Copper and zinc bioavailabilities to ryegrass (*Lolium perenne* L.) and subterranean clover (*Trifolium subterraneum* L.) grown in biosolid treated Chilean soils

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ABSTRACT

The purpose of this study was assessing Cu and Zn availabilities in soils amended with a biosolid through the determination of their sequentially extracted chemical forms and their relationship with the contents of these metals in ryegrass (*Lolium perenne* L.) and subterranean clover (*Trifolium subterraneum* L.) plant tissues cultivated in a greenhouse using four soils classified as Aquic Xerochrepts and Ultic Haploxeralfs representatives of potential areas for biosolids application in the central zone of Chile. The soils were treated with sewage sludge at a rate of 0 and 30 $\mathrm{Mg\,ha^{-1}}$. The greenhouse experiment was carried out through a completely randomized block design in a 2 × 4 (biosolid rate × soil) arrangement, considering three repetitions per treatment. The soils used in the greenhouse experiment before and after cultivation, were sequentially extracted with specific reagents and conditions in order to obtain the following fractions: exchangeable, sodium acetate-soluble, soluble in moderately reducing condition, $K_4P_2O_7$-soluble, soluble in reducing condition, and soluble in strongly acid and oxidizing condition. It was established that Cu and Zn were predominantly found in soils in less available forms, associated to organic matter, oxides and clay minerals. Zinc concentration in ryegrass plants was higher than that found in subterranean clover plants in biosolid-amended soils. Zinc contents in ryegrass shoot and root correlated with the exchangeable, bound-to-carbonate, and bound-to-FeOx metal forms in control soil. Copper and Zn bioavailabilities were estimated through satisfactorily fitted multiple linear regression models, with determination coefficients from 0.77 to 0.99, which showed a positive contribution of the labile metal forms in soils, especially in relation to Zn in both plant species.

1. Introduction

The number of wastewater treatment plants has increased considerably in Chile in the last decade and biosolid disposal constitutes an environmental problem. Biosolid application to agricultural land is an economically and environmentally acceptable form of biosolid disposal. Biosolid applications supply plant nutrients, especially N and P (Mantovi et al., 2005) which are required for plant growth enhancing as well as soil organic matter water holding capacity, improving soil structure, aggregation, and water infiltration (Artiola, 1996; Oberle and Keeney, 1994). Continued sludge application to soils leads to plant heavy metal accumulation, being the main path through which heavy metals may enter the trophic chain (Qian et al., 1996). This fact has compelled different organizations to establish limits on the amount and frequency of biosolid application to soil (Tsadilas et al., 1995). In Chile, the regulation sets maximum allowable rates of 30 $\mathrm{Mg\,ha^{-1}}$ per year in productive soils with severe limitations for its natural fruit production aptitude and/or forestry soils and 50 $\mathrm{Mg\,ha^{-1}}$ per year in degraded soils. On the other hand regulations also establish soil and biosolids concentration limits for the following eight elements: As, Cd, Cu, Hg, Ni, Pb, Se and Zn (DS 123, Chile, 2006).

Heavy metals are associated to several soil components, giving rise to chemical forms to determine their mobility and availability (Ahumada et al., 1999). These metal forms are: soluble, exchangeable, complexed or adsorbed on organic matter, adsorbed or occluded in oxides, associated to clay minerals and to soil primary minerals. The exchangeable and carbonate metal forms are considered readily mobile and available to plants, while metals incorporated in clay crystalline lattices seem to be relatively inert. The other metal forms, associated to Fe, Al and Mn oxides, or bound

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with organic matter, could be considered potentially active or strongly bound, depending on the physical properties of soil (Kabala and Singh, 2001; Ahumada et al., 2004; Arain et al., 2008; Jamali et al., 2006; Jamali et al., 2007a; 2007b).

Total heavy metal content is useful to estimate soil contamination, but it is a poor indicator for bioavailability (Fernández et al., 2000). In this context, it is more convenient to identify the chemical forms in which heavy metals are retained in soil, since they could predict metal availability to plant and the potential metal mobility through the soil profile. Sequential extraction procedures constitute a valid tool to determine the various chemical forms of heavy metals in soil. Metals may be selectively extracted using appropriate chemical reactants so that different heavy metal fractions may be released from the soil components once the binding phase is destroyed (Yong et al., 1993). Although there are a great variety of sequential extraction methods, all of them must handle the problem of choosing the appropriate extracting reactants that will attack only the interest fraction and liberating only the specific heavy metal from it (Filgueiras et al., 2002). In this sense, the accuracy of methods has been questioned, since the lack of an absolutely selective reactant causes selectivity and readsorption problems, affecting analytical results (Howard and Shu, 1996; Bacon and Davidson, 2008).

Metal availability is a chemical form function in which it is found in soil. Plant may change metal availability directly (uptake) and indirectly by different mechanisms such as exudation of complexing agents, respiration roots, which accounts for pH changes, etc. (Hammer and Keller, 2002).

The purpose of this research was assessing the Zn and Cu availabilities in Chilean soils amended with stabilized sewage sludge, or biosolid, through the heavy-metal chemical forms sequentially extracted determination, and establishing their relationship with the contents of these metals in ryegrass (*Lolium perenne* L.) and subterranean clover (*Trifolium subterraneum* L.) plant tissues cultivated in greenhouse.

2. Materials and methods

2.1. Soils and biosolid

Four composite soil samples of surface soil (0–20 cm) were collected from an agricultural zone in central Chile where biosolids could be applied. The soils were two Inceptisols (Aquic Xerochrepts) and two Alfisols (Ultic Haploxerals), named as ICP1, ICP2, ALF1 and ALF2 respectively. Each soil sample was air-dried and sieved through a polyethylene sieve (2 mm-mesh for the chemical characterization and 6 mm mesh for the greenhouse experiments) stored in plastic jars at room temperature. A sample of anaerobically stabilized sludge or biosolid was collected from a monofill at a Santiago sewage treatment plant. This biosolid contained a 7% of water and it was sieved as described above for soils. The biosolid, soil, and soil treated with biosolid, were characterized for pH in water in a 1:2.5 soil-to-water ratio, organic matter was determined by the spectrophotometric method (Sadzawka et al., 2006), and cation exchange capacity (CEC) by the acetate procedure at pH 7. Total metal concentration was determined after acid digestion (4 mL HNO_3, 4 mL HF, and 2 mL H_2O_2) of 200 mg soil sample, using a Milestone/mls Mega microwave oven. The resulting solution was taken to a final volume of 10 mL with 1 M HCl. Metals were determined by flame atomic absorption spectroscopy (F-AAS) using an 1100 B Perkin–Elmer atomic absorption spectrophotometer.

2.2. Greenhouse experiment

Pots containing the equivalent to 2 kg of dried soil were treated with biosolid in equivalent quantities to 0 and 30 Mg ha^{-1}, irrigated to field capacity and let to stand for 30 days before sowing with ryegrass (*L. perenne* L.) and clover (*T. subterraneum* L). The plants did not receive any additional fertilizer. In the experiment with ryegrass 50 seeds were sowed and for subterranean clover 20 seeds (inoculated with *Rhizobium leguminosarun* cv trofolii half a day before sowing) were sowed. After emergence, the ryegrass plants were reduced to 30 and the clover plants to six after the first leave emergence. The irrigation of pots was controlled by weight to 2/3 of the water holding capacity (33 kPa) of each soil. The ryegrass was cut every 30 days three times. The subterranean clover plants were cut 80 days after sowing, when they were flowering and setting the seeds. After cutting the shoots, the roots were separated from soil by soaking the pots one night and washing out the soil with tap water. Shoot and roots were washed with distilled water and dried separately at 60 °C. The dry samples' replicates of shoots and roots of each plant were mixed, ground and homogenized to obtain composite samples for each treatment.

The greenhouse experiment was carried out in a completely randomized block design 2×4 (biosolid rate × soil) for comparing the effect of biosolid rate and soil type in both plants, considering three repetitions per treatment. The plants tissues were analyzed by digesting 500 mg shoot and 200 mg root, with a mixture of 2 mL H_2O_2 and 4 mL HNO_3 in a microwave oven. The resulting solution was taken to a final volume of 20 mL with Milli-Q water. Both metals were determined by ICP-mass spectrometry (ICP-mass Fisons VG Plasmaquad).

2.3. Soil sequential extraction

The following procedure described by Howard and Shu (1996) was used. In a 50 mL centrifuge tube, 3 g soil or biosolid sample, previously ground in an agate mortar, was weighed in triplicate, the proper reactant to each stage was added (20 mL), then it was shaken for the corresponding time period, followed by centrifugation to separate the supernatant, then continue on to the next step; supernatant metal concentration in each step was determined by flame AAS. This method includes the addition of the equivalent to 200 mg L^{-1} of NTA (nitriloacetic acid) to each extractant for preventing metal readsorption and increasing selectivity. Metal in each sample was sequentially extracted according to the following procedure:

Step 1, exchangeable fraction (Exch-F): each sample was shaken for 30 min at 25 °C with 20 mL of 1 M $MgCl_2$ and centrifuged at 4000 rpm for 20 min.

Step 2, carbonate-bound metal fraction (Carb-F): the residue from step 1 was shaken at 25 °C for 5 h with 20 mL of 1 M CH_3COONa adjusted to pH5 with CH_3COOH, centrifuged as above.

Step 3, manganese oxide-bound metal fraction (MnOx-F): twenty millimeters of 0.1 M $NH_2OH \cdot HCl$ in 0.01 M HNO_3 were added to the residue from Step 2. The mixture was shaken at 20 °C for 30 min, and centrifuged as described above.

Step 4, organic matter-bound metal fraction (OM-F): the residue from Step 3 was shaken at 20 °C for 24 h with 20 mL of 0.1 M $K_4P_2O_7$ and then processed as described above.

Step 5, iron oxide-bound metal fraction (FeOx-F): the residue from Step 4 was shaken at 20 °C for 24 h with 20 mL of 1 M $NH_2OH \cdot HCl + 25\%$ (v/v) HOAc (acetic acid). Heated with occasional stirring in a water bath; for 4 h at 85 ± 3 °C and then processed as described above.

Step 6, residual fraction (R-F): the residue from step 5 was dried at 30 °C, and 200 mg of the dry residue were weighed, and subjected to the same procedure as for total metal. In another portion of the dry residue, water content was determined at 105 °C. The supernatant was filtered after each step and then, the tube weight was recorded for correcting volume and metal remaining amount from the previous step.

2.4. Statistical analysis

The results were statistically treated through analysis of variance (ANOVA), where significant *F* values were obtained. Differences between individual means of metals' fractionation experiments were tested using the Tukey-HSD test, mostly for assessing the effect of biosolid application. Multiple correlation test was used to assess relationship between Cu and Zn fractionation in soils and plant metal contents. Both tests were carried out with the Statgraphic 5.0 software.

3. Results and discussion

Table 1 shows some general properties of biosolid, unamended (control) soils, and soils amended with 30 Mg ha^{-1} of biosolid. The biosolid exhibited a high content of organic matter, a high CEC, and a pH nearly neutral, higher than soil pH. Concerning heavy metals in this biosolid, Zn occurred at the highest concentration, followed by Cu, Cr, Ni, and Pb. McLaren and Clucas (2001) found values in the same order of magnitude in a similar biosolid with respect to Zn, Cu and Ni. The total content of the elements under study was lower than the recommended limit values in biosolid, as stated by Chilean regulations. The soils under study were acidic and showed pH values between 5.5 and 6.3. Alfisols (ALF1 and ALF2) soils showed lower pH and CEC values than Inceptisols (INC1 and INC2). Soils exhibited similar organic matter (OM) content values from 2.1 to 2.3%, which increased when amended with biosolid due to the biosolid contribution. As regards to heavy-metal contents (Table 1), in Inceptisol soil samples, exhibited the order: Cr > Zn > Cu > Ni > Pb and Alfisol soil samples presented much lower values except for Pb, being Zn > Cr. Inceptisol soils (INC1 and ICP2) showed a higher total content of copper and zinc than Alfisols, following the same concentration order for both metals: INC1 > INC2 > ALF1 > ALF2.

3.1. Copper

Total copper content did not exceed the maximum limit allowed by Chilean regulation (75 mg kg^{-1}) for soils before biosolid application. Biosolid incorporation to soil excepting INC1 soil caused a total Cu content increase in all soils (Table 1).

Table 1
Selected properties and heavy metals content of control soils and soils amended with 30 Mg ha^{-1} of biosolid before cultivation

Soil and treatment	OM (%)	pH (H_2O) 1:2.5	CEC (cmol kg^{-1})	Total metal content (mg kg^{-1})				
				Cr	Ni	Cu	Zn	Pb
Biosolid	37[a] ± 5[b]	6.7	58 ± 5	189 ± 10	70 ± 4	331 ± 10	1182 ± 52	62 ± 6
INC1								
Control	2.3 ± 0.2	6.3	19 ± 2	133 ± 8	48 ± 2	53 ± 2	106 ± 3	22 ± 1
Amended	2.7 ± 0.1	6.6	19 ± 2	136 ± 2	49 ± 2	54 ± 2	112 ± 4	23 ± 2
INC2								
Control	2.2 ± 0.2	5.9	16 ± 1	112 ± 6	34 ± 4	36 ± 1	69 ± 5	17 ± 3
Amended	2.9 ± 0.1	6.2	17 ± 2	120 ± 4	40 ± 3	47 ± 1	97 ± 5	17 ± 2
ALF1								
Control	2.1 ± 0.1	5.5	11 ± 1	50 ± 8	23 ± 1	13 ± 1	55 ± 2	20 ± 2
Amended	2.3 ± 0.1	5.7	13 ± 2	50 ± 4	23 ± 4	17 ± 4	72 ± 3	20 ± 3
ALF2								
Control	2.2 ± 0.1	5.7	10 ± 2	23 ± 4	13 ± 1	12 ± 1	49 ± 2	14 ± 1
Amended	2.5 ± 0.1	6.2	10 ± 2	23 ± 6	14 ± 2	16 ± 2	66 ± 9	15 ± 2

[a] Mean.
[b] Standard deviation.

Table 2 shows Cu fractionation. This metal was mainly found in its most inert form, so that about a 70% of total content in soils INC1 and INC2 and about a 50% in soils ALF1 and ALF2 is extracted in the last step of sequential extraction. This metal was similarly distributed in all soils, following the order: *Residual-F* > *FeOx-F* > *OM-*F > *Carb-F* > *Exch-F* > *MnOx-F.* Biosolid application significantly increased Cu concentration in the OM- and *FeOx*-fractions.

In general, sludge application in ryegrass – and subterranean clover – cultivated soils increased the exchangeable and bound-to-carbonate Cu fractions (Table 2). These labile forms constitute about a 20% of total Cu extracted, which would indicate that this metal has a high probability of being absorbed by plant, thus entering the trophic chain and constituting a risk for human health (Ahumada et al., 1999). The metal recovery sequentially extracted was always between 89 and 107% of total metal.

3.2. Zinc

Total zinc contents in all soils are within the maximum allowed limit (175 mg kg^{-1}) by Chilean regulations before biosolid application. It was found that biosolid incorporation caused remarkable zinc enrichment in soils due to the high Zn content found in this biosolid (Table 1). Similar results were found by Mbila et al. (2001) in soils repeatedly amended with sewage sludge, where Zn and Cu enrichments were distinctly observed, but not so in the case of Pb and Ni.

Table 3 shows Zn fractionation. In all soils the residual was predominant, accounting for about a 70% of total Zn content in Inceptisols and about a 40% in Alfisols. In INC2, ALF1 and ALF2, Zn followed the same distribution order: *Residual-F* > *FeOx-F* > *Exch-F* > *Carb-F* > *OM-F* > *MnOx-F.* INC1 soil followed the same distribution, but the values for both bound-to-carbonate and exchangeable-Zn fractions were similar. Biosolid incorporation to soils caused a significant increase in most of the Zn fractions in all soils, mainly in the exchangeable and bound-to-carbonate-Zn fractions. In cultivated soils, total Zn content sequentially extracted decreased compared to soils before cultivation; this was mainly observed in biosolid-amended soils. Small changes were observed after cultivation in unamended soils. Biosolid-amended soils showed a significant decrease in Zn contents in the first two fractions after clover cultivation and in the first three fractions in ryegrass cultivated soils. This effect could be caused by Zn uptake by plants, since it was found that about a 15% of total Zn was in easily available forms (exchangeable and bound-to-carbonate fractions), which would account for this metal high content in shoot and root tissues in both plant species, however, no toxicity effects were observed. Similar results were found by Zheljazkov and Warman (2004) in their study to assess the bioavailability of several metals in soils treated with composted biosolid with high metals content. Metal recovery sequentially extracted was always between 92 and 119% of total metal.

3.3. Mobility factor (MF)

Heavy metals mobility in soils may be analyzed on the basis of absolute and relative contents of labile forms in which the metal occurs in soil.

Metal relative mobility index was calculated as a mobility factor (MF) (Narwal et al., 1999), which is equal to the ratio of the sum of labile fractions (exchangeable and bound-to-carbonate) to the sum of all fractions, the result being multiplied by 100 to express the value as a percentage. Table 4 shows in general higher MF values for Cu compared to Zn before and after biosolid application. High MF factors have been interpreted as symptoms of relatively high lability and bioavailability of these metals in soils (Ma and Rao, 1997). In

Table 2

Copper sequential extraction in INC1, INC2, ALF1 and ALF2 soils amended with 0 and 30 Mg ha^{-1} of biosolid, before and after cultivation with ryegrass and subterranean clover.

Sample	Cu–exchang.		Cu–CO_3		Cu–MnOx		Cu–OM		Cu–FeOx		Cu–residual		TMSE[b]
	mg kg^{-1}	RSD[a]	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}
Before cultivation													
INC1 0	1.7	*1.3 e*[c]	2.5	*2.8 e*	nd		3.4	*2.5 d*	6.3	*2.9 f*	36	*3.1e*	50
INC1 30	1.7	*0.5 e*	2.6	*7.2 e*	0.6		4.1	*1.3 e*	8.3	*4.8 g*	37	*1.9 e*	54
INC2 0	0.82	*1.8 bc*	1.8	*3.7 d*	nd		2.6	*1.7 c*	4.3	*2.4 cd*	23	*1.5 b*	32
INC2 30	1.1	*0.2 d*	1.6	*1.6 c*	0.5		3.4	*6.6 d*	5.9	*4.7 e*	29	*1.9 c*	42
ALF1 0	0.66	*2.5 ab*	1.5	*5.1 c*	nd		1.8	*1.6 a*	3.6	*7.5 b*	5.3	*1.0 a*	13
ALF1 30	1.1	*0.5 d*	1.2	*4.0 ab*	nd		2.4	*6.4 bc*	4.6	*5.3 d*	8.3	*3.3 b*	18
ALF2 0	0.53	*0.2 a*	1.3	*3.4 b*	nd		1.8	*6.7 a*	2.7	*8.1 a*	6.5	*2.1 ab*	13
ALF2 30	0.98	*0.3 cd*	1.1	*4.4 a*	0.6		2.3	*6.6 b*	4.0	*3.9 bc*	7.5	*12 ab*	16
Ryegrass cultivated soils													
INC1 0	3.6	*0.1 f*	2.9	*0.2 e*	0.6	*2.7*	3.1	*1.3 d*	6.7	*0.1 e*	34	*1.1 e*	51
INC1 30	2.9	*2.9 c*	3.0	*1.3 e*	nd		3.8	*1.0 f*	7.9	*6.3 f*	32	*0.4 d*	50
INC2 0	2.9	*3 c*	2.7	*6.3 d*	nd		2.6	*1.6 d*	4.7	*1.9 c*	29	*5.8 c*	42
INC2 30	3.2	*1.7 de*	3.4	*0.2 f*	0.6	*2.8*	3.1	*2.8 e*	6.1	*0.9 d*	29	*2.7 c*	45
ALF1 0	1.8	*4.1 a*	1.6	*1.6 a*	nd		1.6	*3.3 a*	3.2	*4 a*	10	*1.8 b*	18
ALF1 30	3.2	*7.4 e*	2.5	*1.1 c*	nd		2.3	*0.5 c*	4.3	*3.6 c*	10	*0.5 b*	22
ALF2 0	2.9	*0.4 cd*	2.3	*1.2 b*	nd		1.8	*0.5 b*	2.8	*8.3 a*	7.2	*11 a*	17
ALF2 30	2.1	*3.2 b*	2.2	*0.7 b*	nd		2.3	*3.5 c*	3.8	*6.5 b*	6.6	*5.6 a*	17
Subterranean clover cultivated soils													
INC1 0	3.6	*4.0 f*	3.1	*2.4 d*	nd		2.9	*0.7 d*	7.2	*11 d*	32	*0.7 e*	49
INC1 30	4.1	*0.9 g*	3.8	*2.5 e*	0.5	*3.1*	3.5	*2.6 e*	7.7	*5.1 d*	35	*0.4 f*	55
INC2 0	2.4	*0.8 b*	2.2	*1.4 b*	nd		2.4	*0.9 b*	4.4	*0.5 c*	24	*5.2 d*	35
INC2 30	3.2	*0.6 e*	3.1	*2.5 d*	nd		2.6	*4.1 bc*	4.7	*1.5 c*	22	*4.5 c*	36
ALF1 0	2.1	*7.7 a*	1.6	*6.2 a*	nd		1.6	*6.3 a*	3.2	*2.4 a*	8.0	*2.3 a*	16
ALF1 30	2.8	*2.4 d*	2.4	*1.0 c*	nd		2.6	*0.3 c*	4.2	*9.5 bc*	12	*3.2 b*	24
ALF2 0	2.6	*1.4 c*	2.2	*3.3 b*	nd		2.6	*4.7 bc*	3.4	*15 ab*	7.1	*3.2 a*	18
ALF2 30	2.7	*2.4 cd*	2.5	*6.2 c*	nd		2.6	*8.1 c*	4.4	*3.6 c*	7.2	*1.3 a*	19

[a] RSD : Relative standard deviation (%).

[b] TMSE: Total metal sequentially extracted.

[c] Comparisons between means were made with the Tukey-HSD test. Means followed by the same letter within each column for each treatment are not significantly different at ($P \geq 0.05$).

Table 3

Zinc sequential extraction in INC1, INC2, ALF1 and ALF2 soils amended with 0 and 30 Mg ha^{-1} of biosolid, before and after cultivation with ryegrass and subterranean clover.

Sample	Zn-exchang.		Zn–CO_3		Zn–MnOx		Zn–OM		Zn–FeOx		Zn–Residual		TMSE[b]
	mg kg^{-1}	RSD[a]	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	mg kg^{-1}	RSD	
Before cultivation													
INC1 0	1.3	*10 a*[c]	1.4	*11 a*	0.54	*2 c*	1.2	*7 c*	9.7	*7 b*	101	*0.2 f*	115
INC1 30	9.6	*5 c*	8.8	*2 d*	1.8	*5 e*	3.3	*9 e*	9.5	*6 b*	82	*9 c*	115
INC2 0	1.5	*8 a*	1.1	*5 d*	0.32	*9 ab*	1.0	*2 bc*	6.7	*0.2 a*	55	*0.2 e*	66
INC2 30	7.5	*8 b*	7.7	*1 e*	1.0	*6 d*	3.0	*7 e*	7.0	*7 a*	71	*0.6 d*	97
ALF1 0	1.2	*6 a*	0.72	*6 a*	0.3	*6 ab*	0.7	*1 a*	14	*6 c*	36	*5 a*	53
ALF1 30	7.1	*5 b*	4.6	*5 b*	0.16	*0.2 a*	1.3	*9 c*	14	*8 c*	47	*6 abc*	74
ALF2 0	1.6	*2 a*	0.93	*3 a*	0.34	*4 b*	1.0	*5 b*	9.1	*7 b*	35	*2 ab*	48
ALF2 30	9.2	*0. 8c*	5.3	*4 b*	0.45	*1 bc*	1.6	*9 d*	7.9	*0.2 ab*	44	*6 bc*	68
Ryegrass cultivated soils													
INC1 0	1.6	*0.8 bc*	0.8	*2 a*	0.5	*7 b*	0.9	*7 c*	8.1	*2 cd*	60	*2 c*	72
INC1 30	5.3	*0.3 d*	2.7	*2 b*	0.8	*4 c*	1.5	*9 e*	10	*0.1 f*	69	*3 c*	89
INC2 0	1.4	*2 ab*	0.7	*6 a*	0.3	*2 a*	0.7	*2 bc*	4.7	*3 a*	43	*8 b*	51
INC2 30	6.7	*2 f*	3.3	*10 c*	0.9	*14 c*	1.9	*7 e*	5.3	*4 ab*	47	*3 b*	65
ALF1 0	1.1	*0.8 a*	0.4	*11 a*	0.2	*3 a*	0.4	*1 a*	9.1	*4 de*	23	*2 a*	34
ALF1 30	6.1	*3 e*	2.4	*10 b*	0.4	*4 b*	1.1	*9 c*	14	*0.2 g*	29	*2 a*	53
ALF2 0	2.1	*2 c*	0.7	*0.2 a*	0.3	*8 a*	0.6	*5 b*	6.4	*2 bc*	25	*8 a*	35
ALF2 30	7.4	*7 g*	3.4	*2 c*	0.9	*8 c*	2.3	*9 d*	8.6	*5 de*	23	*2 a*	46
Subterranean clover cultivated soils													
INC1 0	1.8	*3 a*	0.9	*4 a*	0.5	*6 ab*	1.3	*0.2 b*	6.8	*6 b*	88	*5 c*	99
INC1 30	7.2	*6 e*	5.3	*3 d*	1.1	*13 d*	2.8	*8 f*	12	*4 d*	77	*2 d*	105
INC2 0	1.4	*2 a*	0.7	*0.2 a*	0.3	*6 a*	0.8	*8 b*	4.6	*4 a*	63	*0.2 b*	71
INC2 30	5.3	*0.9 d*	2.5	*2 b*	0.6	*2 bc*	1.6	*0.5 cd*	6.7	*3 b*	66	*6 b*	83
ALF1 0	1.6	*3 a*	1.5	*6 a*	0.3	*2 a*	0.4	*2 a*	11	*2 c*	39	*7 a*	54
ALF1 30	4.8	*4 c*	2.5	*2 b*	0.8	*9 c*	1.9	*7 de*	12	*6 d*	42	*6 a*	64
ALF2 0	2.7	*4 b*	1.3	*7 a*	0.6	*7 bc*	1.4	*12 b*	6.3	*12 b*	43	*12 a*	55
ALF2 30	7.9	*0.5 f*	4.4	*2 c*	1.0	*2 d*	2.1	*12 e*	7.2	*0.6 b*	45	*8 a*	68

[a] RSD: Relative standard deviation (%).

[b] TMSE: Total metal sequentially extracted.

[c] Comparisons between means were made with the Tukey-HSD test. Means followed by the same letter within each column for each treatment are not significantly different at ($P \geq 0.05$).

Table 4
Mobility factor (MF) of Cu and Zn in control soil (0) and amended soils (30 Mg ha^{-1} of biosolid), before (B) and after (A) cultivation (C) with ryegrass (R) and subterranean clover (SC).

Suelo	Treatments					
	BC-0	BC-30	ACR-0	ACR-30	ACSC-0	ACSC-30
Mobility factors of Zn						
ICP1	2.3	16.0	3.3	9.0	2.7	12.0
ICP2	4.3	15.0	4.1	15.0	3.0	9.4
ALF1	3.6	16.0	4.4	16.0	5.8	11.0
ALF2	5.4	21.0	7.7	24.0	7.2	18.0
Mobility factors of Cu						
ICP1	8.3	7.9	13.0	12.0	14.0	14.0
ICP2	8.1	6.5	13.0	15.0	13.0	18.0
ALF1	17.0	13.0	19.0	26.0	22.0	22.0
ALF2	14.0	13.0	31.0	25.0	27.0	27.0

spite of the high MF values found for Cu, this metal mobility suffered almost no effect from sludge application except INC2 soil cultivated with clover with a 38.5% of increase in MF and ALF1 soil cultivated with ryegrass with a 36.8% of increase. For Zn, instead, an increase was observed in MF values in all soils, where ALF1 and ALF2 showed the highest increase. The mobility factor increase of this metal could be partly caused by the contribution of soluble organic matter from sludge, which would complex these metals, thus increasing their solubility (Giusquiani et al., 1992). Ryegrass and subterranean clover cultivations affected Cu predominantly, the MF values of which increased mainly with clover cultivation, ALF2 soil showing the highest MF value, twice as high as before cultivation. This cultivation effect on Cu mobility may be accounted by a rhizospheric environment that may partially change soil properties, producing a pH decrease, which involves a metal solubility increase and thus a metal mobility increase (Alloway, 1990).

3.4. *Copper and zinc contents in ryegrass and subterranean clover plants*

Fig. 1 shows metal contents in different parts of the plant. It may be observed that Zn and Cu accumulate and are retained predominantly in both plant species roots and only a small proportion of these metals can reach the shoot. Metal accumulation in roots may be due to a specific plant strategy for storing and inactivating accumulated toxic elements in root cell walls (Pascual et al., 2004). It was found that Cu and Zn differ in relation to plant uptake; Zn uptake is much higher in both plant species since this metal occurs at a higher concentration than Cu in both substrates and an important amount of copper occurs in labile forms.

Most copper accumulated in the root tissue, with about a threefold accumulation compared to the shoot. Similar results were obtained by Qian et al. (1996) in wheat, where Cu accumulated mostly in roots. Copper plant content varied depending on soil type and treatment. Thus, in ryegrass plants grown in INC1 and INC2 control soils, root metal contents were higher compared to plants grown in ALF1 and ALF2 soils. Biosolid incorporation to soil caused a remarkable copper increase in ryegrass root grown in the two Alfisols under study. Instead, this was not observed in subterranean clover plant roots, but a copper increase was found in subterranean clover grown shoots in all biosolid-amended soils. In relation to zinc, results showed that this occurred in plants at a higher concentration than Cu, which may be attributed to higher Zn soil contents. Like Cu, zinc was mostly distributed in the root of both plant species, where ryegrass showed a higher content than subterranean clover. Biosolid incorporation caused a zinc increase in shoot and root of both plant species, these results being coincident with those found in soils in relation to increase in the labile zinc fractions.

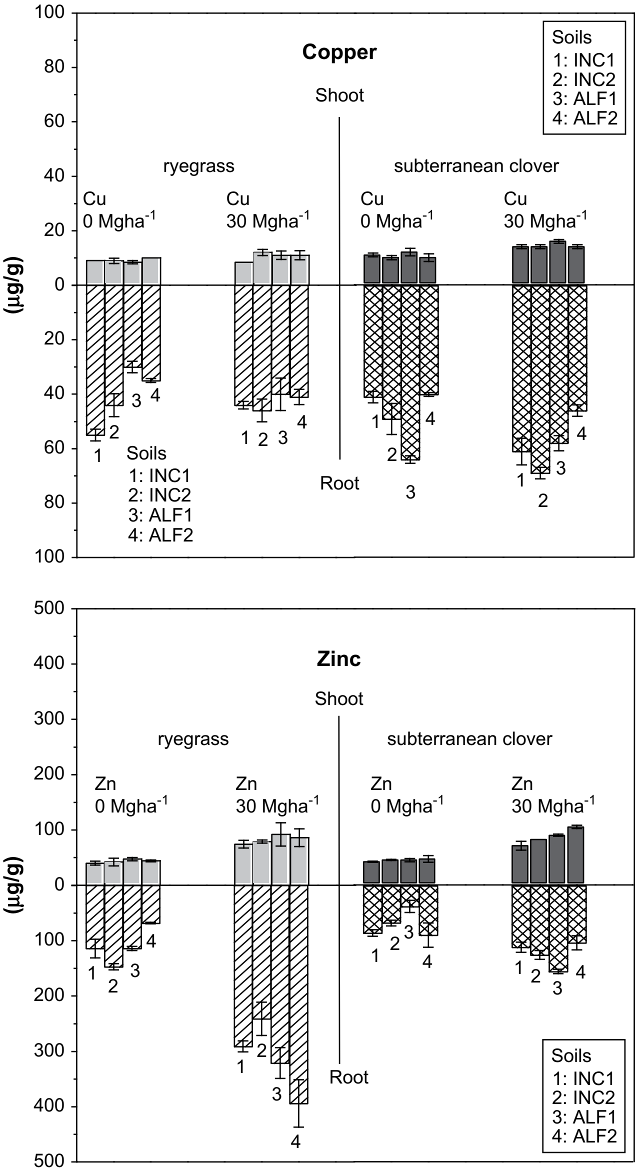

Fig. 1. Copper and zinc contents in shoots and roots of ryegrass and subterranean clover plants cultivated in soils treated with 0 and 30 Mg ha^{-1} of biosolid.

3.5. *Relationship between Cu and Zn fractionation in soils and plant metal contents*

A simple correlation analysis was performed between both variables for identifying a relationship between Zn and Cu plant uptake and chemical fractionation of these metals in soils, which gave statistically significant correlations only for ryegrass (results not shown) with R^2 values higher than 0.7. A statistically significant relationship was found between root Cu contents and OM-bound and FeOx-bound copper. For Zn, instead, a direct relationship was found between shoot and root metal contents and the exchangeable, bound-to-carbonate and bound-to-FeOx zinc fractions. For estimating Cu and Zn bioavailabilities, multiple linear regression models were satisfactorily fitted between these metals' contents in the plant as a dependent variable and these metals' chemical

Table 5
Multiple linear regression equation between copper and zinc contents in roots and shoots of ryegrass and subterranean clover plants and different chemical fractions of these metals in soils amended with 0 and 30 Mg ha^{-1} of biosolid.

Multiple linear regression equation	R^2[c]
Copper	
Cu R-shoot = 6.4 + 1.8 Exch-F	0.69
Cu R[a]-root = 13 + 22 OM-F + 0.38 R-F	0.96
Cu SC[b]-root = 94 − 25 OM-F + 3.1 FeOX-F	0.94
Soils amended with 30 Mg ha^{-1}	
Cu R-shoot = 62 − 5.9 Exch-F + 1.1 Carb-F + 2.0 MnOx-F + 0.65 OM-F − 0.90 FeOX-F − 0.27 R-F	0.99
Cu R-root = −34 + 14 MnOX-F	0.64
Cu SC-root = −49 + 50 Exch-F − 46 MnOX-F − 8.5 FeOX-F	0.96
Zinc	
Zn R-shoot = −280 − 208 Exch-F + 1466 Carb-F + 1547 MnOX-F + 26 FeOX-F	0.96
Zn R-root = −486 − 186 Exch-F + 1110 Carb-F − 2069 MnOX-F + 704 OM-F + 54 FeOX-F	0.97
Zn SC-shoot = 54 + 20 Exch-F − 31 OM-F − 2.5 FeOX-F	0.88
Zn SC-root = 127 − 46 Exch-F + 27 Carb-F	0.89
Soils amended with 30 Mg ha^{-1}	
Zn R-shoot = 258 + 12 Carb-F − 80 MnOX-F − 7.4 FeOX-F − 0.21 F6	0.96
Zn R-root = −218 + 172 OM-F + 25 FeOX-F	0.78
Zn SC-shoot = 110 + 7.7 Exch-F − 29 MnOX-F − 0.83 R-F	0.95
Zn SC-root = 188 + 9.4 Carb-F − 6.8 FeOX-F − 0.62 R-F	0.89

[a] Ryegrass.
[b] Subterranean clover.
[c] Determination coefficient.

fractions (*F*) in the soils as independent variables. Consistently significant multiple linear regression equations are shown in Table 5. The applied models explained metal content variation in both plant species from 69% to 99% for Cu and from 78% to 96% for Zn (Table 5). Independent variables positively contributing to Cu bioavailability in control soils are *OM-F* and *FeOx-F* for ryegrass and subterranean clover roots respectively and, for soils amended with biosolid, *MnOx-F* and *Exch-F* would contribute positively to the contents of this metal ryegrass and subterranean clover roots, respectively. Instead, positive contribution to copper content in ryegrass shoot would correspond to *Exch-F* in control soils and to *Carb-F*, *MnOx-F* and *OM-F* in biosolid treated soils. No significant correlation was found between Cu fractions in soils and metal contents in subterranean clover shoot. As to Zn, the same differences as those for Cu were found in regression models between control and biosolid-amended soils. Fractions contributing positively to the contents of this metal in ryegrass grown in control soils are *Carb-F*, *OM-F*, and *FeOx-F* in root and *Carb-F*, *MnOx-F*, and *FeOx-F* in shoot. In ryegrass grown in biosolid-treated soils the *OM-F* and *FeOx-F* contributed positively in root and only *Carb-F* in shoot. In the case of clover, fractions contributing positively to Zn uptake are *Carb-F* in root and *Exch-F* in shoot both in control and biosolid-treated soils. This finding is in agreement with that obtained by Hseu (2006) who observed that F1 + F2 in sequential extractions was reliable for predicting bioavailability to Chinese cabbages in biosolid-treated soils.

4. Conclusions

Total content of Zn, and Cu was higher in Inceptisol (ICP1 and ICP2) than in Alfisol (ALF1 and ALF2) soils. Copper and zinc concentrations' order in soils was ICP1 > ICP2 > ALF1 > ALF2. The sequential extraction procedure applied allowed establishing that Cu and Zn occur in soils predominantly in the less available forms, associated to iron oxide and residual fraction.

Biosolid application had different effects for both metals. In the case of Zn, biosolid application increased the concentration of the first four fractions. Meanwhile for Cu, the increase occurred in the bound-to-OM and bound-to-FeOx metal forms. Biosolid application increased the more available Zn fractions in all soils, in agreement with the Zn concentration increase for both plant species under study.

Ryegrass plants showed higher uptake of both metals, which concentrated mostly in the root of both species. Ryegrass zinc uptake was higher than that of clover in sludge-amended soils. Zinc concentration in ryegrass shoot and root correlated with the exchangeable, bound-to-carbonate, and bound-to-FeOx metal forms. Copper bioavailability was estimated through satisfactorily fitted multiple linear regression models, with determination coefficients from 0.77 to 0.99, with a positive contribution of the labile metal forms, especially in the case of Zn in both plant species. Ryegrass and subterranean clover would be adequate plant species for future greenhouse research oriented to investigate heavy metals plant uptake (bioavailability) due to biosolid application on different soil types.

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References

Ahumada, I., Escudero, P., Castillo, G., Carrasco, A., Ascar, L., Fuentes, E., 2004. Use of sequential extraction to assess the influence of sewage sludge amendment on metal mobility in Chilean soils. J. Environ. Monitor 6, 327–334.

Ahumada, I., Mendoza, J., Ascar, L., 1999. Sequential extraction in soil irrigated with waste waters. Commun. Soil Sci. Plant Anal. 30, 1507–1519.

Arain, M.B., Kazi, T.G., Jamali, M.K., Jalbani, N., Afridi, H.I., Shah, A., 2008. Total dissolved and bioavailable elements in water and sediment samples and their accumulation in *Oreochromis mossambicus* of polluted Manchar lake. Chemosphere 70, 1845–1856.

Alloway, B.J., 1990. Heavy Metals in Soils. John Wiley and Sons, Inc., New York, USA (pp. 13, 19–21).

Artiola, J.F., 1996. Waste disposal. In: Pepper, I.L., Gerba, C.P., Brusseau, M.L. (Eds.), Pollution Science. Academic Press, NY (Chapter 10).

Bacon, J.R., Davidson, C.M., 2008. Is there a future for sequential chemical extraction? Analyst 133, 25–46.

Decreto Supremo 123. Chile, Reglamento para el manejo de lodos generados en platas de tratamiento de aguas servidas, Ministerio Secretaría General de la presidencia de la República, Santiago 30 de agosto, 2006, 22 páginas.

Fernández, A., Pérez Cid, B., Fernández, E., Falqué, E., 2000. Comparison between sequential extraction procedures and single extractions for metal partitioning in sewage sludge samples. Analyst 125, 1353–1357.

Filgueiras, A.V., Lavilla, I., Bendicho, C., 2002. Chemical sequential extraction for metal partitioning in environmental solid samples. J. Environ. Monitor 4, 823–857.

Giusquiani, P.L., Gigliotti, G., Businelli, D., 1992. Mobility of heavy metals in urban 346 waste-amended soils. J. Environ. Qual. 21, 330–335.

Hammer, D., Keller, C., 2002. Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractants. J. Environ. Qual. 31, 1561–1569.

Howard, J.L., Shu, J., 1996. Sequential extraction analysis of heavy metals in sediments of variable composition using nitrilotriacetic acid to counteract resorption. Environ. Pollut. 91, 89–96.

Hseu, Z.Y., 2006. Extractability and bioavailability of zinc over time in three tropical soils incubated with biosolids. Chemosphere 63, 762–771.

Jamali, M.K., Kazi, T.G., Arain, M.B., Afridi, H.I., Jalbani, N., Adil, R.S.R., 2006. Correlation of total and extractable heavy metals from soil and domestic sewage sludge and their transfer to maize (*Zea mays* L.) plants. Toxicol. Environ. Chem. 88, 619–632.

Jamali, M.K., Kazi, T.G., Arain, M.B., Afridi, H.I., Jalbani, N., Memon, A.R., 2007. Heavy metal contents of vegetables grown in soil, irrigated with mixtures of wastewater and sewage sludge in Pakistan, using ultrasonic-assisted pseudo-digestion. J. Agron. Crop. Sci. 193, 218–228.

Jamali, M.K., Kazi, T.G., Arain, M.B., Afridi, 2007. Heavy metal from soil and domestic sewage sludge and their transfer to Sorghum plants. Environ. Chem. Lett. 5, 209–218.

Kabala, C., Singh, B., 2001. Fractionation and mobility of copper, lead, and zinc in soil profiles in the vicinity of a copper smelter. J. Environ. Qual. 30, 485–492.

Ma, L., Rao, G., 1997. Chemical fractionation of cadmiun, copper, nickel, and zinc in contaminated soils. J. Environ. Qual. 26, 259–264.

Mantovi, P., Baldoni, G., Toderi, G., 2005. Reuse of, liquid, dewatered, and composted sewage sludge on agricultural land: effects of long-term application on soil and crop. Water Res. 39, 289–296.

Mbila, M.O., Thompson, M.L., Mbagwu, J.S.C., Laird, D.A., 2001. Distribution and movement of sludge-derived trace metals in a Nigerian ultisol. J. Environ. Qual. 30, 1667–1674.

McLaren, R.G., Clucas, L.M., 2001. Fractionation of copper, nickel, and zinc in metal-spiked sewage sludge. J. Environ. Qual. 30, 1968–1975.

Narwal, R.P., Singh, B.R., Salbu, B., 1999. Association of cadmium, zinc, copper, and nickel with components in naturally heavy metal-rich soils studied by parallel and sequential extractions. Commun. Soil. Sci. Plant Anal. 30, 1209–1230.

Oberle, S.L., Keeney, D.R., 1994. Interactions of sewage sludge with soil-crop-water systems. Sewage Sludge: Land Utilization and the Environment. SSSA Misc. Publication. ASA, CSSA, SSSA, Inc, Madison, WI, USA (pp. 17–20, 258).

Pascual, I., Antolín, M.C., Sánchez-Díaz, M., 2004. Plant availability of heavy metals in a soil amended with a high dose of sewage sludge under drought conditions. Biol. Fertil. Soils 40, 291–299.

Qian, J., Wang, Z., Shan, X., Tu, Q., Wen, B., Chen, B., 1996. Evaluation of plant availability of soil trace metals by chemical fractionation and multiple regression analysis. Environ. Pollut. 91, 309–315.

Sadzawka, M.A., Carrasco, M.A., Grez, R., Mora, M., Flores, H., Neaman, A., 2006. Métodos de análisis recomendados para suelos de Chile, Serie Actas INIA No. 34, Chile.

Tsadilas, C.D., Matsi, T., Barbayiannis, N., Dimoyiannis, D., 1995. Influence of sewage sludge application on soil properties and on the distribution and availability of heavy metal fractions. Commun. Soil Sci. Plant Anal. 26, 2603–2619.

Yong, R.N., Galvez-Cloutier, R., Phadungchewit, Y., 1993. Selective sequential extraction analysis of heavy metal retention in soil. Can. Geo. J. 30, 834–847.

Zheljazkov, V.D., Warman, P., 2004. Source-separated municipal soil waste compost application to Swiss chard and basil. J. Environ. Qual. 33, 542–552.