM), whereas those in

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

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

327

328 *Metal fractionation in rock samples*

329 Fig. 5 shows the element fractionation in the recovered rock from the control
330 treatment, as well as the bacterial-induced depletion/increase of each fraction relative to the
331 control treatment. The residual fraction was the most important geochemical phase for Al,
332 Cr and Si (representing more than 85% of the total content). This phase was also dominant
333 for Fe and Ni (representing more than 60%) but for these two elements an important
334 fraction was also associated with either amorphous or crystalline Fe oxides (32-35% in
335 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).

360

361 *Pot experiment*

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

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

377 Plant growth induced few changes in physico-chemical soil properties and no
378 significant effects of bacterial inoculation were observed. In plants grown in non-inoculated
379 pots, rhizosphere soil pH was slightly higher than bulk soil pH: 8.4 ± 0.1 compared to $8.3 \pm$
380 0.1 . As expected, the cation exchange complex was dominated by Mg ($8.1 - 9.4$ cmol_c kg⁻¹
381 ¹). The CEC was 10.3 and 10.9 cmol_c kg⁻¹ in bulk and rhizosphere soil, respectively, and
382 Ca/Mg quotients were consistently less than 1 (around 0.2). Water-soluble Ni concentration

383 was significantly higher ($p < 0.01$) in the rhizosphere compared to bulk soil (0.12 and 0.06
384 mg kg^{-1} , respectively). In contrast, $\text{Sr}(\text{NO}_3)_2$ - or $\text{Ca}(\text{NO}_3)_2$ -extractable Ni concentrations
385 tended to be depleted in the rhizosphere (falling from 0.49 to 0.44 mg kg^{-1} and 6.44 to 5.89
386 mg kg^{-1} , respectively). Bacterial inoculation did not lead to any significant changes in these
387 general physicochemical properties.

388

389 **Discussion**

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

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

407 with previously described weathering sequences of serpentine minerals (25). Since strain
408 SBA82 is a siderophore-producer, its mechanism of weathering probably involves involve
409 siderophore-induced mineral dissolution. Siderophores are iron-chelating secondary
410 metabolites which are known to be produced under Fe-limiting conditions. The reduced Fe
411 concentration of the culture medium used in this study will presumably have induced
412 siderophore production by strain SBA82. Previous studies have demonstrated siderophore-
413 promoted dissolution of goethite and hornblende and consequent release of Fe, Si, and Al
414 (26, 27). Although siderophores have a high affinity for Fe(III) they are known to form
415 complexes with other trace elements (28, 29). Their presence in the culture medium would
416 therefore maintain elements such as Al, Fe and Si in solution (which was the case in
417 SBA82 cultures). Siderophore mediated Fe solubilisation from Fe-rich minerals could also
418 lead to the solubilisation and re-distribution of their companion trace metals (Cr, Ni).

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

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

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

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

471 In rhizospheric studies of hyperaccumulators some authors show a depletion of labile
472 metal fractions in the rhizosphere (attributed to plant uptake), while others indicate an
473 increase in such fractions in the rhizosphere. In either case, the differences in the
474 concentration of labile metal fractions do not explain the extreme metal uptake by these
475 plants. It therefore continues to be a point of controversy whether or not these plants are
476 able to access metal fractions not available to non-accumulating plants (thus increasing
477 metal uptake), or if their root activity leads to a faster replenishment of soluble metal pools.
478 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 $\text{Sr}(\text{NO}_3)_2$ - or $\text{Ca}(\text{NO}_3)_2$ -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 ($>1000 \text{ mg Ni kg}^{-1}$) (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

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

529

530 Acknowledgements

531 This research was supported by the Austrian Science Fund (FWF, Project N° 20069-
532 B16 and L561), Ministerio de Ciencia e Innovación (CTM2009-14576-C02-01/02),
533 FEDER and FP7 EC project “Gentle remediation of trace element contaminated land”
534 (Greenland 266124; Theme 2: Food, Agriculture and Fisheries, and Biotechnology).

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670

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 ₂ O ₃	Fe ₂ O ₃	MnO	MgO	CaO	Na ₂ O	K ₂ O	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

679

680 **Table 3**681 Shoot nutrients concentrations (mean \pm SE) of *Alyssum serpyllifolium* subsp. *malacinatum*.682 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 \pm 2.1 <i>a</i>	10.4 \pm 0.7 <i>a</i>	16.5 \pm 1.3 <i>a</i>	1.8 \pm 0.2 <i>a</i>	69 \pm 13 <i>a</i>
LA44	27.1 \pm 1.7 <i>a</i>	12.0 \pm 1.0 <i>a</i>	16.0 \pm 1.6 <i>a</i>	1.7 \pm 0.3 <i>a</i>	75 \pm 4 <i>a</i>
SBA82	33.0 \pm 2.9 <i>a</i>	11.8 \pm 0.9 <i>a</i>	16.0 \pm 1.6 <i>a</i>	2.5 \pm 0.7 <i>a</i>	79 \pm 4 <i>a</i>

683

684

685 **Figure legends**686 **Figure 1**

687 SEM photomicrographs taken from two areas of original ground ultramafic rock (a.1 and

688 b.1), element mapping of these two areas (a.2 and b.2) and results of microprobe analysis

689 from selected spots within each area (a.3 and b.3).

690 **Figure 2**

691 Concentrations of Si (a), Fe (b), Mn (c), Ni (d), K (e) and Co (f) in culture medium during

692 incubation of 14 days (n=5).

693 **Figure 3**

694 Succinate (a) and oxalate (b) concentrations in culture medium during incubation of 14

695 days (n=5).

696 **Figure 4**

697 Score and loading (upper right-hand corner) plots of principal component analysis (PCA) of

698 elements and organic acids concentrations in medium of LA44, SBA82 and non-inoculated

699 treatments.

700 **Figure 5**

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

707 **Figure 6**

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











