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Assessment of chemical, biochemical and ecotoxicological aspects in a mine soil amended with sludge of either urban or industrial origin

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ABSTRACT

A greenhouse pot experiment was conducted to evaluate the effect of sewage sludge (SS), of sugar beet sludge (SBS), or of a combination of both, in the remediation of a highly acidic (pH 3.6) metal-contaminated soil, affected by mining activities. The SS was applied at 100 and 200 Mg ha^{-1} (dry weight basis), and the SBS at 7 Mg ha^{-1}. All pots were sown with Italian ryegrass (*Lolium multiflorum* Lam.). After 60 d of growth, shoot biomass was quantified and analysed for Cu, Pb and Zn. The pseudo-total and bioavailable contents of Cu, Pb and Zn and the enzymatic activities of β-glucosidase, acid phosphatase, cellulase, protease and urease were determined in the soil mixtures. Two indirect acute bioassays with leachates from the soil (luminescent inhibition of *Vibrio fischeri* and *Daphnia magna* immobilization) were also used.

The SS, in particular when in combination with SBS, corrected soil acidity, while increasing the total organic matter content and the cation exchange capacity. The application of SS led to a decrease in the level of effective bioavailable metals (extracted by 0.01 M $CaCl_2$, pH 5.7, without buffer), but caused an increase in their potential bioavailability (extracted by a solution of 0.5 M NH_4CH_3COO, 0.5 M CH_3COOH and 0.01 M EDTA, pH 4.7). Plant biomass increased more than 10 times in the presence of 100 Mg SS ha^{-1}, and more than five times with the combined use of 100 Mg SS ha^{-1} and SBS, but a considerable phytotoxic effect was observed for the application rate of 200 Mg SS ha^{-1}. Copper, Pb and Zn concentrations in the shoots of *L. multiflorum* decreased significantly when using 100 Mg SS ha^{-1} or SBS. The activities of β-glucosidase, urease and protease increased with increasing SS applications rates, but cellulase had a reduced activity when using 200 Mg ha^{-1} SS. Both amendments were able to suppress soil toxicity to levels that did not affect *D. magna*, but increased the soil leachate toxicity towards *V. fischeri*, especially with the application of 200 Mg SS ha^{-1}. This study showed that for this type of mine soils, and when using SS of similar composition, the maximum SS application rate should be 100 Mg ha^{-1}, and that liming the SS amended soil with SBS did not contribute to a further improvement in soil quality.

1. Introduction

Conventional remedial approaches to metal-contaminated soils involve removal and replacement of soil with clean materials or capping the soil with an impermeable layer to reduce exposure to contaminants (Brown et al., 2005). Rehabilitation projects for abandoned mine lands in Portugal rely on that kind of approach (Matos and Martins, 2006), although it is not considered the most economically or environmentally sound solution available. Alternative remedial technologies are currently being developed which involve leaving the contaminated materials in place and using soil amendments to reduce their bioavailability (Basta et al., 2001; Brown et al., 2003). One example is the use of organic biodegradable residues and limestone, or other materials rich in carbonates, to reduce the bioavailability of contaminants and restore the ecological function of metal-contaminated sites (Basta et al., 2001; Brown et al., 2003). Organic biodegradable residues may be able to improve physical, chemical and biological properties of soils by: (i) raising the pH, (ii) increasing the organic matter content, (iii) adding essential nutrients for plant growth, (iv) increasing the water holding capacity, and (v) rendering the heavy metals less bioavailable. Furthermore, in situ immobilization of metals can be combined with phytostabilization, a vegetation cover that reduces wind erosion and water percolation (Vangronsveld et al., 1995).

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The overall improvement in soil quality should be evaluated based not only on soil chemical characteristics, assessed by conventional analytical tests and extraction procedures, but also on additional assays that measure restoration of habitat functions (Brown et al., 2003, 2005; ISO/DIS 17402, 2006). In fact, chemical analysis is insufficient to evaluate potential ecological risks, since the combined effects of the different contaminants are not taken into consideration. The bioavailability of contaminants should also be examined including a range of ecological receptors and relevant pathways (Brown et al., 2005; ISO/DIS 17402, 2006; Alvarenga et al., 2008).

Ecotoxicity assays can overcome these constraints and are recommended for a complete assessment of soil quality (Conder et al., 2001; van Gestel et al., 2001). Bioassays with vascular plants are versatile tools to assess soil contamination, allowing the identification of the effect of pollutants present in the soil as well as the success of remediation processes (van Gestel et al., 2001).

Ecotoxicological tests using aqueous soil extracts, also called leachates or elutriates, can also be used to assess soil quality. They are based on the assumption that soil organisms are affected by chemical compounds present in the aqueous phase. These bioassays (e.g. using the water flea *Daphnia magna* and the marine luminescent bacterium *Vibrio fischeri*) were previously developed to evaluate toxicity in waters, but they can be adapted and used with soil leachates (van Gestel et al., 2001; Loureiro et al., 2005).

Biochemical properties, related to the biocycles of C, N, P and S, can also be used to diagnose soil quality. These include the activities of dehydrogenase and hydrolytic enzymes, such as phosphatase, urease and β-glucosidase (Gil-Sotres et al., 2005). The enzymes selected should represent a range of processes involved in organic matter decomposition and nutrient cycling or reflect microbial activity.

The aim of this study was to evaluate sewage sludge (SS) from an urban wastewater treatment plant, and sugar beet sludge (SBS), an alkaline sludge resulting from the sugar manufacturing process, or a combination of both, as amendments for the reclamation of a degraded metal-rich mine soil. The improvement in soil quality was assessed by the combined use of chemical, biochemical and ecotoxicological indexes.

2. Materials and methods

2.1. Soil characterization

Topsoil (<20 cm depth) from a metal-contaminated site in the Aljustrel mining area (a pyrite mine located in SW Portugal in the Iberian Pyrite Belt) was collected in March 2006. A soil subsample was air-dried, passed through a 2-mm sieve and subjected to chemical characterization. Particle-size distribution was determined by the pipet method (Gee and Bauder, 1986). Soil pH (H_2O) was determined in a soil to deionised water suspension of 1:2.5 (w/v), and electrical conductivity (EC) in a soil to deionised water suspension of 1:5 (w/v). Total oxidizable organic carbon (C_{org}) was determined according to Walkley and Black (1934). Soil cation exchange capacity (CEC) was determined using the ammonium acetate (pH 7) method (Sumner and Miller, 1996). Available P and K were determined using the Egner-Riehm method (Riehm, 1958). All measurements, except particle-size distribution, were carried out in triplicate. Pseudo-total metal (Cd, Cr, Cu, Ni, Pb and Zn) concentrations were determined by either flame or electrothermal atomic absorption spectrometry after digestion of the samples with aqua regia according to ISO 11466 (1995), using a Varian apparatus (SpectrAA 220FS, 220Z, and 110Z). Three independent replicates were performed for each sample and blanks were measured in parallel.

The soil used was a sandy loam, highly acidic, low in organic matter (OM) content, in essential nutrients (N, P and K) and in CEC (Table 1). The total concentrations of Cu, Pb and Zn present in the soil were high and they exceeded many times their limit value in agriculture soils for SS application allowed by Portuguese Legislation (Decreto-Lei n° 118/2006). However, the referred metals were mostly structurally bound and, therefore, scarcely bioavailable, as discussed elsewhere (Alvarenga et al., 2008).

2.2. Characterization of organic and inorganic amendments

The residues tested as soil amendments were an anaerobically digested SS, from an urban wastewater treatment plant located in Portalegre (Portugal), and SBS, an industrial wastewater treatment sludge, resulting from the sugar manufacturing process of a plant located in Coruche (Portugal). The SS was chosen due to its high organic matter content and the SBS due to its liming capacity, since it consisted mostly of $CaCO_3$ (80–85% dry weight).

Three replicates of each sample were analysed using methodologies previously described by Alvarenga et al. (2007): pH and EC were measured in a suspension with a residue/water ratio of 1:5 (w/v), after 1 h stirring. OM was determined by loss on ignition at 550 °C for 8 h. Total nitrogen was analysed using the Kjeldahl method and total phosphorous was measured colorimetrically as molybdo-vanadate phosphoric acid. Calcium and Mg were analysed spectrometrically by atomic absorption, using a VARIAN apparatus (SpectrAA 220FS) after digestion of the samples with 3 M HCl. This same digested solution was analysed for Na and K by flame photometry (Corning Flame Photometer 410). Total heavy metal content (Cd, Cr, Cu, Ni, Pb and Zn) was determined as described above for the soil characterization. All these parameters were determined on dry samples (approximately 95% dry matter content for both sludges), as they were used in the soil amendment. All analytical results were corrected to dry matter basis.

Both sludges had total metal contents below the established limits for SS (Decreto-Lei n° 118/2006), allowing their use in agricultural soils.

Table 1
Characteristics of the soil and of the sewage sludge (SS) and sugar beet sludge (SBS) used in the study (range values or mean ± standard deviation, $n = 3$)

Parameter	Soil	SS	SBS
pH	3.4–3.8	6.5–6.8	8.3–8.9
EC (dS m^{-1})	0.207 ± 0.07	2.90 ± 0.09	1.33 ± 0.08
Organic matter (% DW)	0.37 ± 0.02	72 ± 2	11.6 ± 2.7
CEC (cmol kg^{-1})	5.6 ± 0.5	–	–
Texture (g kg^{-1})			
Sand	704	–	–
Silt	177	–	–
Clay	119	–	–
$N_{Kjeldahl}$ (% DW)	0.07 ± 0.1	7.2 ± 0.4	0.37 ± 0.08
Extractable-P (mg kg^{-1} DW)	n.d.	–	–
Extractable-K (mg kg^{-1} DW)	52 ± 2	–	–
Total P (% DW)	–	1.36 ± 0.06	0.6 ± 0.2
Na (g kg^{-1} DW)	–	4.6 ± 0.9	0.75 ± 0.07
K (g kg^{-1} DW)	–	1.3 ± 0.3	0.21 ± 0.05
Ca (g kg^{-1} DW)	–	21.7 ± 0.8	345 ± 22
Mg (g kg^{-1} DW)	–	3.4 ± 0.2	9.3 ± 0.7
Cd (mg kg^{-1} DW)	2.6 ± 0.2	1.46 ± 0.04	< 0.5
Cr (mg kg^{-1} DW)	21.8 ± 0.6	15.3 ± 0.2	6.4 ± 0.2
Cu (mg kg^{-1} DW)	362 ± 23	98 ± 5	27 ± 8
Ni (mg kg^{-1} DW)	15.4 ± 0.4	10.0 ± 0.1	< 1
Pb (mg kg^{-1} DW)	4350 ± 169	37 ± 1	12 ± 2
Zn (mg kg^{-1} DW)	245 ± 64	491 ± 12	51 ± 4

DW: dry weight; EC: electrical conductivity; CEC: cation exchange capacity; n.d.: not detected.

2.3. Experimental set-up

The soil was homogenised, without sieving, and stored at field moisture content. Pots were prepared with 3000 ± 100 g of soil (dry weight basis), plus the amendments. The SS was applied at 100 and 200 Mg ha^{-1} (51 and 102 g kg^{-1}, respectively, dry weight basis), and the SBS at 7 Mg ha^{-1} (4 g kg^{-1}, based on its neutralizing value and soil buffer capacity). The amendments were tested alone, or in combination. A control, which did not receive any amendment, was included in the study. The soil mixtures were then adjusted to 70% of their maximum water holding capacity, with deionised water, transferred to a greenhouse, and allowed to equilibrate for 28 d prior to sowing. The experiment had a completely randomised design, with three replicates per treatment. After the incubation period, Italian ryegrass (*Lolium multiflorum* Lam.) was sown (2 g of seeds in each pot), and allowed to grow for 60 d. The pots were daily watered, throughout the experiment to maintain their initial water content.

2.4. Plant analysis

At harvest, the shoots were removed from the pots, washed thoroughly with tap water to remove any attached particles, and then rinsed three times with deionised water. The samples were dried at 70 °C for 48 h, weighed, and ground in an electric mill. Approximately 1 g of dried plant sample was ashed in a muffle furnace at 500 °C for 6 h, dissolved with 10 mL of 3 M HCl and evaporated to near dryness twice, dissolved again with the same acid solution, filtered (blue ribbon filter 589/3, Schleicher and Schuell Filters), and adjusted to a volume of 100 mL with ultra-pure water. The samples were analysed for total Cu, Pb and Zn, by flame atomic absorption spectrometry, using a Varian apparatus (SpectrAA 220FS).

2.5. Soil characterization after remediation

2.5.1. Chemical analysis

The soil mixtures were air-dried, passed through a 2 mm sieve and analysed as described before.

Metal bioavailable fractions were determined using two different single step extractions: a mobile fraction (extracted by 0.01 M $CaCl_2$, pH 5.7, without buffer), sometimes referred as the "effective bioavailable metal fraction", and a mobilisable fraction (extracted by a solution of 0.5 M NH_4CH_3COO, 0.5 M CH_3COOH and 0.01 M EDTA, pH 4.7), considered as a "potentially bioavailable metal fraction" (Alvarenga et al., 2008). Extractions were performed with 2 h horizontal reciprocate shaking, on a 1:10 (w/v) soil to solution ratio, at room temperature. The extract was separated from the solid residue by centrifugation at 3000*g* for 10 min.

2.5.2. Enzymatic activities

Soil sub-samples were kept refrigerated (4 °C) at their "field moisture content". Before analysis, samples were sieved through a 2 mm sieve, and their dry matter content was determined to express the enzymatic activity on a dry matter basis.

Acid phosphomonoesterase (EC 3.1.3.2) and β-glucosidase (EC 3.2.1.21) activities were measured by incubating the soil with a substrate containing a *p*-nitrophenyl group in its structure, according to Eivazi and Tabatabai (1977, 1988). Cellulase activity was determined according to Hope and Burns (1987). Cellulases are enzyme systems that degrade cellulose and release reducing sugars as the end product. In the context of this work, the term refers to the combined action of endo-1,4-β-D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21) on Avicel, a purified depolymerised alpha cellulose. Urease (EC 3.5.1.5), which catalyses the hydrolysis of urea to CO_2 and NH_3, was determined according to Kandeler and Gerber (1988). Protease activity was determined spectrophotometrically after the incubation of the soil with sodium caseynate during 2 h at 50 °C (Ladd and Butler, 1972; Alef and Nannipieri, 1995). All measurements were carried out in quadruplicate.

2.5.3. Ecotoxicity bioassays

Two indirect exposure bioassays were performed: luminescent inhibition of *V. fischeri* (ISO 11348-2, 1998) and *D. magna* immobilization (ISO 6341, 1996). Soil leachate was obtained according to DIN 38 414-S4 (1984), using a batch test with a single leaching cycle (deionised water in a 1:10 (m/v) solid-to-liquid ratio, 24 h under constant agitation, at room temperature). The leachate was separated by centrifugation, filtered through a membrane filter of pore size 0.45 μm and analysed for pH, EC and Cu, Zn and Pb contents by flame atomic absorption spectrometry using a Varian apparatus (SpectrAA 220FS).

The *D. magna* acute immobilization test was performed according to the standardised method ISO 6341 (1996). Soil leachates, and their dilutions, 12.5%, 25%, 50% and 75% (v/v), were tested. Holding and dilution water was prepared according to ISO 6341 (1996) and used also as a negative control. Five young daphnids, aged less than 24 h at the start of the test, were exposed to 20 mL of the test solution at different concentrations for a period of 48 h. Tests were conducted in environmental chambers at 20 ± 2 °C. A 16 h light and 8 h dark cycle was used. Immobilization was recorded after 24 and 48 h exposure and compared with the control.

Inhibitory effects of soil leachate on the light emission of *V. fischeri* (NRRL B-11177) were determined according to ISO 11348-2 (1998). Soil leachates, and their dilutions with a non-toxic control (2% NaCl solution), 6.25%, 12.5%, 25% and 50% (v/v), were tested and compared with the control. The decrease of luminescence was measured after 15 and 30 min contact using a LUMIStox 300 equipment. All measurements were carried out in duplicate. Whenever possible, the EC_{20} and EC_{50} values (leachate concentration, % v/v, at which a toxic effect on 20% or 50% of the population of organisms can be observed) were calculated.

2.6. Statistical treatment of data

All data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. Data not satisfying assumptions for ANOVA were analysed non-parametrically using Kruskal–Wallis ANOVA by Ranks test. Whenever significant differences where found ($p < 0.05$) a post-hoc Tukey honest significant difference (HSD) test was used to further elucidate differences among means ($p < 0.05$).

For statistical purposes, results below the detection limit, although reported as "not detected", were assumed to be equal to the detection limit. Pearson correlation coefficients (r) were calculated between soil physico-chemical properties and their enzymatic activities. Three levels of significance were considered: $p < 0.05$, $p < 0.01$ and $p < 0.001$. All statistical analysis was carried out with the software Statistica 6.0 (StatSoft, Inc., 2001). The EC_{20} and EC_{50} values were calculated using the Trimmed Spearman-Karber method (Hamilton et al., 1977).

3. Results and discussion

3.1. Changes in soil chemical properties

The SS application rates used led to significant increases in pH, OM content, CEC, and EC values (Fig. 1a–d) compared with control soil ($p < 0.05$). Comparing the limed and the correspondent

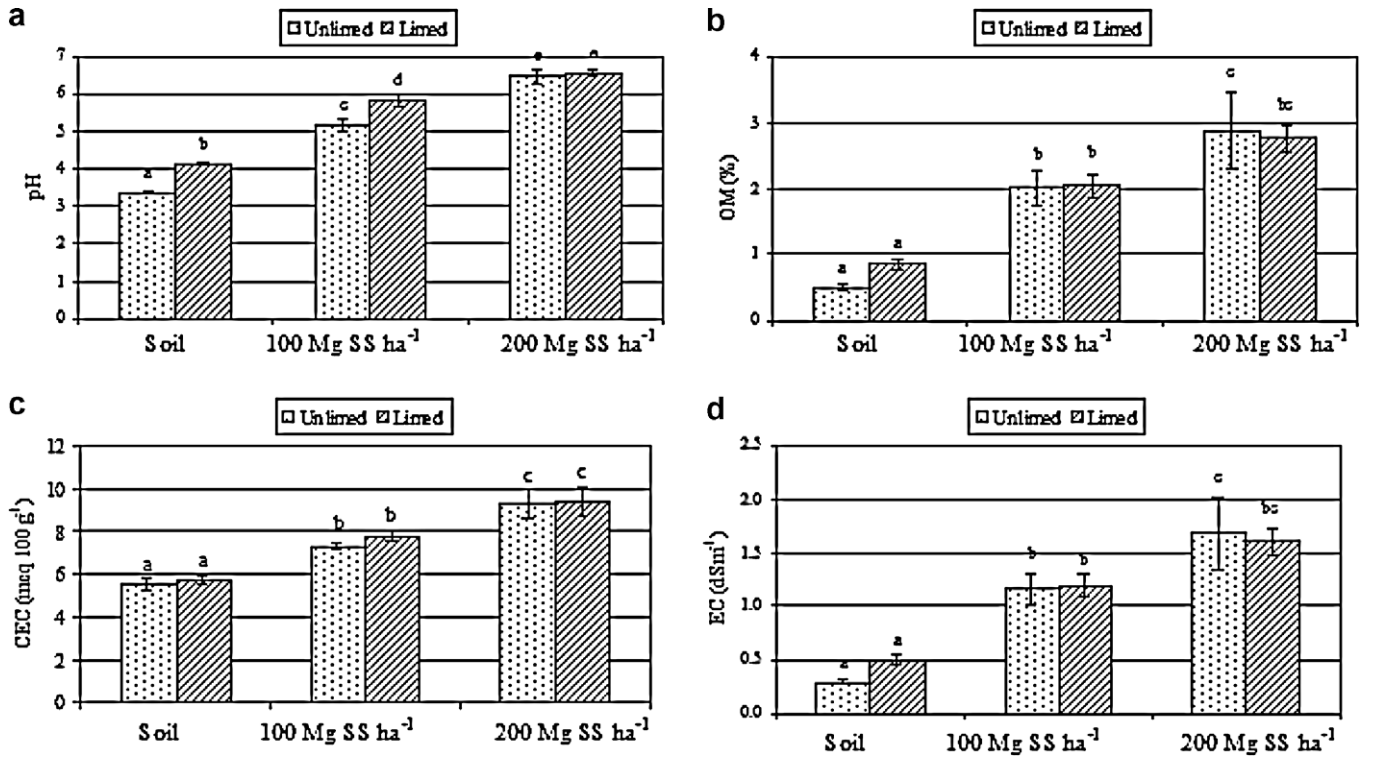

Fig. 1. Soil chemical characteristics obtained with the different amendments tested (mean ± standard deviation, $n = 3$): (a) pH, (b) total organic matter content (OM) (%), (c) cation exchange capacity (CEC) (meq 100 g^{-1}), and (d) electrical conductivity (EC) (dS m^{-1}). Columns marked with the same letter are not significantly different (Tukey test, $p > 0.05$). SS: sewage sludge.

unlimed treatment, the values for OM content, CEC and EC were not significantly different between treatments. pH values close to 6.0–6.5 were obtained by the application of 200 Mg SS ha^{-1} (with or without SBS) or with 100 Mg SS ha^{-1} plus SBS.

Pseudo-total concentrations of Cu, Pb and Zn, did not change significantly with SS or SBS application (data not shown) and, consequently, the heavy metal load into the mine soil from these particular types of sludges and application rates can be neglected.

The application of SBS significantly decreased Cu and Zn effective bioavailable fractions, relative to the unamended soil, but the strongest effect was obtained with the addition of SS, that led to levels of effective bioavailable Cu and Zn undetected by the analytical technique used (Table 2). These results can be explained by the fact that acidity is one of the most important factors controlling solubility and adsorption–desorption of metals in soils (Ross, 1994; Walker et al., 2003). Consequently, the residues decreased the effective bioavailability of metals, as they raised soil pH. This could be attributed to the high $CaCO_3$ content of SBS and to the basic cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) present in the amendments (Pérez-de-Mora et al., 2006). Another important factor controlling metal bioavailability, is the quantity and quality of OM (Ross, 1994). Organic amendments that contain a large proportion of humified OM, can also decrease the bioavailability of heavy metals due to the formation of stable chelates (Walker et al., 2003; Clemente et al., 2006). In fact, the greatest decrease in effective bioavailable Cu and Zn was achieved with SS application. On the other hand, Pb effective bioavailability was already small in the unamended soil and its level was undetected in all treatments with amendments.

Basta and Sloan (1999), among others, reported that the application of organic residues can promote an increase in soil pH, and, as a consequence, can decrease effective metal bioavailability.

Table 2
Metal concentrations in the soil (mg kg^{-1} DW) obtained with the different amendments tested: effective bioavailable and potentially bioavailable (mean ± standard deviation, $n = 3$)

	Cu (mg kg^{-1} DW)			Pb (mg kg^{-1} DW)		Zn (mg kg^{-1} DW)	
	SS application rate (Mg ha^{-1})	Effective bioavailable[A]	Potentially bioavailable[B]	Effective bioavailable[A]	Potentially bioavailable[B]	Effective bioavailable[A]	Potentially bioavailable[B]
Unlimed	0	3.4 ± 0.1^{a}	7.6 ± 0.3^{ab}	<QL	21 ± 2^{ab}	5.8 ± 0.3^{a}	6.8 ± 0.4^{a}
	100	<QL	12.3 ± 1.7^{bc}	<QL	51 ± 12^{ab}	1.4 ± 0.7^{bc}	19.4 ± 1.6^{ab}
	200	<QL	14.6 ± 1.4^{cd}	<QL	80 ± 14^{bc}	<QL	39.3 ± 11.2^{bc}
Limed	0	0.6 ± 0.1^{b}	5.5 ± 0.4^{a}	<QL	20 ± 2^{a}	2.2 ± 0.6^{b}	4.0 ± 0.5^{a}
	100	<QL	13.5 ± 0.7^{c}	<QL	50 ± 2^{ab}	0.5 ± 0.1^{c}	20.3 ± 2.0^{ab}
	200	<QL	13.7 ± 1.9^{cd}	<QL	65 ± 8^{ab}	<QL	48.6 ± 19.3^{c}

QL: quantification limit; DW: dry weight; QL (Cu) = 0.5 mg kg^{-1} DW; QL (Pb) = 5.0 mg kg^{-1} DW; QL (Zn) = 0.5 mg kg^{-1} DW.
Means within each column marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$).
[A] Extracted by 0.01 M calcium chloride solution.
[B] Extracted by 0.5 M ammonium acetate, 0.5 M acetic acid and 0.01 M EDTA, pH 4.7.

However, they can also be responsible for large metal loadings into the soil. The potential risk derived from their application can be estimated from the potential bioavailable metal fraction (Table 2). In the present experiment, the SBS did not contribute to an increase in potentially bioavailable Cu, Pb and Zn, but the application of SS caused a significant increase in their values. Hence, although SS application was in accordance with sustainable management practices, it has to be applied at the right rates and the soil should be monitored regularly.

3.2. Changes in soil enzymatic activities

Both β-glucosidase and cellulase are extracelular enzymes related to the C-cycle, with an important role in OM degradation: cellulases are enzyme systems that degrade cellulose and release reducing sugars as the end product, and β-glucosidase is involved in the final step of cellulose degradation, catalysing the hydrolysis of carbohydrates with β-D-glucoside bonds like cellobiose, providing energy substrates for soil heterotrophic microorganisms (Tabatabai, 1982; Eivazi and Tabatabai, 1988). β-Glucosidase has been widely used to assess soil quality. It is usually impaired by agronomic practices that cause a rapid loss of soil OM (e.g. tillage), and enhanced following application of organic amendments (Gil-Sotres et al., 2005; Pérez-de-Mora et al., 2005). Some authors reported that β-glucosidase activity is strongly reduced by the presence of heavy metals (Hinojosa et al., 2004a), but Pérez-de-Mora et al. (2005) suggested that the major factor that influences its activity is soil OM. In the present study, both factors could have positively influenced β-glucosidase activity, which increased with increasing SS applications rates (Fig. 2a): an increase in soil OM content and a concomitant decrease in metal effective bioavailability. For cellulase, the only treatments where a significantly higher activity was found, compared with unamended soil, were those with 100 Mg ha^{-1} SS, with or without liming (Fig. 2c). The greatest SS application rate tested (200 Mg ha^{-1}) led to adverse conditions for this enzyme, as for other parameters, as discussed below.

Acid phosphomonoestearase (acid phosphatase) is one of the many phosphatases in soils and it is largely responsible for the mineralization of organic phosphate compounds in acid soils (Eivazi and Tabatabai, 1977; Huang and Shindo, 2000). Among

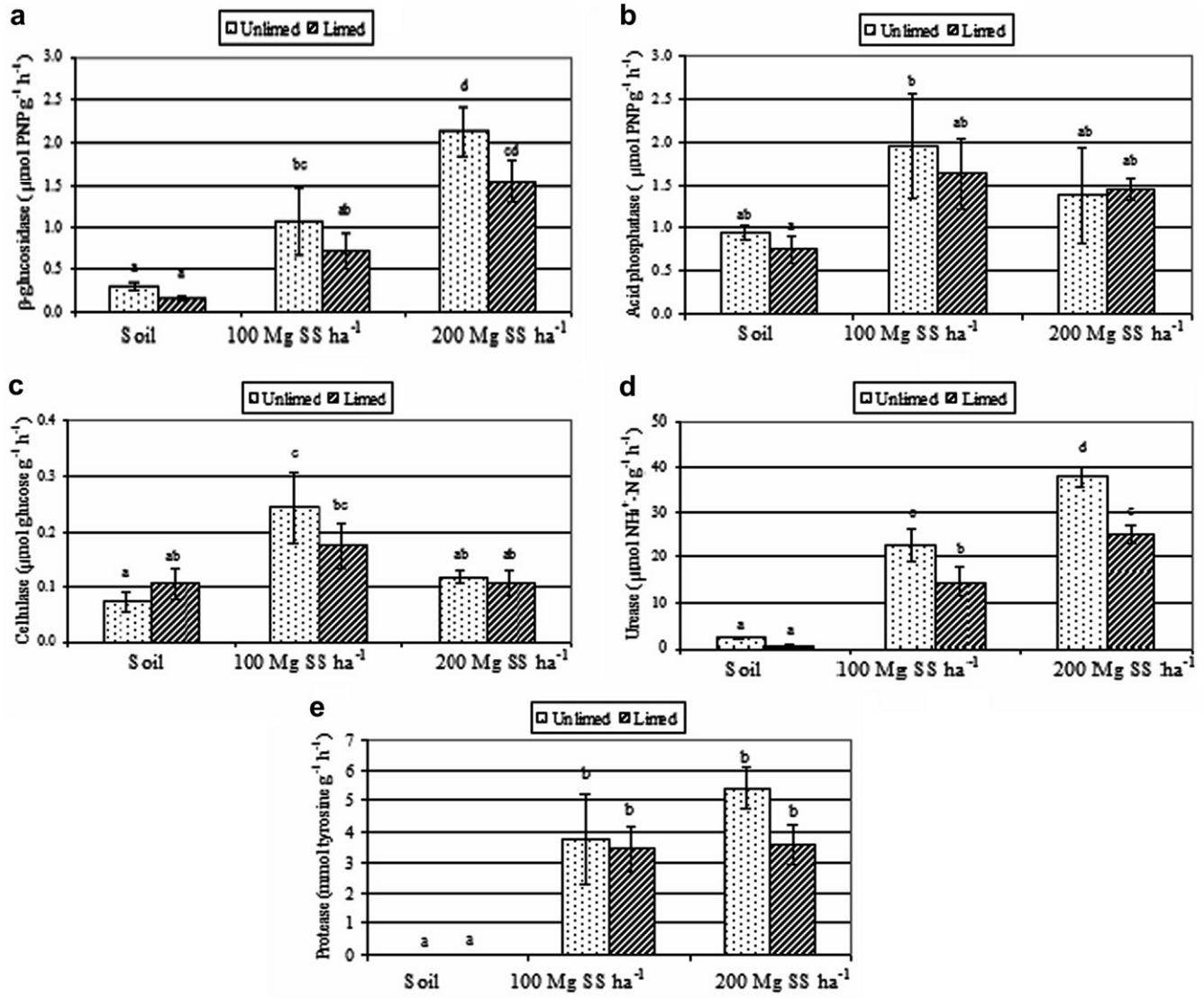

Fig. 2. Values of enzymatic activities obtained in the different soil treatments (mean ± standard deviation, $n = 12$): (a) β -glucosidase (μmol PNP g^{-1} h^{-1}), (b) acid phosphatase (μmol PNP g^{-1} h^{-1}); (c) cellulase (μmol glucose g^{-1} h^{-1}); (d) urease (μmol NH_4^+–N g^{-1} h^{-1}) and (e) protease (mmol tyrosine g^{-1} h^{-1}). Columns marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$). PNP: *p*-nitrophenol; SS: sewage sludge.

Table 3
Pearson's correlation coefficients between soil chemical properties and their enzymatic activities ($n = 18$)

	pH	EC	OM	CEC	Cu(EB)	Cu(PB)	Pb(T)	Pb(PB)	Zn(EB)	Zn(PB)	β -gluc	Ac-phos	Cellu	Prot
EC	0.95***	–	–	–	–	–	–	–	–	–	–	–	–	–
OM	0.95***	1***	–	–	–	–	–	–	–	–	–	–	–	–
CEC	0.88***	0.81***	0.81***	–	–	–	–	–	–	–	–	–	–	–
Cu(EB)	−0.79***	−0.74***	−0.74***	−0.50*	–	–	–	–	–	–	–	–	–	–
Cu(PB)	0.87***	0.86***	0.86***	0.70**	−0.55*	–	–	–	–	–	–	–	–	–
Pb(T)	0.52*	0.51*	0.51*	0.47*	−0.39	0.43	–	–	–	–	–	–	–	–
Pb(PB)	0.89***	0.96***	0.96***	0.79***	−0.61**	0.86***	0.48*	–	–	–	–	–	–	–
Zn(EB)	−0.92***	−0.87***	−0.87***	−0.72***	0.95***	−0.69**	−0.47*	−0.77***	–	–	–	–	–	–
Zn(PB)	0.82***	0.77***	0.77***	0.86***	−0.49*	0.70***	0.23	0.72***	−0.65**	–	–	–	–	–
β-gluc	0.83**	0.86*	0.86*	0.77	−0.51*	0.77**	0.26	0.89*	−0.68**	0.85***	–	–	–	–
Ac-phos	0.48	0.52	0.52	0.18	−0.43	0.65**	0.14	0.51*	−0.47	0.37	0.49*	–	–	–
Cellu	0.24	0.29	0.29	−0.16	−0.48*	0.35	0.17	0.30	−0.38*	−0.01	0.14	0.77**	–	–
Prot	0.88***	0.91***	0.91***	0.69**	−0.67**	0.89***	0.38	0.92***	−0.79***	0.74***	0.89***	0.69*	0.47*	–
Ure	0.86	0.91	0.91	0.72	−0.60	0.87	0.34	0.94	−0.73	0.76	0.96	0.56	0.30	0.94***

EC: electrical conductivity; OM: total organic matter; CEC: cation exchange capacity; Pb(T): pseudo-total Pb content; Cu(EB) and Zn(EB): effective bioavailable metal content; Cu(PB), Pb(PB) and Zn(PB): potential bioavailable metal content; β-gluc: β-glucosidase; Ac-phos: acid phosphatase; Cellu: cellulase; Prot: protease; Ure: urease; Marked correlations are significant at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

hydrolases, acid phosphomonoesterase activity has been frequently used to estimate changes in soil quality, due to management practices or presence of contaminants (Gil-Sotres et al., 2005). Its activity is impaired by Pb (Gil-Sotres et al., 2005) and other heavy metals, such as Cd, Zn and Cu (Renella et al., 2003). As it is considered a good index of the quality and quantity of OM in soils, it was predicted that if the OM content of a degraded soil were to increase, this enzymatic activity should also rise. However, in the present experiment, the activity of acid phosphatase was not affected by the treatments (Fig. 2b), with no significant differences between its activities in the different pots. Likely, the level of heavy metals was still too high, impairing its activity, or the raise in soil pH did not had a positive effect on its activity.

Protease is the enzyme that catalyses protein hydrolysis to peptides and aminoacids (Ladd and Butler, 1972; Alef and Nannipieri, 1995), and urease is the enzyme that catalyses the hydrolysis of urea to CO_2 and NH_3 (Kandeler and Gerber, 1988). The activity of both enzymes, related to the N-cycle, increased compared to the unamended soil following the application of SS (although with no significant differences between the four amended treatments in the case of protease) (Fig. 2d and e). Moreover, comparing the relative increase in all enzymatic activities, these two enzymes had the highest positive response to soil amendments. This could be a consequence of the fact that the SS used had a high organic nitrogen content, 7.2% DW (Table 1), which improved the activity of enzymes related to the N-cycle.

Significant differences between the unlimed and the limed SS treated pots were found only in the case of the urease activity, with smaller activities when SBS was applied. In fact, although with no significant differences, liming with SBS seems to have contributed to a decrease in the activity of all the enzymes tested.

3.3. Pearson's correlations

Table 3 shows the Pearson's correlation coefficients between soil chemical properties and their enzymatic activities. In general, chemical parameters (pH, EC, OM and CEC) and potentially bioavailable Cu, Pb and Zn were highly positively correlated with each other ($p < 0.001$). This was predictable, as the application of SBS and SS promoted an increase in these soil properties. In contrast, negative correlations were found between effective bioavailable Cu and effective bioavailable Zn and the chemical parameters. Pseudo-total contents of Cu and Zn and effective bioavailable Pb were not correlated with any other soil properties (data not shown). With the exception of cellulase, all soil enzymatic activities were positively correlated with soil chemical properties, and negatively correlated with effective bioavailable Cu and Zn. Other authors, studying the potential use of soil enzymes as indicators of changes in soil microbial activity in response to heavy metals and remedial measures, reported similar results (Huang and Shindo, 2000; Kızılkaya et al., 2004; Hinojosa et al., 2004a,b; Pérez-de-Mora et al., 2005, 2006). Enzyme reactions are inhibited by metals which may complex with the substrate, combine with the active site of enzymes, or react with the enzyme–substrate complex (Hinojosa et al., 2004a). However, high levels of heavy metals are not always translated into lower enzymatic activities for all soil enzymes. Examples were reported by Hinojosa et al. (2004b), who found that arylsulfatase and alkaline phosphatase activities were similar in polluted and restored soils and by Kızılkaya et al. (2004), who found that urease activity was not negatively correlated with total heavy metal content in agricultural soils. Aoyama et al. (1993) also reported that β -glucosidase was only marginally affected by high Cu and Zn concentrations. According to Hinojosa et al. (2004a), soil type (e.g. CEC, OM content) modulates the nature and degree of inhibition of soil enzymes by heavy metals, explaining the referred contradictions. Kızılkaya et al. (2004) stated that extracellular enzymes, like urease, which is strongly adsorbed by clay and humus, are more resistant to environmental impacts than other enzymes and their activities (e.g. dehydrogenase and catalase).

3.4. Plant analysis

Plant biomass increased more than 10 times in the presence of 100 Mg SS ha^{-1}, and more than five times with the combined use of 100 Mg SS ha^{-1} and SBS (data not shown). A considerable phytotoxic effect was registered for the 200 Mg SS ha^{-1} application rate,

Table 4
Metal concentrations (mg kg^{-1} DW) in the aboveground plant material of *L. multiflorum* (mean ± standard deviation, $n = 3$)

	SS application rate (Mg ha^{-1})	Cu (mg kg^{-1} DW)	Pb (mg kg^{-1} DW)	Zn (mg kg^{-1} DW)
Unlimed	0	63.3 ± 0.2[a]	41 ± 7[a]	499 ± 35[a]
	100	11.5 ± 0.9[b]	<QL	26 ± 1[b]
Limed	0	5.9 ± 0.5[b]	<QL	27 ± 4[b]
	100	16 ± 2[b]	23 ± 4[a]	27 ± 3[b]

Means within each column marked with the same superscript letter are not significantly different (Tukey HSD test, $p > 0.05$).
SS: sewage sludge; DW: dry weight; QL: quantification limit; QL (Pb) = 5.0 mg kg^{-1} DW.

Table 5
Results from the acute toxicity bioassays using soil leachates (EC_{xx} ± standard deviation, $n = 6$)

	Luminescent inhibition (*V. fischeri*) % (v/v)					Immobilization (48 h) (*D. magna*) % (v/v)	
	SS application rate (Mg ha^{-1})	15 min		30 min		EC_{20}	EC_{50}
		EC_{20}	EC_{50}	EC_{20}	EC_{50}		
Unlimed	0	n.t.	n.t.	n.t.	n.t.	20 ± 3	31 ± 2
	100	22.0 ± 0.3	n.t.	5.0 ± 0.3	n.t.	n.t.	n.t.
	200	15.8 ± 0.2	47.0 ± 0.2	16.3 ± 0.1	45.5 ± 0.1	n.t.	n.t.
Limed	0	n.t.	n.t.	50.5 ± 0.1	n.t.	n.t.	n.t.
	100	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	200	21.0 ± 0.2	62.0 ± 0.2	22.7 ± 0.3	63.8 ± 0.2	n.t.	n.t.

n.t.: no toxic effect detected; SS: sewage sludge.
EC_{20}: leachate concentration (% v/v) at which a toxic effect on 20% of the exposed organism can be observed, considering the selected endpoint.
EC_{50}: leachate concentration (% v/v) at which a toxic effect on 50% of the exposed organism can be observed, considering the selected endpoint.

with no plant growth in these pots. This probably derived from soluble salts present in the sludge, rather than to the presence of heavy metals that, as discussed above, were less bioavailable following soil amendment. The same explanation was put forward by Rodgers and Anderson (1995), who recommended that sludge amendments should not exceed a one-time application of 100 Mg ha^{-1} to prevent growth inhibition during the first year, following field application. Ye et al. (2002) pointed out that at an EC of 4 dS m^{-1}, yield of many crops will be restricted due to inhibition of plant growth and seed germination.

Copper, Pb and Zn concentrations in the shoots of *L. multiflorum* decreased significantly using 100 Mg SS ha^{-1} or SBS, but liming the SS-amended soil with SBS did not contribute to a further reduction in the level of these metals in the plant (Table 4).

3.5. Bioassays results

The leachate from the unamended soil, at the end of the experiment, was toxic to *D. magna* but not to *V. fischeri* (Table 5). As the original mine soil presented toxicity towards *V. fischeri*, EC_{20} (15 min) of 45.2% and EC_{20} (30 min) of 10.7% (v/v) (Alvarenga et al., 2008), the decreased toxicity of the unamended soil towards *V. fischeri* can only be ascribed to its vegetation cover.

Both amendments were able to suppress soil toxicity to levels that did not affect *D. magna*, but increased the toxicity towards *V. fischeri*, especially with the application of 200 Mg SS ha^{-1}. This result corroborates the phytotoxicity results discussed above: 200 Mg SS ha^{-1} was excessive, resulting in toxicity towards some organisms.

As pointed out by other authors, no single bioassay is sufficient to monitor a remediation process, since toxicity trends during remediation differ according to the assay used (Knoke et al., 1999). In this study, we found that *V. fischeri* was extremely sensitive to high doses of SS applied to soil. Chaîneau et al. (2003) also found that *V. fischeri* and plant growth were very sensitive indicators of soil toxicity, and were more reliable than other bioassays (e.g. worm survival and seed germination).

4. Conclusions

The amendments used had positive effects on the soil chemical properties. The SS used, in particular when in combination with SBS, was able to correct soil acidity, while increasing the soil total OM content and its CEC. In particular, SS proved to be very efficient in raising soil pH, decreasing effective bioavailable heavy metals, so that liming was not needed for the SS higher application rate (200 Mg ha^{-1}). However, concomitantly, all amendments led to a significant increase in EC values, which, in some cases, were phytotoxic.

The highest SS application rate should be 100 Mg ha^{-1}, as a higher application rate (200 Mg SS ha^{-1}) was toxic to plants and *V. fischeri* and led to a lower soil cellulase activity. Liming the SS-amended soil with SBS did not contribute to a further improvement in soil quality, as evaluated by its bioavailable metal content and by its enzymatic activities.

This study showed that the application of SS, at a rate of 100 Mg ha^{-1}, improved the quality of the mine soil, as evaluated by the combined use of chemical, biochemical and ecotoxicological parameters. More studies are needed to separate the effects of amendment application from the effects of a vegetation cover on soil characteristics, especially in soil effective bioavailable heavy metals, and in soil biochemical and ecotoxicological properties.

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