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

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Abstract

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

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

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

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

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

1 Introduction

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

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

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

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

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

2 Materials and methods

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_2) and other metallic and
109 polymetallic sulphides as chalcopyrite ($CuFeS_2$), 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_2 (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^{-1}, 1:2.5 ratio); 24% water content at field capacity; HIX 1.16;
118 SUVA (L $g^{-1}cm^{-1}$) 10.8. Otherwise, this soil contents high total concentrations of potential
119 hazardous elements (mg kg^{-1}) (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^{-1}, 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_L) received
127 Carbocal at an equivalent rate of 1.5% (w/w) in $CaCO_3$. 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^{-1}. 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,

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

2.3 Soil induced respiration and microbial biomass C

Soil induced respiration (SIR) measurements were performed in an automatic equipment (μTrac 4200, Sy-Lab, Gomensoro, Madrid, Spain), after defrozing the samples at ambient temperature. Briefly, ca. 5 g soil was mixed with 50 mg of talc:glucose (10:1 ratio) and weighed into a plastic tube, which was introduced into a measuring cell containing 2 mL of a 2% KOH solution. The tightly closed cell was maintained at 30ºC during 20 h and CO_2 evolution was monitored every 5 min, through the measurement of solution impedance decrease. Results are expressed as mg CO_2 100 g^{-1} h^{-1}.

Soil microbial biomass C (SMBC, mg C 100 g^{-1} soil) was estimated from the SIR assay. SMBC was calculated from the CO_2 generated during 6 h of soil incubation as follows: $SMBC = 40.04 \times CO_2 + 0.37$ (Anderson and Domsch 1978).

2.4 Enzyme activities

Enzyme activities are used as an index of microbial functional diversity if they reflect changes in microbial activities. Since microbial functional diversity includes many different metabolic processes, a representative set of enzyme activities was assessed: During incubation dehydrogenase activity (DHA), proposed as a measure of overall microbial activity, was determined by incubating soil samples during 20 h at 25°C with 0.2 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 (β-glucosidase), N cycle (protease), P cycle (alkaline
161 phosphatase) and S cycle (arylsulphatase). The enzyme activities β-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 μg PNP g^{-1} 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 μg tyrosine g^{-1}
168 h^{-1}. All enzyme activities were determined in defrozen samples in triplicate and expressed
169 on an oven-dried (105ºC) 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 H_2O_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, L g^{-1} 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

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

2.6 Pot experiments

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

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

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

the pigment concentrations are given in μg mL^{-1} extract solution (Lichtenthaler and Buschmann 2001)

$$[\text{Chlorophyll a}] = (16.75 * A_{665,2}) - (9.16 * A_{652,4})$$

$$[\text{Chlorophyll b}] = (34.09 * A_{652,4}) - (15.28 * A_{665,2})$$

$$[\text{Carotenoids}] = \frac{((1000 * A_{470}) - (1.63 * [\text{Chlorophyll a}]) - (104.96 * [\text{Chlorophyll b}]))}{221}$$

2.7 Statistical treatment of the data

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

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

3 Results and discussion

3.1 Evolution of soil physicochemical properties along incubation

Addition of Carbocal as a liming agent effectively raised soil pH with an average value of 6.8 ± 0.18 for the whole assayed period. The increase in pH as a result of liming has been 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 ± 0.15) was slightly higher than that of non-amended soil (pH 6.8 ± 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 ± 0.1) and of SSL2 (2.5 ± 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 ± 0.14) and amended with

259 2% SSL (1.9 ± 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 mg CO_2 100 g^{-1} h^{-1}), and
273 increased with liming (3.49 mg CO_2 100 g^{-1} 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 ± 0.31) than for the higher doses (3.21 ± 0.43

285 for SSL5 and 3.45 ± 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 μg INTF g^{-1} h^{-1} for NC and 0.036 μg INTF g^{-1} h^{-1} for NC_L), 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 ($GMea = (DHA \times Glu \times AlkP \times \Pr o \times Aryl)^{1/5}$) 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 μg PNP g^{-1} h^{-1} for

glucosidase and 4.2-59.8 μg PNP g^{-1} h^{-1} for arylsulfatase) (Trasar-Cepeda et al. 2000) relative to agricultural soils.

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

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

SIR did not follow any clear trend at the end of incubation, indicating that a SSL dose above 2% would decrease microbial activity. De Andrés et al. (2012) also reported that 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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Figure caption sheet

Fig. 1 Evolution during incubation of electrical conductivity (left) and soil organic carbon (right) for limed Nerva soil (NC_L) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%

Fig. 2 Evolution during incubation of soil induced respiration (left) and dehydrogenase activity (right) for limed Nerva soil (NC_L) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%

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

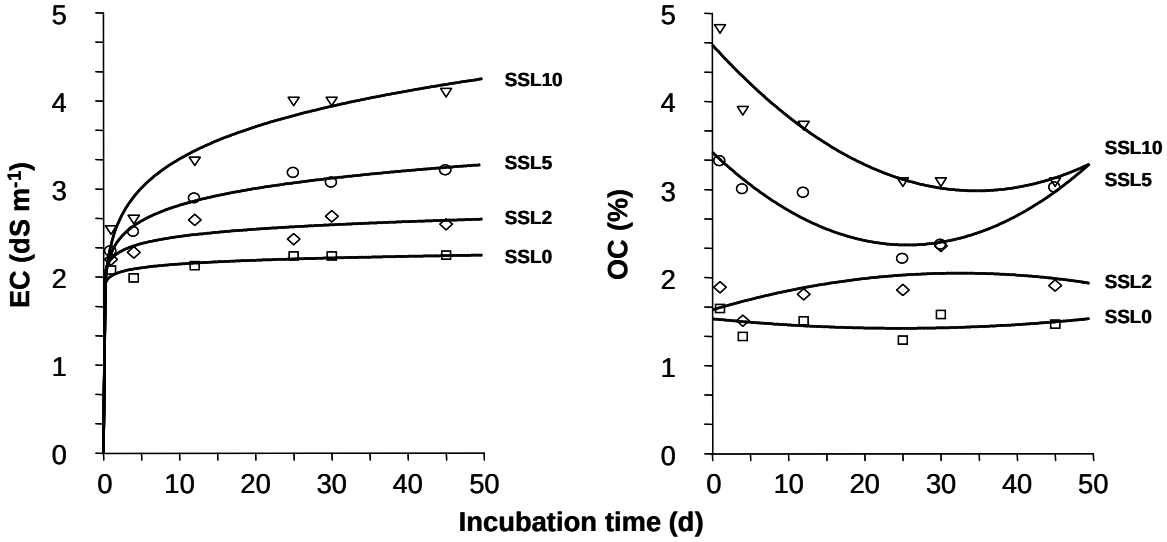

Figure 1. Evolution during incubation of electric conductivity (left) and soil organic carbon (right) for limed Nerva soil (NC_L) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%.

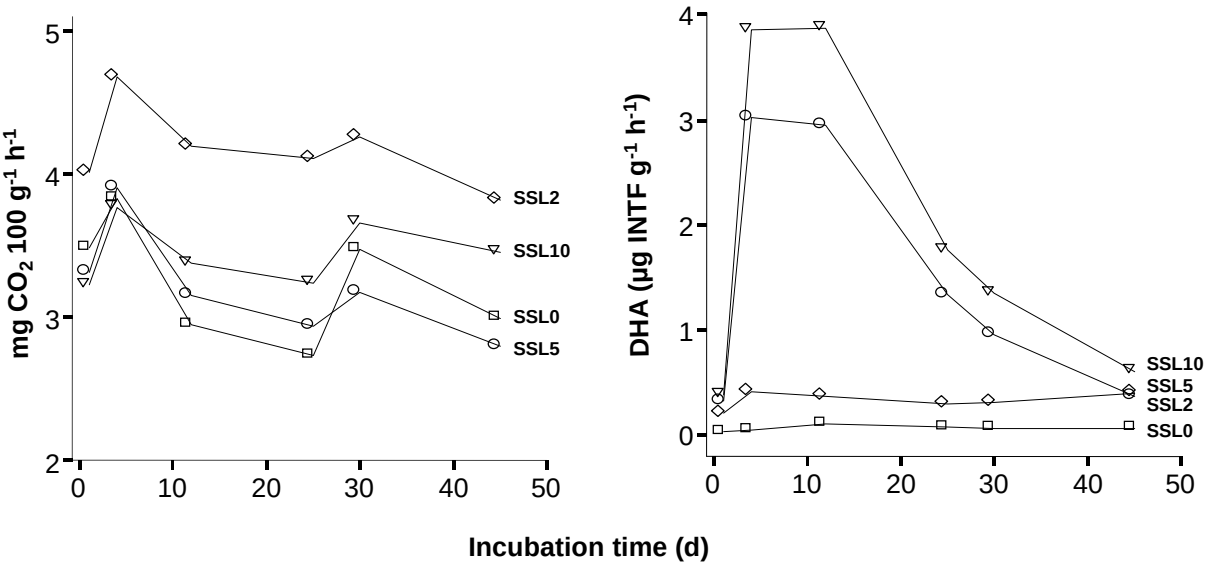

Figure 2. Evolution during incubation of soil induced respiration (left) and dehydrogenase activity (right) for limed Nerva soil (NC_L) amended with stabilized sewage sludge (SSL) at 0, 2, 5 and 10%.

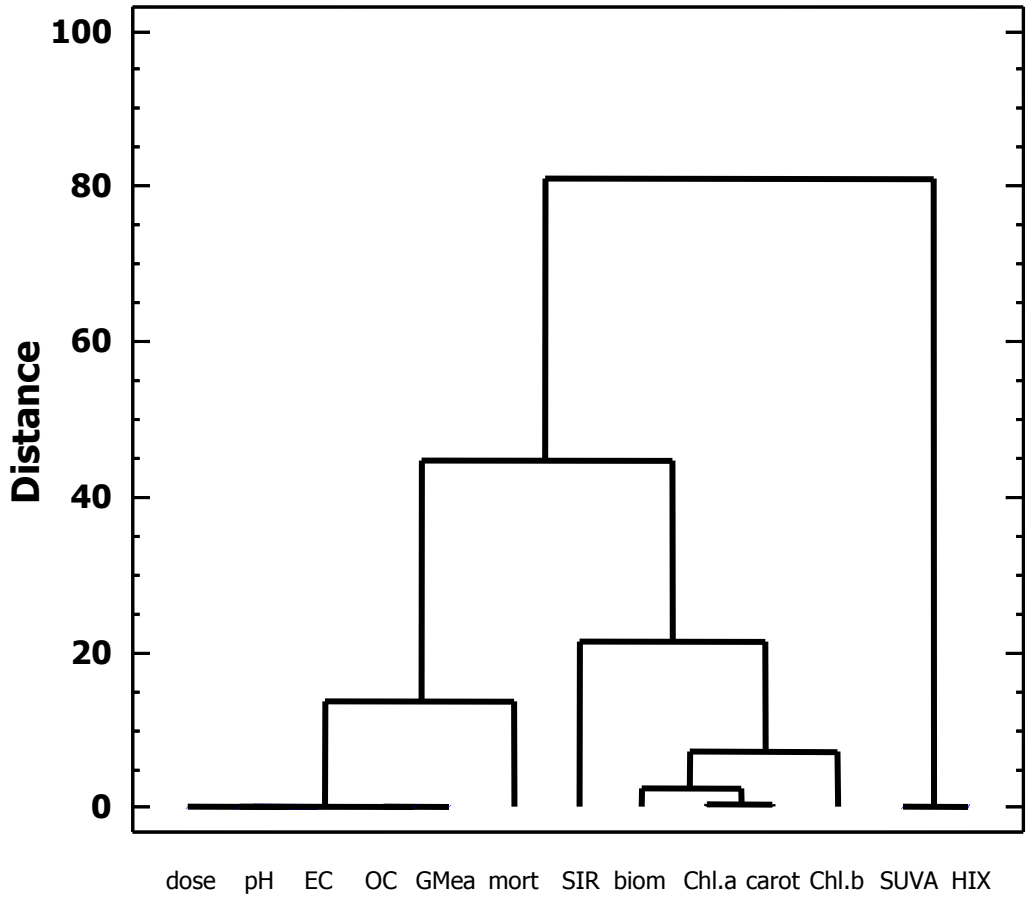

Figure 3. Dendrogram for hierarchical clustering of soil and plant variables

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.

	Chemical properties			**Enzyme activities** μg substrate g^{-1} h^{-1}						**Microbial properties**		**Soil solution**	
SSL dose (%)	pH	EC dS m^{-1}	OC %	DHA	Glu	AlkP	Pro	Aryl	GMea	SMBC mg C 100 g^{-1} soil	SIR mg CO_2 100g^{-1} 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: β-glucosidase. AlkP: alkyl phosphatase. Pro: protease. Aryl: arylsulfatase. GMea: geometric mean of the enzyme actitivies. 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 ± standard deviation.

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