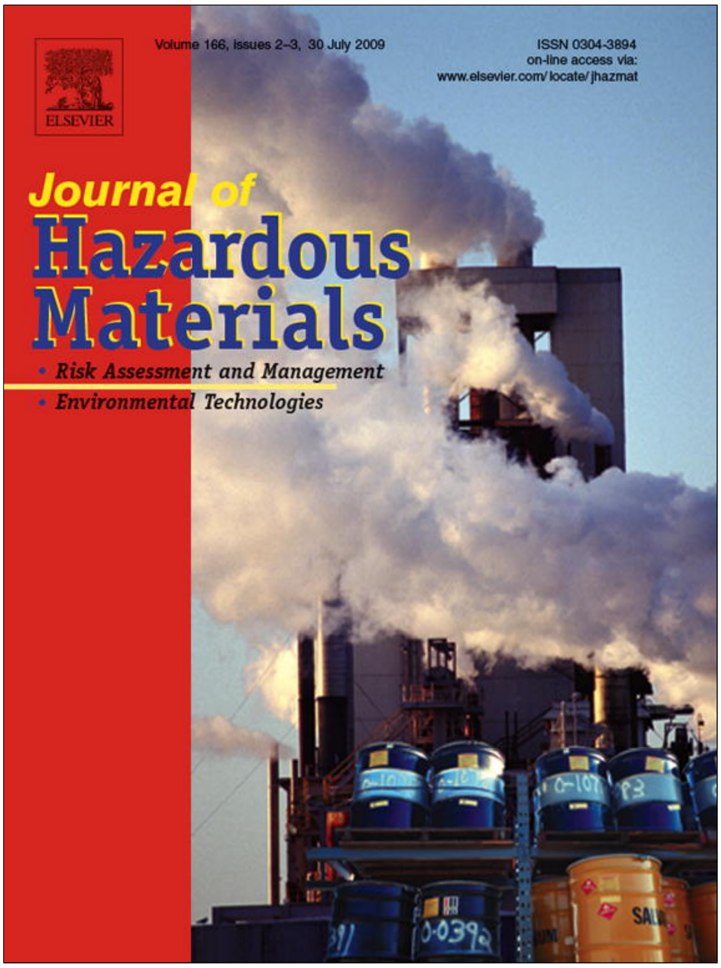

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Plant absorption of trace elements in sludge amended soils and correlation with soil chemical speciation

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ABSTRACT

The aim of the present study was to investigate the relationship between *Lolium perenne* L. uptake of Cd, Cu, Pb, and Zn in sludge amended soils and soil availability of these elements assessed by soil sequential extraction. A greenhouse experiment was set with three representative soils of the Pampas Region, Argentina, amended with sewage sludge and sewage sludge enriched with its own incinerated ash. After the stabilization period of 60 days, half of the pots were sampled for soil analysis; the rest of the pots were sown with *L. perenne* and harvested 8, 12, 16 and 20 weeks after sowing, by cutting just above the soil surface. Cadmium and Pb concentrations in aerial tissues of *L. perenne* were below detection limits, in good agreement with the soil fractionation study. Copper and Zn concentration in the first harvest were significantly higher in the coarse textured soil compared to the fine textured soil, in contrast with soil chemical speciation. In the third harvest, there was a positive correlation between Cu and Zn concentration in aerial biomass and soil fractions usually considered of low availability. We conclude that the most available fractions obtained by soil sequential extraction did not provide the best indicator of Cu and Zn availability to *L. perenne*.

1. Introduction

Potentially toxic elements (PTE) content is one of the major factors limiting the application of sewage sludge to agricultural land [1,2]. The environmental hazards of trace element pollution depend on the geochemical and biochemical properties of the element considered. Pollution problems may arise if PTE are mobilized into the soil solution and are either taken up by plants or transported in drainage waters. Risks for human health may then occur through direct or indirect consumption of these crops or intake of contaminated waters. A large number of investigations has been done to find a reliable method that can estimate bioavailability of trace metals and thereby predict their impact on the soil ecosystem. It is well known that total PTE concentration in soils provides very limited information about the element's chemical behaviour and potential fate [3]. Several approaches have been commonly used for the quantitative estimation for the quantitative estimation of chemical species of PTE that strongly affect their mobility, reactivity and availability to plants. The most important methods include: single batch extraction [4,5]; sequential extraction [6,7]; and column leaching experiments [8]. Sequential extraction methods have been widely used in an attempt to quantitatively estimate PTE chemical forms that strongly affect their mobility, reactivity and availability to plants. In this technique, the soil is subjected to a series of chemical reagents of increasing reactivity, with phytoavailability and mobility of PTE decreasing in the order of the sequential extraction step. In this way, the amount of PTE extracted from the more bioavailable fractions gives an idea of the size of the pool that might be depleted by a plant during the growing period. On the other hand, the degree of metal association with distinct geochemical phases is strongly dependent upon the physico-chemical conditions of the soils, such as pH, cation exchange capacity (CEC), organic matter content, mineralogy and the nature and amount of the trace element [9,10].

The relationships between the speciation of trace elements in soils and plant uptake have been estimated by simple or multiple correlation procedures. In some of the investigations, consistent correlations between specific metal fractions and plant metal contents were found: exchangeable [11,12] and organically bound [13] trace elements were found to predict reasonably well plant tissue concentrations. In other studies, no correlation with these fractions was observed, and therefore, multiple regression analysis between soil metal fractions, soil parameters and plant metal uptake were applied [14–16]. However, PTE absorption by plants may not only depend on soil properties or the nature of the trace element. Plants may induce changes in the biochemical, chemical and physical properties of the rhizosphere that increase PTE diffusion into the root [17,18]. In this way, PTE uptake by plants through their roots depends not only on the amount of PTE present in available forms, or the ability of the plants to transfer the metals across the soil–root

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interface, but on the soil chemical processes that occur as a result of root activity as well. Moreover, the soil environment immediately adjacent to the roots can be strongly influenced by root exudates so that chemical processes of dissolution, chelation, and precipitation outside the root also occur. Besides, graminaceous plant species secrete phytosiderophores which can form stable complexes with Cu, Fe or Zn [19]. The underlying changes in the rhizosphere may also arise from other processes for which roots are not directly responsible, like the activity of microorganisms that are stimulated in the vicinity of the roots as a consequence of the release of rhizodeposits [20].

Plants have been employed in recent years as a means of stabilising PTE in sludge amended soils by reducing metal mobility through rhizosphere-induced adsorption and precipitation processes [21]. *Lolium perenne* L. is considered a suitable species for this purpose, producing high dry matter yields and accumulating moderate to high levels of PTE in its biomass from readily extractable and soluble forms [22], fitting the definition of a facultative metallophyte [23]. The present study aims to investigate the relationship between *Lolium perenne* L. uptake of Cd, Cu, Pb, and Zn in soils amended with sewage sludge or sewage sludge enriched with its own incinerated ash and soil availability assessed by a soil sequential extraction method.

2. Materials and methods

2.1. Soils and sludge

A pot experiment was conducted using three representative Mollisols (U.S. Soil Taxonomy) of the Pampas Region, Argentina. The soils are classified as Typic Hapludol, Typic Natraquol and Typic Argiudol, sampled near C. Casares, Pila and S.A. de Areco towns, respectively. Composite soil samples (10 sub samples, 0–15 cm depth) were collected from fields with no previous history of fertilization or contamination, and were thoroughly homogenized, air dried and passed through a stainless steel sieve with 2-mm openings. Water holding capacity (WHC) was determined in the three soils according to the method proposed by Mizuno et al. [24].

Non-digested sewage sludge was obtained from Aldo Bonzi wastewater treatment plant located at the SW outskirts of Buenos Aires City. The sludge (SS) was dried at 60 °C before grinding and sieving (<2 mm) and then split into two portions. A portion was incinerated at 500 °C. The ash obtained was thoroughly mixed with a portion of the sieved sewage sludge, resulting in a new mixed waste which contained 30% DM as ash (AS).

2.2. Greenhouse experiment

Plastic pots were filled with 2.5 kg (air-dry equivalent) of soil. The rate of application of SS and AS to each soil was an equivalent field application rate of 150 dry t/ha (6:100, w/w, SS or AS: soil ratio). Both amendments were thoroughly mixed with the soils (day 1). Unamended soils were used as control. The pots were arranged in completely randomized blocks and housed in a greenhouse sheltered from rain or direct sunlight. Pots were left undisturbed and allowed to settle down over 60 days. During that period, the moisture content of the pots was kept at 80% of water holding capacity by daily replenishing with distilled water to a constant weight. In all, 18 treatments: 3 soil materials × (2 amendments + control) × (plant, no plant) were each replicated four times.

After the stabilization period of 60 days, four pots per treatment were sampled, air-dried and passed through a 2 mm sieve for soil analysis. 2.00 g seeds with average germination rate over 95% were sown in the rest of the pots. Soil moisture was maintained in all pots at 80% of WHC by daily adding distilled water and weighing. The pots were moved around at regular intervals to compensate for light differences. *L. perenne* was harvested 8, 12, 16 and 20 weeks after sowing, by cutting just above the soil surface. Aerial biomass was oven dried at 65 °C for 48 h to reach constant dry weight.

2.3. Plant analysis

Plant aerial tissue was weighed for biomass determination, and further processed for chemical analysis by grinding in a stainless steel mill to pass a 0.5-mm sieve. Samples were stored in paper bags, and placed in an oven at 65 °C until constant mass was achieved to remove moisture added during grinding and handling of the samples. As the above-ground parts of the plants are more relevant to grazing animals, root studies of the plants were excluded in this study. Aerial tissue of the first and third harvest (0.5 g) was digested using a mixture of HNO_3 and $HClO_4$ [25]. The extracts were analyzed for Cd, Cu, Pb, and Zn by AAS. Detection limits for Cd, Cu, Pb and Zn were 0.02, 0.03, 0.1 and 0.01 mg kg^{-1}, respectively. Total concentrations of metals were also analyzed in certified plant standards (spinach leaves, NIST 1570a) as a quality control procedure.

2.4. Soil fractionation

The sequential extraction scheme proposed by McGrath and Cegarra [26] was used to partition Cd, Cu, Pb and Zn in control and sludge treated soils into several fractions. The fractions were defined as (1) EXCH: water-soluble and exchangeable fraction, extracted by 0.1 M $CaCl_2$; (2) organic matter (OM) bound fraction, extracted by 0.5 M NaOH; (3) inorganic fraction (INOR), extracted by 0.05 M Na_2EDTA; and (4) residual fraction (RES), digested by conc. HNO_3 + conc. HCl + conc. HF [27]. Concentrations of Cd, Cu, Pb and Zn were measured in each filtrate using atomic absorption spectrophotometer (AAS). Cd, Cu, Pb and Zn concentrations in the whole soil sample were determined by acid digestion [27]. Blanks were used for background concentrations. For quality control purposes, certified soil standards (Montana soil, NIST 2711; National Institute of Standards and Technology, Gaithersburg, MD) were subjected to the same treatment and included in the over-all analytical process.

2.5. Statistical analysis

All results reported are the mean of four replicates. The statistical analysis was done with the Statistics 7.0 (2000) package, processing the data for Analysis of Variance (ANOVA) for a completely randomized design. One-way ANOVA was carried out to compare the means of different treatments; where significant *F* values were obtained, differences between individual means were tested using Tukey's test. Statistical significance was defined as $p < 0.05$. The relationship between PTE content in plant and soil fractions was evaluated by simple and stepwise regression analysis.

3. Results

3.1. PTE concentration in soil fractions

Physicochemical properties for soils and sludge are summarized in Tables 1 and 2. Total soil concentration and distribution of Cd, Cu, Pb and Zn among soil fractions in controls and in SS or AS amended soils at day 60 is shown in Table 3.

3.2. Dry matter yield

The germination of *L. perenne* in amended soils was delayed for 15 days. After that, *L. perenne* grew uniform in all sludge treatments along the growing period, showing no visible symptoms of metal

Table 1
Main physical and chemical characteristics of the three untreated soils (A horizon, 0–15 cm) used for pot experiment.

	Typic Hapludoll	Typic Natraquoll	Typic Argiudoll
Clay (%)	19.2	27.6	30.3
Silt (%)	23.2	43	53.6
Sand (%)	57.6	29.4	16.1
pH	5.12	6.21	5.72
Organic carbon (g kg^{-1})	28.6	35.31	24.5
Electrical conductivity (dS m^{-1})	0.61	1.18	0.7
Cation exchange capacity ($cmol_{(c)}$ kg^{-1})	20.3	22.3	24.5
Exchangeable cations			
Ca^{2+} ($cmol_c$ kg^{-1})	10.2	9.1	12.6
Mg^{2+} ($cmol_c$ kg^{-1})	2	5.4	4.3
Na^{+} ($cmol_c$ kg^{-1})	0.3	2.1	0.2
K^{+} ($cmol_c$ kg^{-1})	2.8	1.6	2.1

Table 2
Selected properties of sewage sludge (SS) and 70:30 DMW mixture of sewage sludge and sewage sludge ash (AS).

	SS	SSA
pH	5.82	6.17
Moisture content (%)	5	4.5
Total organic carbon (mg g^{-1})	251	176
Total N (mg g^{-1})	19.3	22.5
Total P (mg g^{-1})	0.052	0.086
Electrical conductivity (dS m^{-1})	0.90	0.89
Cation exchange capacity ($cmol_c$ kg^{-1})	11.95	nd
Ca (mg g^{-1})	22.5	nd
Mg (mg g^{-1})	5.6	nd
K (mg g^{-1})	10.7	nd
Total Cd (mg kg^{-1})	10.08	13.08
Total Cu (mg kg^{-1})	750.8	894.7
Total Pb (mg kg^{-1})	334.2	365.9
Total Zn (mg kg^{-1})	2500	3200

nd = not determined.

toxicity or nutrient imbalances. Partial and total dry matter yields of *L perenne* grown in each treatment in the three soils are shown in Table 4. No significant differences ($p < 0.05$) in terms of aboveground biomass yields were observed between SS treatment, AS treatment and control in the first and second harvest. After that, plants grown in the amended soils exhibited a clear better growth compared to controls. Irrespective of soil amendment, dry matter yield at the end of the experimental period was, in certain cases, more than 300% as compared to control.

3.3. *PTE concentration in aerial plant tissues of L. perenne*

Potentially toxic elements concentration of the studied metals in the first and third harvest of *L. perenne* followed the order Zn ≫ Cu ≫ Cd, Pb in all treatments. Cadmium and lead concentrations were below detection limits in aerial part of *L. perenne*.

In the first harvest, Cu concentration in aerial biomass did not seem to depend on soil treatment, for no significant differences were observed between control and SS or AS treatments for the

Table 3
Sequential extraction of cadmium, copper lead and zinc (mg kg^{-1}) from pristine soils (C) and soils amended with sewage sludge (SS) and 70:30 DMW mixture of sewage sludge and sewage sludge ash (AS) (150 t ha^{-1}), before cultivation (day 60).

	Typic Hapludoll			Typic Natraquoll			Typic Argiudoll		
	C (mg kg^{-1})	SS (mg kg^{-1})	AS (mg kg^{-1})	C (mg kg^{-1})	SS (mg kg^{-1})	AS (mg kg^{-1})	C (mg kg^{-1})	SS (mg kg^{-1})	AS (mg kg^{-1})
EXCH-Cd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OM-Cd	nd	nd	nd	nd	nd	nd	nd	nd	nd
INOR-Cd	nd	nd	nd	nd	nd	nd	nd	nd	nd
RES-Cd	nd	0.37	0.39	nd	0.40	0.43	nd	0.39	0.45
EXCH-Cu	nd	nd	nd	nd	nd	nd	nd	nd	nd
OM-Cu	3.85 c	18.02 ab	17.68 ab	3.46 c	16.57 b	24.94 a	7.01 c	18.19 ab	23.61 ab
INOR-Cu	2.73 e	11.95 c	13.86 bc	3.35 e	16.04 ab	14.80 abc	6.56 d	16.35 ab	17.65 a
RES-Cu	15.43 bc	22.63 ab	32.26 a	4.19 d	11.64 c	13.71 c	2.43 d	12.41 c	16.89 bc
EXCH-Pb	nd	nd	nd	nd	nd	nd	nd	nd	nd
OM-Pb	nd	nd	nd	nd	nd	nd	nd	nd	nd
INOR-Pb	5.83 c	26.61 ab	23.62 ab	7.24 c	29.31 a	20.40 b	8.26 c	25.25 ab	23.49 ab
RES-Pb	12.16 cd	19.93 b	33.18 a	1.76 f	8.22 de	27.40 a	4.75 e	16.28 bc	28.31 a
EXCH-Zn	1.61 c	28.71 a	24.58 a	1.07 c	12.35 b	10.83 b	1.00 c	22.43 a	21.59 a
OM-Zn	2.62 c	9.61 ab	9.61 ab	1.75 c	13.10 ab	24.07 a	1.97 c	8.14 b	13.29 ab
INOR-Zn	3.90 c	71.83 b	93.18 ab	4.14 c	135.63 a	123.01 a	6.22 c	86.83 ab	103.51 ab
RES-Zn	46.87 bc	101.11 a	124.51 a	40.04 c	45.55 bc	85.96 ab	49.81 bc	97.86 ab	117.49 a

Soil fractions: water-soluble and exchangeable (EXCH), bound to organic matter (OM), inorganic precipitate (INOR) and residual (RES). Different letters in the same row are significantly different at the 0.05 probability level ($n = 4$).

Table 4
Partial and total mean values and standard deviation of aerial dry weight (in g) of *L. perenne* grown in control and sludge-treated pots over four harvests ($n = 3$, ±S.E.).

	Aerial dry weight (in g) of *L. perenne*/pot				Total DM yield
	1° Harvest	2° Harvest	3° Harvest	4° Harvest	
Hapludoll—C	2.43 ± 0.055 ab	2.66 ± 0.116 ab	3.01 ± 0.138 bc	1.73 ± 0.103 b	9.83 BC
Hapludoll—SS	2.45 ± 0.078 ab	3.01 ± 0.108 a	5.49 ± 0.149 a	4.38 ± 0.301 a	15.32 A
Hapludoll—AS	2,82 ± 0.105 ab	2.99 ± 0.067 a	5.82 ± 0.103 a	3.14 ± 0.072 ab	14.78 A
Natraquoll—C	1.97 ± 0.094 b	1.76 ± 0.133 b	1.11 ± 0.138 c	0.84 ± 0.068 c	5.68 C
Natraquoll—SS	2.22 ± 0.137 ab	2.45 ± 0.136 ab	4.99 ± 0.229 ab	4.65 ± 0.257 a	14.32 A
Natraquoll—AS	2.32 ± 0.128 ab	2.35 ± 0.095 ab	4.31 ± 0.308 ab	4.17 ± 0.446 a	13.15 AB
Argiudoll—C	2.51 ± 0.163 ab	1.96 ± 0.162 b	1.58 ± 0.161 c	0.78 ± 0.024 c	6.82 C
Argiudoll—SS	3.07 ± 0.070 a	3.28 ± 0.020 a	5.28 ± 0.354 a	4.50 ± 0.264 a	16.13 A
Argiudoll—AS	2.87 ± 0.134 ab	2.96 ± 0.054 a	4.74 ± 0.220 ab	3.33 ± 0.239 ab	13.90 AB

Soils: Typic Hapludoll, Argiudoll and Natraquoll. Treatments: C = control, SS = sewage sludge amended soils, AS = soils amended with the 70:30 DMW mixture of sewage sludge and sewage sludge ash. Groups in a column detected as different at the 0.05 probability level (Tukey test) were marked with different letters (a, b, c, etc. for partial harvest; A, B, C, etc. for total yield).

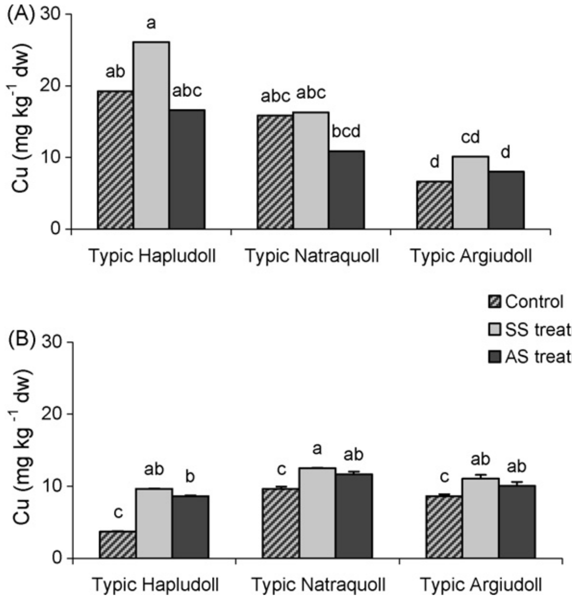

Fig. 1. Accumulation of Cu (mg kg^{-1} dw) in shoot of *Lolium perenne* L. grown in unamended and 150 dry t/ha SS or AS—amended soils (Mean ± SE) in the 1° harvest (A) and in the 3° harvest (B). Error bars show the standard deviations. Bars with different letters in each group show significant difference at $p < 0.05$ (Tukey).

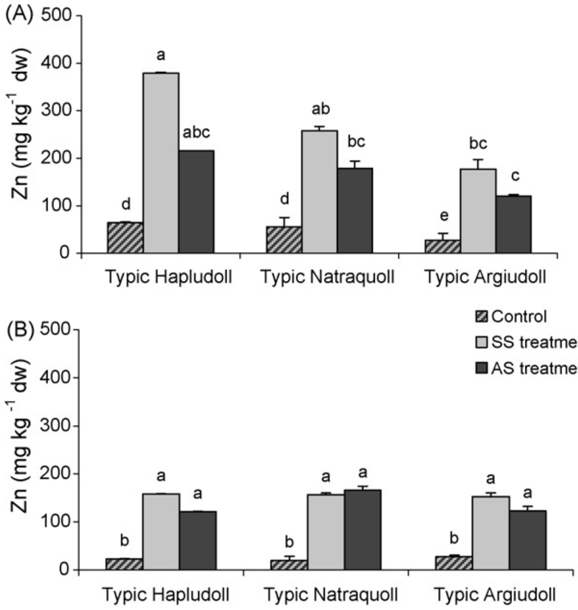

Fig. 2. Accumulation of Zn (mg kg^{-1} dw) in shoot of *Lolium perenne* L. grown in unamended and 150 dry t/ha SS or AS—amended soils (Mean ± SE) in the 1° harvest (A) and in the 3° harvest (B). Error bars show the standard deviations. Bars with different letters in each group show significant difference at $p < 0.05$ (Tukey).

same soil (Fig. 1A). In the third harvest, Cu concentration in aerial tissue was significantly higher in amended soils compared to controls, irrespective of soil type or amendment (Fig. 1B). No significant differences ($p < 0.05$) were observed among soils for the same treatment.

The addition of SS or AS amendments significantly increased Zn concentration in the aerial part of *L. perenne* compared to controls. In the first harvest, no significant differences ($p < 0.05$) in aerial biomass concentration of Zn between SS or AS treatments were observed for the same soil. The highest Zn concentration was detected in the aerial part of *L.perenne* grown on the Hapludol SS-amended soil (378.9 mg Zn kg^{-1} DMW), a significantly higher concentration compared to the SS-amended Argiudol soil (177.6 mg Zn kg^{-1} DMW). In the AS treatments, no significant differences ($p < 0.05$) among soils were observed (Fig. 2A). In the third harvest, plants sown in the three amended soils did not show any significant difference in terms of Zn concentration in aerial tissue, regardless soil or sludge amendment (Fig. 2B).

4. Discussion

Although the soils presented differences in particle size distribution, clay had the same origin and mineralogical composition [28]. Soils in the Pampas region are moderately acid, low in available P, and have high organic carbon content (29–46 g kg^{-1}). The region shows no signs of contamination with PTE, with concentrations and dispersion values of PTE similar to other non-contaminated soils of the world [29].

The contents of Cd, Cu, Pb and Zn in SS and AS did not exceed ceiling concentrations for land application recommended by Argentine regulation [30].

The growth of *L. perenne* in the amended soils was initially conditioned by the phytotoxic potential of SS or AS [31]. These results are in opposition with a previous phytotoxic assay on seed germination, in which no germination delay was observed among sludge treatments for this species [32]. This difference in results was probably due to the methodology used. In the phytotoxic assay, aqueous extracts of the sludge amended soils were prepared immediately after sludge application (day 0). In this assay, seeds were sown 60 days after sludge application. The delay in seed germination observed herein for both amendments may be the result of a combination of several factors. These factors include an increase in Zn availability over incubation time [33], the intense mineralization of the labile organic matter pool of the sludge [34], which may have originated ammonia, low molecular weight organic acids and/or salts, all of which have been shown to have inhibitory effects [35–37]. Other studies have also reported that this toxic effect is normally of an ephemeral nature, disappearing within 14–21 days [38]. Both sludge amendments resulted in an increase in plant aerial biomass during the experimental period compared with plants grown in unamended soils. Several factors may have contributed to improve growth in both sludge amended soils, especially the increased supply of N and P, in agreement with [39] and [40], together with an increasing limitation on nutrient supply in control soils with time. In addition, an improvement in the amended soils' physical and biological properties cannot be ruled out. Although AS had a significantly higher total PTE content than SS (Table 2), no significant differences in terms of total or partial dry matter yield were observed between both treatments for each soil.

The application of either SS or AS amendments had no effect on Cd concentration in the above ground tissue of *L. perenne* compared to control. Conversely, other authors found that application of sewage sludge to soil clearly resulted in elevated Cd concentrations in pasture plant species [41] and [42]. In our case, Cd was only extracted from the residual fraction (Table 3), considered inactive in terms of chemical processes.

Lead concentration in aerial biomass was below detection limits in all treatments, in agreement with soil sequential extraction. Moreover, Jones et al. [43] concluded that roots of actively growing *L. perenne* provided a barrier which restricted the movement of lead to the above-ground parts of plants. Possibly, the low Pb availability in the amended soils combined with a physiological restriction of translocation to shoots resulted in Pb concentrations below analytical detection limits in aerial biomass.

Copper and Zn concentration in shoots was below the range of critical concentration in plants described by Kabata-Pendias and

Table 5
Significant correlations between Cu and Zn contents in plant samples of *L. Perenne* and Cu or Zn contents in soil fractions or soil properties before cultivation.

			Adjusted r^2	F statistic	$P > F$
Cu	3° cut	3.14 + 0.37 OM-Cu	0.7565	81.78	0.0000
		2.34 + 0.54 INOR-Cu	0.8021	106.36	0.0000
		2.31 + 0.15 OM-Cu + 0.35 INOR-Cu	0.8252	129.977	0.0000
Zn	1° cut	64.84 + 7.19 EXCH-Zn	0.45	24.38	0.0000
		-212.16 + 7.68 EXCH-Zn + 13.70 CEC	0.62	45.3	0.0000
	3° cut	50.08 + 4.01 EXCH-Zn	0.48	24.52	0.0000
		50.41 + 5.71 OM-Zn	0.51	27.57	0.0000
		34.01 + 1.03 INOR-Zn	0.70	62.94	0.0000
		19.97 + 1.77 EXCH-Zn + 2.35 OM-Zn + 0.55 INOR-Zn	0.82	113.07	0.0000

Pendias [44]. Moreover, the concentration of these elements in aerial tissue was found to be under the threshold values specified by the National Research Council [45] suggesting that consumption of *L. perenne* grown on sludge amended soils would pose no risk to grazing animals. This issue is crucial in order to avoid the threat of transfer of metals to the food chain. Physiological mechanisms that regulate the internal translocation of PTE have been postulated for this species [46].

In the first harvest, the concentration of Cu in aerial biomass was within the range of 6.6–26.1 mg kg^{-1}. Although it is generally accepted that, at least in the short term the exchangeable fraction of a metal is the most mobile and consequently the most bioavailable phase present in soils [47,48], this fraction was below the detection threshold of AAS. It might be expected that Cu would show a great increase in the labile fraction over time due to organic carbon mineralization [34]. However, the largest proportion of Cu was initially found in the residual fraction in the three amended soil samples [49]. On the other hand, Cu concentration in the first harvest was significantly higher in the coarse textured soil compared to the fine textured soil. Several studies indicated that crops grown on sandy, low organic matter status soils are likely to have a greater uptake of certain PTE compared with crops grown on soils with higher clay and organic matter contents [50,51]. Other studies on Cu adsorption by individual soil components have indicated relatively strong bonding and high capacity of silicate minerals to adsorb Cu, whereas the amounts of Cu that can be readily desorbed is very small [52]. In our study, the higher concentration of clay in the Argiudol soil might have supplied more Cu-binding sites, reducing Cu availability. Furthermore, AS treatment did not increase Cu concentration in aerial biomass compared to SS treatment in the three soils, in good agreement with soil fractionation study (Table 3).

In the third harvest, mean Cu concentration in aerial biomass of all treatments was lower compared to the first harvest. In all cases, there was a positive correlation between Cu concentration in aerial biomass and OM-Cu and INOR-Cu obtained in the soil sequential extraction, alone or combined together through a stepwise regression (Table 5). It is usually considered that soil fractions are, ideally, indicative of the potential bioavailability of PTE, with phytoavailability decreasing in the order of the sequential extraction step. However, our findings indicate that plant available Cu in sludge amended soils came from organic and inorganic fractions, frequently considered of low availability [53]. Soil micro organisms are known to affect PTE availability, enhancing organic matter mineralization or through the release of chelating agents and phosphate solubilization [54–56]. Alternatively, the complexity of the soil–plant relationship may induce changes in the properties of the soil rhizosphere through changes in pH [57] or variations in redox potential [19,58], and consequentially induce changes in metal speciation [59]. Although we did not measure rhizosphere's pH, we observed an increase up to 0.2 units in the Hapludol sown with *L. perenne* compared with soil without plant (data not shown). However, metals are mobile under acidic conditions [57]. In this way, it appears that pH is unlikely to be the reason for copper mobilization from the inorganic fraction. We conclude that the positive correlation established between Cu concentration in *L. perenne* aerial biomass and INOR-Cu herein obtained by sequential extraction suggests that root-activity has induced INOR-Cu dissolution in sludge amended soils.

Zn was the PTE absorbed by *L. perenne* in the largest proportion from all three amended soils, showing the greatest degree of mobility as observed by Fuentes et al. [60] and Pueyo et al. [61]. In the first harvest, Zn concentration in aerial biomass was significantly higher in the coarse textured soil compared to the fine textured soil. Egiarte et al. [62] stated that sludge-borne Zn compounds are relatively highly soluble and that exchange reactions are the main way of retention for Zn in soils. The coarse textured soil (Hapludol) would most likely provide a low total surface area and therefore few sorption sites, which could promote Zn mobility compared to the fine textured soil [63]. Plant Zn concentration in the first harvest significantly correlated with EXCH-Zn measured in soils at day 60. The coefficient of determination (r^2) was calculated to be 0.49. This indicates that the model explained 49% of the variability in the data. When CEC was included in a stepwise regression, the model explained 64% of the variability in the data (Table 5). No correlation was observed between plant Zn concentration and soil pH of the three soils before cultivation, in spite that EXCH-Zn in sludge amended soils was largely dependent on soil pH [33]. Moreover, the Natraquol exhibited the highest pH before cultivation and its Zn contents in aerial biomass was in-between those of the Hapludol and the Argiudol soils, both with lower pH. The results obtained here indicate that, like Cu, Zn availability in the first harvest of sludge amended soils was higher in the coarse compared to the fine textured soil, in good agreement with the results reported by Canet et al. [64].

In the third harvest, a significant decrease of Zn in aerial biomass concentration compared to the first harvest was observed in the amended soils, irrespective the soil considered (Fig. 2B). The decrease in Zn concentration was probably originated by an initial depletion of available sludge-borne Zn. Nevertheless, Zn concentration in the above ground tissue of the amended soils was still significantly higher than controls, indicating a high Zn availability. It has been postulated that at very high application rates, the sludge matrix would begin to exert its influence on PTE binding [65]. In this way, sludge properties would predominate in PTE chemistry in the short amount of time, although these properties would have a smaller influence over longer periods of time, the control of the soil characteristics becoming stronger [66]. This hypothesis is partially supported by the results herein obtained for Zn. After a first depletion of sludge-available Zn, the uptake of this element might be determined by its binding to the sludge matter. These results may also indicate that there is a continuous equilibrium process happening in soil, causing the shift of sludge-borne Zn from one chemical species to another.

Linear correlation of data indicated that Zn uptake by *L. Perenne* in the third harvest was positively and linearly related to EXCH-Zn, OM-Zn and INOR-Zn in sludge-soil mixtures. When a step-wise multiple regression analysis was performed, the correlation was largely improved (Table 5). Analysis of the regression model revealed that INOR-Zn was the most significant parameter ($p \leq 0.0037$) followed by EXCH-Zn ($p \leq 0.0111$) and OM-Zn ($p \leq 0.0210$), with an r^2 value of 0.82, indicating that 82% of the variability was explained by the model. Accordingly, a large proportion of Zn could be absorbed by *L. perenne* from the inorganic fraction in sludge amended soils, indicating that this fraction is a potential reservoir for plant-available Zn. EDTA solution in this fractionation study was assumed to extract principally carbonate-bound fractions of Zn by forming strong soluble complexes, and Zn included in Fe amorphous oxides [67]. Exchangeable Zn fractions were also easily absorbed by *L. perenne*, in agreement with other studies [68]. The significant correlation herein observed between Zn concentration in aereal tissue and organic Zn suggest that Zn existed in labile organic fractions in sludge amended soils, which were easily released by soil microorganisms and adsorbed by plants. This finding is in agreement with that obtained by He et al. [69] in maize plants. On the other hand, Hseu [70] found positive correlations between EXCH-Zn and INOR-Zn and plant uptake. Therefore, our results suggest that, after a first initial depletion of sludge-borne available Zn by *L. perenne*, a dynamic equilibrium among Zn fractions of different availability was established.

5. Conclusions

The results of the present study show that *L. perenne* induced changes in Cu and Zn bioavailability in sludge amended soils. Initially, Cu and Zn concentration in aerial tissue was higher in the coarse than in the fine textured soils. Conversely, soil chemical speciation did not exhibit significant differences between soils in Cu or Zn availability. In the third harvest, there was a positive correlation between Cu and Zn concentration in aerial biomass and soil fractions usually considered of low availability. Based on these results, *L. perenne* increased Cu and Zn availability through the mobilization of these metals from less available to more available fractions. In this way, the amounts of Cu and Zn in water-soluble or exchangeable fractions do not relate directly to the proportion taken up by *L. perenne*. Therefore, soil speciation does not seem appropriate for estimating plant bioavailability of some trace elements in sludge amended soils.

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