

1 POTENTIALLY HARMFUL ELEMENTS IN SOIL-PLANT INTERACTIONS

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3 **Stabilized municipal sewage sludge addition to improve properties of an acid mine**  
4 **soil for plant growth**

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30

31 **Abstract**

32 Purpose: Degraded soils, such as those encountered in areas of mine activities, need to be  
33 ameliorated by liming to correct soil acidity and by addition of organic inputs to improve  
34 soil properties and fertility.

35 Materials and methods: Non amended mine soil and soil amended with stabilized sewage  
36 sludge were incubated for 45 d. Soil physicochemical and biological indicators were  
37 periodically measured along incubation and other enzyme activities at the end of  
38 incubation. In improved soils a study of plant development in 250-g pots was carried out  
39 with three vegetal species: tomato, rye grass and ahipa. Germination and mortality rates,  
40 biomass production and photosynthetic pigments were measured.

41 Results and discussion: Soil incubation with sewage sludge slightly increased soil pH and  
42 led to an enhancement of soil electrical conductivity, organic carbon and dehydrogenase  
43 activity, especially for the higher doses (5 and 10%). However soil respiration was more  
44 promoted with the 2% dose, pointing to a possible toxic effect of the sludge. At the end of  
45 incubation physicochemical and biological properties were in general enhanced. Biomass  
46 production was improved in tomato and rye grass by sewage sludge addition (more at the  
47 2% dose) whilst ahipa growth was not affected by sewage sludge treatments. Tomato  
48 mortality reached 73% with high sludge doses (10%).

49 Conclusions: According to this set of parameters, amendment with SSL of a limed acid  
50 mine soil would be considered as a good strategy for soil amelioration in view of plant  
51 establishment and development.

52

53 **Keywords** Ahipa • Mine soil • Organic amendment • Rye grass • Tomato

54 **1 Introduction**

55 The Iberian Pyrite Belt occupies the southwestern corner of the Iberian Peninsula,  
56 extending from Seville, in Spain, to the Atlantic Ocean, south of Lisbon, in Portugal,  
57 making up a belt of about 230 km in length and 40 km in width (Sáez et al. 1996). It  
58 constitutes the world largest massive sulphide deposit (mainly Cu-Pb-Zn), which has been  
59 mined since the Metal Age, according to archeometallurgical evidences, but reaching a  
60 peak between the 19th and the 20th centuries, when most mines were closed due to  
61 exhaustion of the ore (Salkield 1987).

62 The soils in this belt are characterised by high level of acidity, poor physical structure  
63 and also contain toxic concentrations of metals and low levels of major plant nutrients  
64 (Fernández-Caliani et al. 2009). Even if some communities of pioneer plants colonized this  
65 area, it needs to be improved by different measures, which include correction of soil  
66 acidity and improvement of soil properties, because the extreme environmental conditions  
67 are not suitable to promote plant establishment. Besides, it is essential to assess the  
68 efficacy of the implemented measures by evaluating physical, chemical and biological  
69 properties, in order to gain a full understanding of constraints and opportunities.

70 Remediation of mine tailings by revegetation is an interesting approach with obvious  
71 economic and environmental advantages. However some of the limitations to revegetation  
72 on an acid soil include pH values out of physiological values, low nutrient status, low  
73 organic matter and microbial activity and low water holding capacity. Addition of organic  
74 amendments can increase organic matter, nutrient status, microbial activity and water  
75 holding capacity (Jones et al. 2012; González-Ubierna et al. 2012). The use of organic  
76 amendments together with liming or other materials rich in carbonates is a way of restoring  
77 the ecological function of metal-contaminated sites, given that these approaches improve  
78 the physicochemical and biological soil conditions and favour plant growth (Haynes and  
79 Swift 1988; Alvarenga et al. 2008, 2009; de Varennes et al. 2010)

80 Organic amendments from different sources have been assayed for the improvement of  
81 mine soil conditions, from agricultural, industrial or miscellaneous origins (Alvarenga et  
82 al. 2008; de Varennes et al. 2010, 2011; Arocena et al. 2012). Furthermore, the use of  
83 organic wastes from water treatment plants is an alternative to the disposal of these  
84 residues, generated in huge quantities. The applications of these biowastes, whose  
85 production is estimated up to 138 million tonnes per year in the EU and with high potential  
86 added value, are regulated by a European Directive (Council Directive, ECC 1986) which  
87 considers both environmental and soil protection, encouraging the agricultural use of  
88 sewage sludge in such a way as to avoid harm to vegetation, animals or humans. In 2007,  
89 1.17 Mt of dry urban sewage sludges were produced in Spain, as a result of the treatment  
90 of almost 85% of the total residual water, in compliance with European guidelines  
91 (Council Directive, ECC 1991). The use of treated sewage sludges provides economic,  
92 agronomic and environmental benefits since they are low-cost amendments, have great  
93 fertilising ability and are a source of macro (N and P) and micro (Fe, Zn, Cu, etc) nutrients.  
94 Additionally they increase soil organic matter content thus improving soil water-holding  
95 capacity, microbial activity, and other physical, chemical, and biological properties (Hueso  
96 et al. 2012).

97 The aims of this work were (a) to assess the effect of the addition of a stabilized  
98 sewage sludge of urban origin on physicochemical and biological properties of a severely  
99 degraded mine soil and (b) to study the effect of the amendment on the establishment of  
100 three plant species (tomato and rye grass, of agricultural interest and ahipa, a species which  
101 could be employed for biofuel production) and therefore their suitability to be used for  
102 revegetation.

103

## 104 **2 Materials and methods**

### 105 **2.1 Site description and properties of the soil and the amendment**

106 The soil (NC) was collected in a mine waste situated in the proximity of the village of  
107 Nerva, province of Huelva (37° 42' 4.5" N 6° 33' 35.1" W), located in the Iberian Pyrite  
108 Belt, which includes one of the largest deposits of pyrite (FeS<sub>2</sub>) and other metallic and  
109 polymetallic sulphides as chalcopyrite (CuFeS<sub>2</sub>), sphalerite ((Zn,Fe)S) and galena (PbS)  
110 (Chopin and Alloway 2007). Soil samples, characterised as sandy loam, were collected at  
111 random from the selected site. They were air dried for 2 weeks, sieved through 3-mm for  
112 incubation assays, or passed through a 2-mm mesh sieve or ground to 50 µm, for analysis  
113 following standard methodology.

114 According to X-ray fluorescence analysis SiO<sub>2</sub> (46.9%) and Fe (23.0%) and Al  
115 (12.7%) oxides represent more than 80% of the soil mineralogical composition. It is a very  
116 acid soil (pH 2.4) with low organic carbon content (OC, 1.4%), and high electrical  
117 conductivity (EC, 1.3 dS m<sup>-1</sup>, 1:2.5 ratio); 24% water content at field capacity; HIX 1.16;  
118 SUVA (L g<sup>-1</sup> cm<sup>-1</sup>) 10.8. Otherwise, this soil contents high total concentrations of potential  
119 hazardous elements (mg kg<sup>-1</sup>) (As, 3951; Cu, 694; Pb, 3976) and also of S (8320).

120 Stabilized sewage sludge (SSL) from the wastewater treatment plant of Granada (SE  
121 Spain), was used for the amendment of NC soil. The main properties are: pH 6.9; EC (dS  
122 m<sup>-1</sup>, 1:10 ratio) 2.8; OC (%) 35.5; HIX 0.43.

123

## 124 **2.2 Soil incubation**

125 Mine soil (NC) was first limed with Carbocal (Azucarera Ebro), a residue rich in calcium  
126 carbonate (83.4%) with an OC content of 5.1%. The limed Nerva soil (NC<sub>L</sub>) received  
127 Carbocal at an equivalent rate of 1.5% (w/w) in CaCO<sub>3</sub>. After liming the organic waste  
128 was applied at 2, 5 and 10% (w/w) (SSL2, SSL5 and SSL10, respectively), corresponding  
129 to approximately 40, 100 and 200 Mg ha<sup>-1</sup>. The mixtures, carried out with air-dried soil and  
130 the amendment, were placed in plastic trays covered with aluminium foil to avoid  
131 desiccation, their moisture adjusted to 40% of the soil field capacity with deionised water,

132 and allowed to stand in the dark at ambient temperature ( $20 \pm 2$  °C), up to 45 d. Water was  
133 supplied as required to maintain soil humidity. One subsample was periodically withdrawn  
134 (0, 2, 5, 7, 14, 21, 29, 34, 42 and 49 days) for determination of pH, moisture, conductivity  
135 and organic carbon (OC) content. Another subsample was kept frozen (-18°C) until  
136 analysis for enzyme activities and soil induced respiration (SIR).

137

### 138 **2.3 Soil induced respiration and microbial biomass C**

139 Soil induced respiration (SIR) measurements were performed in an automatic equipment  
140 ( $\mu$ Trac 4200, Sy-Lab, Gomensoro, Madrid, Spain), after defrozing the samples at ambient  
141 temperature. Briefly, ca. 5 g soil was mixed with 50 mg of talc:glucose (10:1 ratio) and  
142 weighed into a plastic tube, which was introduced into a measuring cell containing 2 mL of  
143 a 2% KOH solution. The tightly closed cell was maintained at 30°C during 20 h and CO<sub>2</sub>  
144 evolution was monitored every 5 min, through the measurement of solution impedance  
145 decrease. Results are expressed as mg CO<sub>2</sub> 100 g<sup>-1</sup> h<sup>-1</sup>.

146 Soil microbial biomass C (SMBC, mg C 100 g<sup>-1</sup> soil) was estimated from the SIR  
147 assay. SMBC was calculated from the CO<sub>2</sub> generated during 6 h of soil incubation as  
148 follows: SMBC = 40.04 × CO<sub>2</sub> + 0.37 (Anderson and Domsch 1978).

149

### 150 **2.4 Enzyme activities**

151 Enzyme activities are used as an index of microbial functional diversity if they reflect  
152 changes in microbial activities. Since microbial functional diversity includes many  
153 different metabolic processes, a representative set of enzyme activities was assessed:  
154 During incubation dehydrogenase activity (DHA), proposed as a measure of overall  
155 microbial activity, was determined by incubating soil samples during 20 h at 25°C with 0.2  
156 mL of 0.4% 2-p-iodophenyl-3 p-nitrophenyl-5 tetrazolium chloride as a substrate. The

157 iodonitrotetrazolium formazan (INTF) formed was measured spectrophotometrically at 490  
158 nm, according to García et al. (1997).

159 At the end of the incubation period various enzymes were analyzed: enzymes  
160 participating in the C cycle ( $\beta$ -glucosidase), N cycle (protease), P cycle (alkaline  
161 phosphatase) and S cycle (arylsulphatase). The enzyme activities  $\beta$ -glucosidase (Glu),  
162 alkaline phosphatase (AlkP) and arylsulphatase (Aryl) were spectrophotometrically  
163 quantified at 400 nm by estimating the *p*-nitrophenol (PNP) released by incubating the soil  
164 with a substrate containing a *p*-nitrophenyl moiety (Tabatabai and Bremner 1970; Ladd  
165 and Butler 1972; Tabatabai 1994) and expressed as  $\mu\text{g PNP g}^{-1} \text{ h}^{-1}$ . Protease activity (Pro)  
166 determined the released tyrosine reacted with Folin-phenol reagent measuring at 700 nm  
167 by spectrophotometry (Ladd and Butler 1972). This activity is expressed as  $\mu\text{g tyrosine g}^{-1}$   
168  $\text{h}^{-1}$ . All enzyme activities were determined in defrozen samples in triplicate and expressed  
169 on an oven-dried (105°C) soil basis.

170

## 171 **2.5 Analytical methods**

172 The soil particle size distribution was determined by sieving and sedimentation, applying  
173 the Robinson's pipette method after organic matter had been removed with  $\text{H}_2\text{O}_2$ , using  
174 sodium hexametaphosphate as dispersing agent. Field capacity was obtained from water  
175 retention of disturbed soil samples using ceramic pressure plates at an air pressure of 0.03  
176 MPa. The pH and EC determinations were carried out in sample/deionised water  
177 suspensions 1/2.5 (w/v) for soil and mixtures of soil and sludge and 1/10 (w/v) for sludge.  
178 Organic C (OC) content was determined by a modified Walkey and Black method  
179 (Mingorance et al. 2007). The humification index (HIX) (Zsolnay 2003) and the specific  
180 UV absorbance (SUVA,  $\text{L g}^{-1} \text{ cm}^{-1}$ ) (Hernández-Soriano et al. 2011) were determined in  
181 sample/deionised water suspensions 1/4 (w/v) at the end of the incubation period. Samples

182 were analysed in triplicate. Estimation of metal content was accomplished by X-ray  
183 fluorescence analysis.

184

## 185 **2.6 Pot experiments**

186 Three plant species were selected for the experiments: two of agricultural interest, tomato  
187 (*Lycopersicon esculentum* Mill.) and rye grass (*Lolium perenne* L.), and ahipa  
188 (*Pachyrhizus ahipa* (Wedd.) Parodi), whose tuberous root could be used for biofuel  
189 production. The experiment was carried out in pots of 250 g and under greenhouse  
190 conditions (average temperature 21.8°C and humidity 67%). Non-amended limed soil  
191 (NC<sub>L</sub>) as a control and NC<sub>L</sub> amended with stabilized SSL at 2 and 10% (SSL2; SSL10)  
192 were the treatments tested.

193 In each pot the number of seeds was planted according to previous germination assays.  
194 The seeds showed the following germination rate: tomato 85%, rye grass 95% and ahipa  
195 70%. A total of ten plants per pot were cultivated. Tap water was periodically added to  
196 maintain soil field capacity. In order to reduce soil compactness, 40 g of glass beads (4  
197 mm) were mixed in each pot.

198 Germination and mortality rates, biomass production and photosynthetic pigments were  
199 measured. Germination was monitored at the beginning and mortality at the end of the  
200 assays. The experiment was carried out during 18 days for rye grass and for one month for  
201 tomato and ahipa harvesting plants at vegetative stage. Fresh weight of the plant aerial  
202 vegetative part was only measured at the end of the experiment. Photosynthetic pigments  
203 were determined using a spectrophotometer (Thermo, Helios Gamma). A piece of leaf was  
204 taken and pigments were extracted with pure methanol for 24 hours and then the extract  
205 was measured. The concentrations of Chlorophyll a (Chl. a), b (Chl. b) and the sum of leaf  
206 carotenoids were calculated with the following equations given for pure methanol, where



207 the pigment concentrations are given in  $\mu\text{g mL}^{-1}$  extract solution (Lichtenthaler and  
208 Buschmann 2001)

$$\begin{aligned} 209 \quad & [\text{Chlorophyll a}] = (16.75 * A_{665,2}) - (9.16 * A_{652,4}) \\ 210 \quad & [\text{Chlorophyll b}] = (34.09 * A_{652,4}) - (15.28 * A_{665,2}) \\ 211 \quad & [\text{Carotenoids}] = \frac{((1000 * A_{470}) - (1.63 * [\text{Chlorophyll a}]) - (104.96 * [\text{Chlorophyll b}]))}{221} \end{aligned}$$

212

213

## 214 **2.7 Statistical treatment of the data**

215 Exploratory analysis was carried out to check normality of the data sets. Differences  
216 between treatments were determined by ANOVA of normal data sets and Kruskal-Wallis  
217 test of non-normal data sets. Otherwise, the post-hoc Tukey-t or Fisher's LSD (least  
218 significant difference) tests were used for comparison of several means. Comparison  
219 between two sample means was performed by *t*-test or Mann-Whitney test. The  
220 relationship between variables was performed by either correlation or regression analysis.  
221 Hierarchical cluster analysis was used to arrange the soil and plant properties into groups  
222 using the Ward's method as linking algorithm and the square Euclidean distance as  
223 similarity measurement. The clustering results were shown in a dendrogram to provide  
224 grouping of variables.

225 Values with  $p < 0.05$  were considered significant. SPSS v.17.0 (Illinois, USA) was  
226 used for statistical data analysis.

227

## 228 **3 Results and discussion**

### 229 **3.1 Evolution of soil physicochemical properties along incubation**

230 Addition of Carbocal as a liming agent effectively raised soil pH with an average value of  
231  $6.8 \pm 0.18$  for the whole assayed period. The increase in pH as a result of liming has been  
232 linked with a change in the charge characteristics of soil OC (Curtin and Smillie 1983;

233 Chan and Heenan 1999). The pH increased 0.009 units per incubation day ( $R^2= 0.812$ )  
234 reaching a value of 7.1 at the end of this period.

235 The addition of SSL modified soil pH depending on the applied dose. The pH for the  
236 SSL2 treatment did not change with incubation time ( $p > 0.05$ ) and the overall pH average  
237 ( $6.9 \pm 0.15$ ) was slightly higher than that of non-amended soil ( $pH 6.8 \pm 0.16$ ), which  
238 neither varied along the incubation ( $p > 0.05$ ). However, addition of 5% SSL increased pH  
239 linearly with incubation time ( $R^2=0.846$ ;  $pH = 6.71 + 0.02 \times t$ ) and in the case of SSL10  
240 pH also increased linearly during 17 d ( $R^2=0.812$ ;  $pH = 6.39 + 0.07 \times t$ ) and then  
241 remained constant at a value of 7.7.

242 Soil EC also increased for limed soil, from 1.2 to 2.2  $dS m^{-1}$ , due especially to a strong  
243 enhancement of  $CO_3^{2-}$  and  $Ca^{2+}$  concentrations because of the Carbocal addition and of  
244 formation of  $SO_4^{2-}$  from the pyrite due to the pH increase (Curtin and Smillie 1983).  
245 Amendment addition resulted in a further soil solution EC increase in comparison with  
246 non-amended ( $NC_L$ ) soil. The EC of  $NC_L$  ( $2.2 \pm 0.1$ ) and of SSL2 ( $2.5 \pm 0.2$ ) remained  
247 constant during incubation while that of SSL5 ( $EC = 2.3 \times t^{0.09}$ ) and SSL10  
248 ( $EC = 2.4 \times t^{0.15}$ ) increased with increasing incubation time following a power function  
249 reaching final values of 3.2 and 4.1  $dS m^{-1}$ , respectively (Fig. 1). This increase was  
250 probably as a consequence of the production of low molecular weight organic ions or of  
251 the release of salts during decomposition of organic substances and agrees with soil EC  
252 increases after addition of high doses of organic amendments to soil (González-Ubierna et  
253 al. 2012). The increase in EC coincides with the increase in soil pH indicated above  
254 ( $r=0.844$ ). It is important to note that 4.0  $dS m^{-1}$  has been reported as an EC value which  
255 may inhibit plant growth and seed germination (Ye et al. 2002).

256 Soil OC was initially enhanced, as expected from the OC content of the organic waste,  
257 and was proportional to the applied dose ( $OC = 1.5 + 0.34 \times dose$ ;  $R^2=0.981$ ) (see Fig. 1).  
258 Along incubation OC content of the non-amended  $NC_L$  soil ( $1.5 \pm 0.14$ ) and amended with

259 2% SSL ( $1.9 \pm 0.27$ ) decreased slightly but without significant variations ( $p > 0.05$ ). On the  
260 contrary, the OC of soils amended with 5 and 10% SSL decreased with time following a  
261 quadratic regression ( $R^2 = 0.850$ ,  $OC_{SSL5} = 3.4 - 0.08 \times t + 0.002 \times t^2$ ;  $R^2 = 0.902$ ,  
262  $OC_{SSL10} = 4.6 - 0.09 \times t + 0.001 \times t^2$ ) likely as a result of OC mineralization in a  
263 stabilization process of the sludge (see Fig. 1).

264

### 265 **3.2 Evolution of soil biological properties with incubation**

266 To assess the results of soil remediation it is also necessary to observe the microbial  
267 processes, since soil biological investigations (such as soil respiration, biomass, enzyme  
268 activities, microbial counts) can give information on the presence of viable  
269 microorganisms. Biological methods can therefore be a good complement of  
270 physicochemical methods for the evaluation of amendment addition or for the assessment  
271 of the success of a remediation strategy.

272 Evolution of  $CO_2$  was low for untreated NC soil ( $0.76 \text{ mg } CO_2 \text{ } 100 \text{ g}^{-1} \text{ h}^{-1}$ ), and  
273 increased with liming ( $3.49 \text{ mg } CO_2 \text{ } 100 \text{ g}^{-1} \text{ h}^{-1}$ ). The low NC respiration could be related  
274 with high soil metal pollution. Tyler (1974) showed a severe reduction of soil respiration  
275 with increasing concentrations of Cu and Zn. On the contrary, lime application to acid soils  
276 is known to induce a temporary stimulation of soil biological activity and has been  
277 reported to enhance microbial biomass content, soil respiration rate, soil enzyme activities  
278 and net mineralization of soil organic N and S (Haynes and Swift 1988; Haynes and Naidu  
279 1998). However it is also possible that dissolution of  $CaCO_3$  from the liming agent could  
280 partially contribute to the emitted  $CO_2$  as has been recently indicated for soil carbonates  
281 (Tamir et al. 2011).

282 The evolution of  $CO_2$  from soil amended with SSL was similar to that corresponding to  
283 non-amended soil (Fig. 2). At the beginning of the incubation, the fact that soil respiration  
284 increased more for the 2% amendment ( $4.18 \pm 0.31$ ) than for the higher doses ( $3.21 \pm 0.43$ )

285 for SSL5 and  $3.45 \pm 0.22$  for SSL10), which displayed a similar behaviour, could be an  
286 indication of SSL toxicity. Sewage sludges from the treatment of domestic waters may  
287 contain organic pollutants and potentially toxic elements (Smith 2009; Passuello et al.  
288 2010), which could interfere or even inhibit the effective mineralization of labile organic  
289 compounds by soil microorganisms.

290 Dehydrogenase is an oxidoreductase only present in viable cells, therefore it has been  
291 considered to represent the average activity of the active microbial population of a soil  
292 (Nannipieri et al. 2002). Dehydrogenase activity (DHA) slightly increased with liming  
293 ( $0.030 \mu\text{g INTF g}^{-1} \text{h}^{-1}$  for NC and  $0.036 \mu\text{g INTF g}^{-1} \text{h}^{-1}$  for NC<sub>L</sub>), in agreement with  
294 previous reports (Badalucco et al. 1992), and was greatly promoted by addition of SSL (see  
295 Fig. 2), already after the first incubation day, proportionally to the SSL dose ( $R^2 = 0.991$ ;  
296  $DHA = 0.045 + 0.086 \times dose - 0.0052 \times dose^2$ ), suggesting that this fresh amendment  
297 provided C which could be metabolised by most soil microorganisms. Addition of 2% SSL  
298 increased DHA values after 4 days of incubation, keeping the values constant along the  
299 incubation period, whereas SSL5 and SSL10 provided a strong increase with maximum  
300 DHA activity occurring between 4 and 12 days after amending (see Fig. 2). Then a  
301 concomitant reduction in DHA values was observed, which should be related to the  
302 decrease of easily-degradable substrates (Serra-Wittling et al. 1996; Saviozzi et al. 2002),  
303 in coincidence with a decline of the OC concentration in the soil (see Fig. 1).

304

### 305 **3.3 Soil properties at the end of the incubation period**

306 Table 1 shows the enhancing effects of SSL on soil properties after 45 days of incubation.  
307 Soil pH increased with SSL addition, and the increases in EC and OC content were  
308 proportional to the added dose ( $R^2 = 0.999$ ,  $EC = 2.25 + 0.19 \times dose$ ;  $R^2 = 0.812$ ,  
309  $OC = 0.17 + 0.17 \times dose$ ). The characterization of the more available OC fraction for soil  
310 microorganisms and plants, dissolved OC (DOC), showed that SSL addition diminished its

311 aromaticity and humification degree. Both HIX and SUVA indexes displayed low values  
312 for amended soils, decreasing proportionally to the dose ( $R^2=0.962$ ,  
313  $SUVA = 1.83 - 0.27 \times dose + 0.02 \times dose^2$ ).

314 The enzyme activities of non-amended soil without liming (data not shown) were  
315 below those corresponding to agricultural soils (Trasar-Cepeda et al. 2000) probably  
316 reflecting the toxic effect of some metals in this soil (Kandeler et al. 1996). It has been  
317 shown that increasing metal concentrations (Ni, Cd or Pb), reduce some biochemical  
318 indicators such as enzyme activities (Tejada et al. 2008; Khan et al. 2010). It is long known  
319 that incorporation of organic matter modifies soil biochemical activity and can change the  
320 effects of heavy metal to selected soil biochemical parameters. However, the influence of  
321 OM on soil biological properties depends upon amount, type, size and dominant  
322 component of added organic materials (Tejada et al. 2008).

323 As can be seen in Table 1, changes in biological properties by SSL incorporation were  
324 also noteworthy. However, the consideration of soil properties individually has proven  
325 generally to be unsatisfactory in providing an appropriate estimate of soil quality. It is  
326 difficult to draw meaningful conclusions about soil quality using individual soil enzyme  
327 activities, because a particular soil enzyme activity is not strongly related to a specific soil  
328 property but rather to a range of soil properties. Therefore, an overall index, i.e. the  
329 geometric mean of the assayed enzyme activities  
330 ( $GMea = (DHA \times Glu \times AlkP \times Pro \times Aryl)^{1/5}$ ) was used to assess soil functioning (García-  
331 Ruíz et al. 2008; Paz-Ferreiro et al. 2012). GMea was significantly higher in the  
332 organically-amended soil than in non-amended soil (see Table 1). This value was highly  
333 dependent on the dose ( $R^2 = 0.965$ ,  $GMea = 3.43 \times dose$ ), confirming the improvement of  
334 soil quality due to the addition of increasing SSL doses. In fact the enhanced enzyme  
335 activities fell into the range of the values found in earlier studies (63-202  $\mu\text{g PNP g}^{-1} \text{h}^{-1}$  for

336 glucosidase and 4.2-59.8  $\mu\text{g PNP g}^{-1} \text{ h}^{-1}$  for arylsulfatase) (Trasar-Cepeda et al. 2000)  
337 relative to agricultural soils.

338 A better understanding of the role of these enzymes on soil functioning is obtained by  
339 re-examining them individually, as they are related to the mineralization of relevant  
340 nutrients. Glu, an extracellular enzyme related to the C-cycle, catalysing the hydrolysis of  
341 cellobiose and other disaccharides, releasing sugars that act as energy source for  
342 microorganisms and Pro, which is related to the N-cycle and involved in the release of  
343 inorganic N from simple peptidic substrate, strongly increased with SSL addition (see  
344 Table 1), in agreement with Alvarenga et al. (2009) who reported increases in both  
345 enzymatic activities after addition of SSL between 25 and 100  $\text{Mg ha}^{-1}$  to an acid mine  
346 soil. It is clear that the N cycle was modified when the mine soil was treated with SSL,  
347 since Pro was stimulated even at the lowest SSL dose (Table 1). Glu reflects the state of  
348 the organic matter and the processes occurring therein (García et al., 1994). The activity  
349 was low for control soil and increased with amendment addition, likely as a consequence  
350 of the higher content of labile C in the soil, as corroborated by SUVA and HIX values for  
351 soil DOC (Table 1).

352 AlkP, used to describe a wide group of enzymes which catalyze the hydrolysis of  
353 organic-P compounds to phosphates, and DHA also showed an increase with SSL at the  
354 end of the incubation period (see Table 1). The supply of readily metabolizable C in SSL  
355 may have been responsible for the stimulation in the synthesis of soil AlkP activity. The  
356 variations of the Aryl behaviour, an enzyme which hydrolyzes sulfate esters with an  
357 aromatic radical, were lower without showing a relationship with SSL addition, as  
358 previously reported (Paz-Ferreiro et al. 2012).

359 SIR did not follow any clear trend at the end of incubation, indicating that a SSL dose  
360 above 2% would decrease microbial activity. De Andrés et al. (2012) also reported that  
361 sewage sludge application generally increased soil respiration, although in a manner not

362 proportional to the quantity applied. On the other hand, Paz-Ferreiro et al. (2012) attributed  
363 the decrease in soil respiration by sewage sludge addition to the solubility of heavy metals  
364 in soil, while enzymatic activities were enhanced.

365 In general, the organic amendment stimulated soil enzymatic activities because the  
366 added material may contain intra- and extracellular enzymes, as well as labile organic  
367 matter fractions which may also improve microbial activity in the amended soil. The large  
368 OC content of SSL would provide an energy source for soil microorganisms, but in  
369 particular, the metal load of this mine soil, with toxic effects on soil microbiota, could be  
370 counterbalanced in SSL-amended soil by the chemical composition of its OM, which  
371 would be effective in binding and chelating the metals from the soil. Similar results were  
372 reported by Tejada et al. (2008) in an acid-amended soil. However, due to the intrinsic  
373 chemical complexity of DOC, it is difficult to predict DOC reactivity (Weisshaar et al.  
374 2003)

375

### 376 **3.4 Screening of plant growth**

377 The growth of ahipa was not inhibited by SSL treatments and none of the parameters  
378 studied were affected ( $p > 0.05$ ). This means that this species does not need any soil  
379 correction to grow, except Carbocal addition to raise the soil pH.

380 In rye grass, SSL amendment increased significantly ( $p < 0.05$ ) the biomass production  
381 in comparison with non-amended soil, presenting significantly greater growth at 2%, but  
382 without significant differences between treatments ( $p > 0.05$ ) in photosynthetic pigments,  
383 germination or mortality rate (Table 2).

384 Soil added with SSL2 (Fig. 3) led to an increase of biomass production of tomato ( $p =$   
385 0.013), but SSL10 produced a growth inhibition, resulting in biomass values similar to  
386 non-amended soil ( $p = 0.695$ ). Actually, at 0 and 2% SSL no mortality was recorded but it  
387 reached values ranging from 58 to 100% with addition of SSL10. At the highest SSL

388 application, tomato stunted growth and later plant death might have resulted by the  
389 significant increase in soil EC (Table 1) (the species is more sensitive to salinity at  
390 germination and early seedling stage, see Foolad 1996). Inhibition of plant growth in soils  
391 of high salinity has been reported for other vegetal species when added with sewage sludge  
392 or other organic amendments (Gascó and Lobo 2007; Pardo et al. 2011). However, other  
393 effects should not be discarded like overcoming toxicity thresholds either from metals in  
394 the sludge or in the contaminated soil. In tomato, the SSL treatment had a positive effect  
395 on photosynthetic pigments (see Table 2), being significantly higher in plants growing in  
396 amended soil as compared to non amended soil ( $p < 0.05$ ) without showing significant  
397 differences between doses ( $p > 0.05$ ). Estimation of chlorophyll content is often  
398 accomplished to assess the impact of most environmental stresses as the pigment content is  
399 linked to toxicity/deficiency symptoms and photosynthetic plant productivity (Gupta and  
400 Sihna 2007). Our results are in agreement with earlier reports (Gupta and Sihna 2007;  
401 Singh and Sihna 2005) and may be attributed to the improved bioavailability in the soil of  
402 elements required for chlorophyll biosynthesis or the additional supply of nutrients from  
403 the sludge. Carotenoids, protective pigments associated to chlorophylls, increase  
404 sometimes under stress conditions (Kenneth et al. 2000; Rossini et al. 2010) and the results  
405 suggest the metal contamination of the soil and the metal contribution of sludge  
406 application.

407 An exploratory cluster data analysis (see Fig. 3), carried out to discover similarities  
408 among the main soil properties and the plant growing indicators, revealed that plant  
409 mortality was related with soil properties and the dose of SSL; the biomass and pigment  
410 content were related with soil respiration, an indicator of soil fertility; finally, the third  
411 group is related with the quality of DOC but seems not to be related with plant growth.  
412 There is clear evidence that addition of SSL had a strong effect on plant establishment and  
413 at the same time a dose increase resulted in an improvement of the soil biological activity



414 and microbial efficiency and, depending on the species, of the plant growth.

415 Finally, if we focus on individual plant species, ahipa and rye grass grow well up to  
416 SSL10, while a dose > 2% negatively affects tomato establishment.

417

#### 418 **4 Conclusions**

419 The addition of organic amendments to degraded soils, such as those from acid mining  
420 areas, should be selected taking into consideration not only soil fertility, but also soil  
421 ability to protect the underlying saturated zone from pollution and the ability to constitute  
422 an optimum ecosystem for the introduction of appropriate plants for revegetation purposes.

423 Addition of SSL from urban treatment plants resulted in an initially enhanced soil OC,  
424 though it decreased with time as a result of sludge stabilization, and a general increase of  
425 biological and biochemical parameters (enzymatic activities and soil respiration).

426 According to this set of parameters, soil amended with SSL would be considered as a good  
427 candidate for soil revegetation and plant establishment and development. All the species  
428 tested (tomato, rye grass and ahipa), with different economic and agricultural interest,  
429 could be used for revegetation after liming this soil for pH correction and the use of the  
430 amendment at 2%, which implies less economic investment, might be recommended for  
431 the establishment of tomato plants. However to confirm the observed effects attained with  
432 the present approach, based on a screening carried out with the three plant species in small  
433 pots, a future test under field conditions will be required.

434

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439

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572

573 **Figure caption sheet**

574

575 **Fig. 1** Evolution during incubation of electrical conductivity (left) and soil organic carbon  
576 (right) for limed Nerva soil (NC<sub>L</sub>) amended with stabilized sewage sludge (SSL) at 0, 2, 5  
577 and 10%

578

579 **Fig. 2** Evolution during incubation of soil induced respiration (left) and dehydrogenase  
580 activity (right) for limed Nerva soil (NC<sub>L</sub>) amended with stabilized sewage sludge (SSL) at  
581 0, 2, 5 and 10%

582

583 **Fig. 3** Dendrogram for hierarchical clustering of soil and plant variables



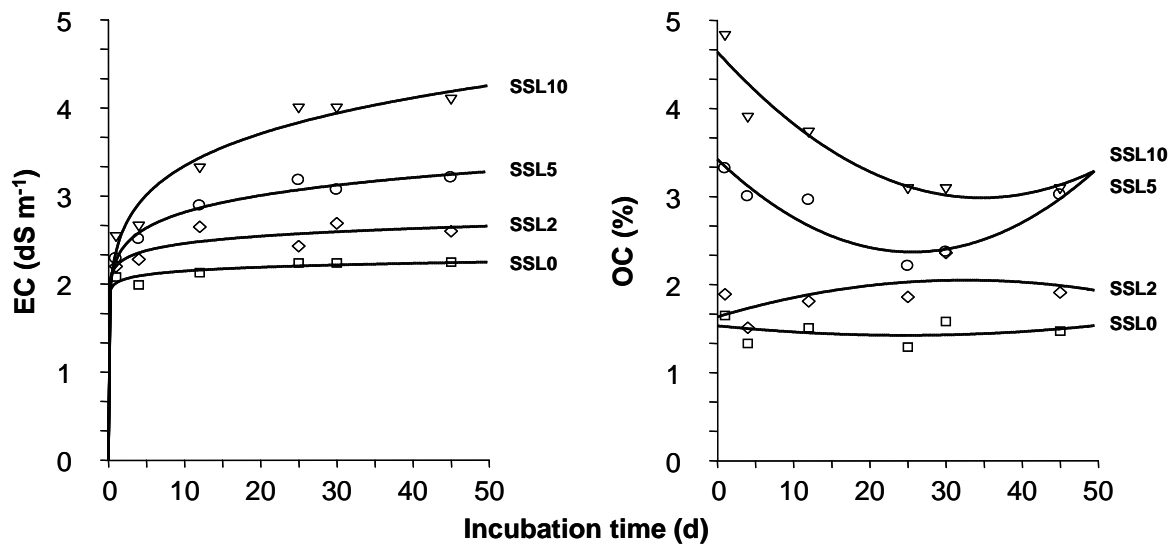


Figure 1. Evolution during incubation of electric conductivity (left) and soil organic carbon (right) for limed Nerva soil ( $\text{NC}_L$ ) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%.

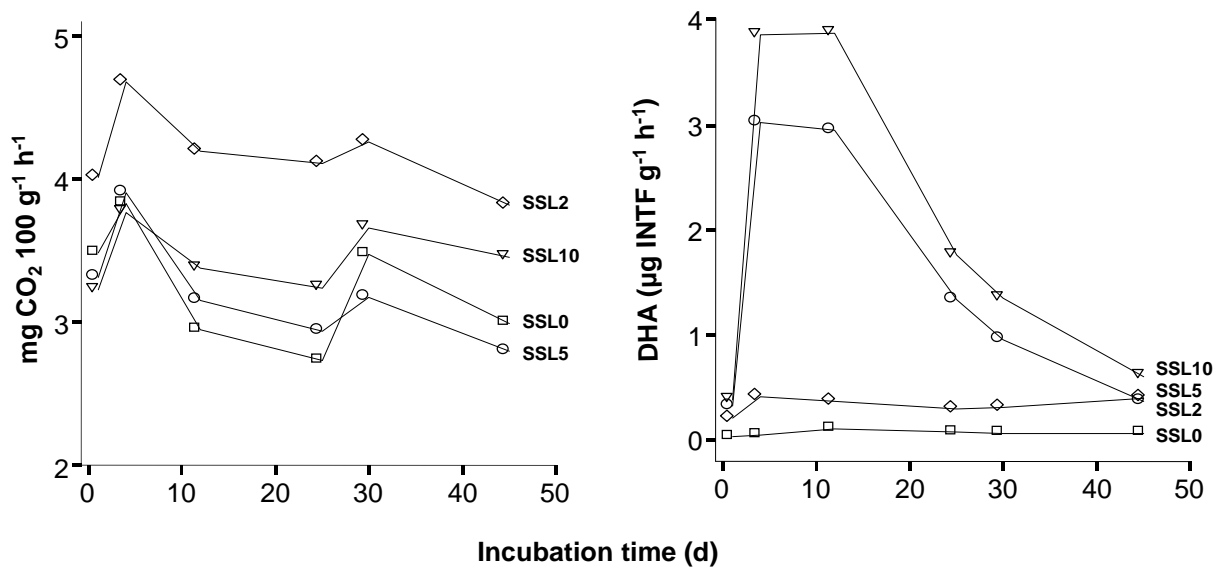


Figure 2. Evolution during incubation of soil induced respiration (left) and dehydrogenase activity (right) for limed Nerva soil (NC<sub>L</sub>) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%.

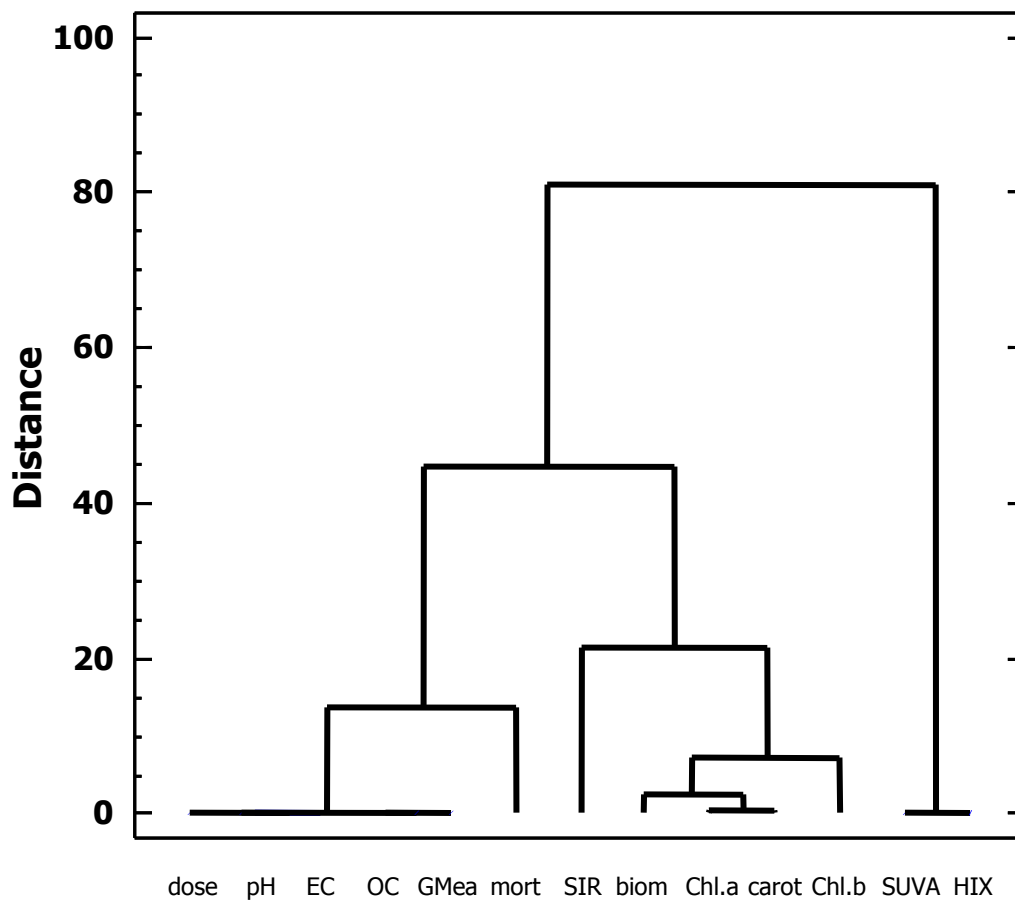


Figure 3. Dendrogram for hierarchical clustering of soil and plant variables

Table 1. Selected properties of non-amended and amended mine soil with stabilized sewage sludge (SSL) sampled at the end of the incubation study. Values are means of 3 replicates.

SSL dose (%)	Chemical properties			Enzyme activities $\mu\text{g substrate g}^{-1} \text{h}^{-1}$						Microbial properties		Soil solution	
	pH	EC $\text{dS m}^{-1}$	OC %	DHA	Glu	AlkP	Pro	Aryl	GMea	SMBC $\text{mg C } 100 \text{ g}^{-1} \text{ soil}$	SIR $\text{mg CO}_2 100\text{g}^{-1} \text{ h}^{-1}$	SUVA	HIX
0	7.14	2.25	1.47	0.060	10.2	2.63	0.28	0.185	0.61	67.4	2.99	1.88	5.74
2	7.24	2.60	1.91	0.396	108	78.4	48.2	6.39	15.9	85.9	3.82	1.25	1.91
5	7.58	3.21	3.02	2.798	109	305	98.4	16.2	43.1	63.2	2.80	1.01	1.35
10	7.69	4.11	3.10	3.664	173	530	181	14.9	61.9	77.7	3.45	0.94	1.02

EC: electrical conductivity. OC: organic carbon. DHA: dehydrogenase activity. Glu:  $\beta$ -glucosidase. AlkP: alkyl phosphatase. Pro: protease. Aryl: arylsulfatase. GMea: geometric mean of the enzyme activities. SMBC: soil microbial biomass C. SIR: soil induced respiration. SUVA: specific UV absorbance. HIX: humification index.

Table 2. Parameters in the plants growing in non-amended and amended mine soil with stabilized sewage sludge (SSL). Values are means of 3 replicates  $\pm$  standard deviation.

Species	SSL dose (%)	Germination (%)	Mortality (%)	Biomass (g)	Chl. a (mg g <sup>-1</sup> )	Chl. b (mg g <sup>-1</sup> )	Carotenoids (mg g <sup>-1</sup> )
Ahipa	0	87 $\pm$ 0	3 $\pm$ 4	4.78 $\pm$ 1.13	2.56 $\pm$ 0.45	0.83 $\pm$ 0.15	0.65 $\pm$ 0.11
	2	64 $\pm$ 28	3 $\pm$ 4	4.76 $\pm$ 2.19	2.47 $\pm$ 0.23	0.76 $\pm$ 0.08	0.63 $\pm$ 0.04
	10	69 $\pm$ 4	9 $\pm$ 9	3.38 $\pm$ 1.10	2.43 $\pm$ 0.48	0.77 $\pm$ 0.14	0.59 $\pm$ 0.17
Rye grass	0	91 $\pm$ 9	3 $\pm$ 6	0.07 $\pm$ 0.01	1.08 $\pm$ 0.16	0.56 $\pm$ 0.05	0.23 $\pm$ 0.09
	2	94 $\pm$ 5	0 $\pm$ 0	0.24 $\pm$ 0.02	1.57 $\pm$ 0.27	0.67 $\pm$ 0.10	0.38 $\pm$ 0.06
	10	88 $\pm$ 5	0 $\pm$ 0	0.13 $\pm$ 0.01	1.76 $\pm$ 0.33	0.92 $\pm$ 0.23	0.32 $\pm$ 0.06
Tomato	0	92 $\pm$ 8	6 $\pm$ 11	0.38 $\pm$ 0.08	0.99 $\pm$ 0.14	0.34 $\pm$ 0.06	0.23 $\pm$ 0.04
	2	81 $\pm$ 13	0 $\pm$ 0	1.12 $\pm$ 0.30	2.04 $\pm$ 0.28	0.71 $\pm$ 0.09	0.53 $\pm$ 0.08
	10	92 $\pm$ 8	73 $\pm$ 24	0.23 $\pm$ 0.05	2.21 $\pm$ 0.43	0.75 $\pm$ 0.13	0.52 $\pm$ 0.09