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Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED_{50})

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Abstract

The ecological dose (ED_{50}) of Cd on alkaline and acid phosphatase activity and the ATP content of three contrasting forest soils was measured with or without Cu and Zn to assess the additive toxic effects of these two metals. Soils polluted with Cu and/or Zn were treated with increasing Cd concentrations to give the following metal combinations: Cd, Cd + Cu, Cd + Zn and Cd + Cu + Zn. Alkaline and acid phosphatase activities and ATP content of the three soils were analysed 4 h, 7 and 28 days after the metal additions. The ED_{50} values were obtained by interpolating the enzyme activities or ATP data with a kinetic model and the goodness of fit was satisfactory.

Generally, the ED_{50} values of both acid and alkaline phosphatase activities for Cd were lower (higher toxicity) with than without Cu and Zn and the effect of Cu and Zn was particularly adverse when these two metals were both added to soils. The alkaline phosphatase was more sensitive in the acid and neutral soil whereas the acid phosphatase was more sensitive in the alkaline soil. Both phosphatase activities and the ATP content were more sensitive in the sandy than in the finer textured soils. The ATP content was less sensitive to the additive effects. Increasing toxicity was observed during the incubation.

Analysis of 1 M NH_4NO_3-extractable Cd, Cu and Zn revealed that Cd competed with Zn for the adsorption sites but not with Cu. However, the lower ED_{50} values for Cd of the two phosphatase activities and of the ATP content in the presence of heavy metal combinations could be not explained by the heavy metal solubility data. It is concluded that the ED_{50} may be a sensitive tool for assessing additve toxic effects to soil biochemical parameters.
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Keywords: Soil phosphatase activity; Heavy metals; Ecological dose; Additive effects

1. Introduction

Soil enzyme activity is involved in nutrient cycling and availability to plants and can be used as an index of soil functioning (Nannipieri et al., 2003). The negative effects of heavy metals on soil enzyme activity have been recognised (Tyler, 1974; Deng and Tabatabai, 1995) and the urgent problem of the continuous enrichment of soil with heavy metals has also led to exploit the potential use of enzyme activities as biochemical indicators of soil pollution and soil quality (Nannipieri, 1995).

The use of dose-response curves for quantifying the effects of heavy metals on soil biochemical parameters was proposed by Babich et al. (1983). Several studies have been carried out on the effects of heavy metals on soil enzyme activities, in relation to soil properties such as pH, texture, organic matter content (Haanstra et al., 1985; Doelman and Haanstra, 1986; Haanstra and Doelman, 1991; Speir et al., 1995, 1999; Moreno et al., 2001). Doelman and Haanstra (1989) studying the phosphatase activity in function of Cd, Cu and Zn found very similar ED_{50} values regardless of the metal added.

Both Zn and Mg ions are activators whereas Cd, Cu and Hg ions act as inhibitors of pure phosphatases in vitro and the inhibiting effect of Cd can be suppressed by the addition of Zn (Blum and Schwedt, 1998). However, soil is a heterogeneous system in which the reactivity of colloids may interfere with the inhibition and/or activation effects of any compounds on enzyme activity (Nannipieri, 1995).

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Toxic effects of heavy metals on enzyme activity have been studied in soil by calculating the ED_{50} value (Haanstra et al., 1985; Doelman and Haanstra, 1986; Haanstra and Doelman, 1991; Speir et al., 1995, 1999; Moreno et al., 2001). Only single additions of each heavy metal have been studied and no information is available in the additive effects of more heavy metals. Among the heavy metals polluting soil, Cd is one of the most toxic, whereas Cu and Zn may be less toxic but generally are present in higher concentrations.

The aim of this work was to study the effects of Cd on the ED_{50} of acid and alkaline phosphatase activities and ATP content of three contrasting soils with or without Cu and/or Zn.

2. Materials and methods

Three unpolluted forest soils with contrasting pH and texture were used in this work (Table 1). All soils were sampled between October 2000 and January 2001 from the surface layer (0–10 cm). Moist soils were sieved (<2 mm) and incubated at 40% water holding capacity (WHC) and 25 °C for 7 days prior to use. Soils were treated with water, with Cu, Zn and Cu + Zn solutions, to give final metal concentrations of 140 and 300 mg kg^{-1} of soil, respectively. After this pretreatment the soils were left 1 h to equilibrate before the addition of Cd. Pre-treatments with Cu or Zn did not reduce the enzyme activities significantly (data not shown). The following metal combinations were investigated: no metals, Cd, Cd + Cu, Cd + Zn, Cd + Cu + Zn. Cadmium concentrations used for calculating the ED_{50} were: 0, 3, 10, 50, 300, 1000 and 5000 mg Cd kg^{-1} dry weight soil. All metals were added as sulphates and after the treatments the soil moisture was 55% WHC for all soils. Soils were incubated at 25 °C in the dark and after 4 h and 7 and 28 days they were analyzed for the acid and alkaline phosphatase activities (EC 3.1.3.2 and EC 3.1.3.1, respectively) according to Tabatabai and Bremner (1969), for the ATP content according to Ciardi and Nannipieri (1990), and for heavy metal availability by 1 M NH_4NO_3 according to Preuβ (1998). All treatments and measurements were replicated three times.

The kinetic approach proposed by Speir et al. (1995) based on the Michaelis–Menten hyperbolic model was used for calculating ED_{50} values for Cd of the acid and alkaline phosphatase activities and ATP content. The model describes a partial inhibition in which values never fall to zero but to an asymptote parallel but above the *x*-axis. This model is more suitable to study enzyme kinetics in an heterogeneous environment like soil than a model describing a full enzyme activity inhibition mechanism. Indeed, enzymes in soils can be physically and chemically protected by soil constituents (organic and inorganic ligands), which interact with heavy metals. Thus, inhibition by heavy metals can be lower in soil than in homogeneous solutions. A detailed description of the models used here to calculate the ecological dose (ED_{50}) values was given by Speir et al. (1995) and Moreno et al. (2001).

Significances of differences of the means ($n = 3$) were calculated by the Tukey–Kramer test at a significance level of 0.05 and calculations were done by computer packages (Statwiew5, SAS Institute).

3. Results

3.1. Heavy metal availability

Values of 1 M NH_4NO_3-extractable Cd, Cu and Zn in the three soils are reported in Tables 2–4. Generally, in the Vallombrosa acid soil Cd availability was significantly higher ($P < 0.05$) in the Cd + Zn and Cd + Cu + Zn treatments than in the Cd and Cd + Cu treatments, regradless of the incubation time (Table 2). Moreover, Cd availability decreased during the incubation in the 0–50 mg Cd kg^{-1} treatments whereas no temporal trends were observed for higher Cd concentrations (Table 2). Copper availability was always very low (below 1 mg kg^{-1}) regardless of the metal treatment and incubation time (Table 2). The available concentration of Zn was increased by increasing Cd concentration in the Cd + Zn and Cd + Cu + Zn treatments, reaching a maximum value of 10% of the added Zn (Table 2).

In the Romola sandy neutral soil availability of Cd was significantly higher in the Cd + Zn and Cd + Cu + Zn treatments than in Cd and Cd + Cu treatments after 4 h and 7 days of incubation (Table 3). Availability of Cd decreased over time becoming similar for all treatments. Copper availability was very low (below 1 mg kg^{-1}) for all the metal treatments and incubation time (Table 3). Generally, availability of Zn was increased by higher Cd concentrations

Table 1
Soil characteristics

Soil	pH	Sand (%)	Silt (%)	Clay (%)	TOC (%)	N tot (%)	Cd (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
Vallombrosa	5.1	77.8	20.0	2.2	3.7	2.1	0.09	87.2	16.7	111.3	14.1	56.9
Romola	7.2	81.9	6.7	11.4	0.7	0.07	0.17	132.4	47.9	89.8	21.6	128.1
Vicarello	8.1	20.5	33.0	42.2	2.2	0.22	0.28	168.2	26.7	146.5	24.5	107.3

Table 2
NH_4NO_3-extractable Cd, Cu and Zn in the Vallombrosa soil incubated for 4 h, 7 days and 28 days

(Cd) (mg kg^{-1})	Metal treatments											
	(Cd)			(Cd + Cu)			(Cd + Zn)			(Cd + Cu + Zn)		
	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn
4 h extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.0004	0.0003	0.0003	0.0003	0.003	0.0003	0.0008	0.0003	0.013	0.0004	0.001	0.002
3	0.002	0.0003	0.0003	0.002	0.003	0.0003	0.003	0.0003	0.014	0.005	0.001	0.010
10	0.004	0.0003	0.0003	0.006	0.003	0.0003	0.006	0.0003	0.017	0.008	0.005	0.012
50	0.027	0.0006	0.0003	0.021	0.003	0.0003	0.027	0.0003	0.085	0.037*	0.004	0.017
300	0.189	0.0006	0.0006	0.207	0.003	0.0004	0.323*	0.0003	0.136	0.366*	0.006	0.060
1000	3.67	0.0006	0.0007	3.375	0.003	0.0006	4.213*	0.0003	0.180	4.177*	0.007	0.211
5000	12.960	0.0006	0.002	16.24	0.005	0.0012	22.10	0.001	0.285	23.53*	0.007	0.374*
7 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.0014	0.04	0.0003	0.0004	0.002	0.0003	0.0005	0.0003	0.001	0.0002	0.0001	0.002
3	0.002	0.04	0.0003	0.001	0.002	0.0003	0.003	0.0003	0.012	0.004	0.0001	0.008
10	0.004	0.04	0.0003	0.004	0.002	0.0003	0.004	0.0003	0.012	0.006	0.003	0.010
50	0.021	0.04	0.0003	0.019	0.003	0.0006	0.025	0.0003	0.063	0.035*	0.003	0.014
300	0.143	0.03	0.0004	0.163	0.003	0.0004	0.284*	0.0003	0.081	0.316*	0.005	0.050
1000	1.147	0.03	0.0008	1.238	0.004	0.0006	2.776*	0.0003	0.099	3.200**	0.006	0.163
5000	10.799	0.03	0.001	16.828	0.006	0.001	20.123*	0.002	0.111	18.674*	0.011	0.485*
28 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.0009	0.0006	0.0003	0.0008	0.002	0.0003	0.0006	0.0003	0.003	0.0003	0.001	0.002
3	0.002	0.0006	0.0003	0.002	0.002	0.0003	0.002	0.0003	0.003	0.003	0.002	0.006
10	0.003	0.0006	0.0003	0.003	0.002	0.0003	0.003	0.0003	0.009	0.005	0.002	0.007
50	0.016	0.0005	0.0003	0.014	0.002	0.0003	0.023	0.0003	0.058	0.027*	0.002	0.008
300	0.154	0.0006	0.0003	0.168	0.002	0.0005	0.258*	0.0004	0.061	0.350*	0.002	0.043
1000	1.280	0.0006	0.0003	1.200	0.002	0.0008	2.793*	0.0003	0.075	1.516	0.003	0.174
5000	12.352	0.0004	0.002	14.962	0.002	0.001	18.837*	0.002	0.115	17.805*	0.005	0.414*

Symbols (* and **) indicate values significantly different at $P < 0.05$ and $P < 0.01$, respectively in the same line.

in the Cd + Zn and Cd + Cu + Zn treatments at all incubation times (Table 3).

In the Vicarello clay alkaline soil Cd availability was again significantly higher in the Cd + Zn and Cd + Cu + Zn treatments than in the Cd and Cd + Cu treatments at all incubation times when Cd was added at rate of 50 mg kg^{-1} (Table 4). Availability of Cd did not change substantially throughout (Table 4). Copper availability was very low for all the treatments and incubation times (Table 4). Availability of Zn was increased by higher Cd concentrations in the Cd + Zn and Cd + Cu + Zn treatments at all incubation times (Table 4).

3.2. ATP content of soils

In the Vallombrosa soil at time zero (4 h after the metal treatments), the ATP content was significantly reduced ($P < 0.05$) only in the Cd + Cu + Zn treatments while the ATP content was only slightly decreased in the other Cd treatments (Fig. 1).

The ATP content of the Romola soil was significantly reduced ($P < 0.05$) by the Cd + Cu and Cd + Cu + Zn treatments as compared to Cd and Cd + Zn treatments at every incubation time (Fig. 1). No particular trends were displayed during the incubation (Fig. 1).

In the Vicarello soil the ATP content at time zero was not significantly different in the different treatments. After 7 and 28 days the ATP content was significantly lower ($P < 0.05$) in soils treated with Cd + Cu + Zn, with no differences observed in the other treatments (Fig. 1).

3.3. Acid and alkaline phosphatase activity

Both the acid and alkaline phosphatase activities were differently affected by heavy metal combinations, soil properties, and incubation time. Interpolation of values of the the acid and alkaline phosphatase activities with the model was satisfactory as the r^2 values were higher than 0.9. The asymptote values were expressed as mg *p*-NP kg^{-1} soil h (Table 5).

In the Vallombrosa acid soil at time zero (4 h after the metal treatments) the acid phosphatase activity was not reduced to 50% of the initial value when Cd was the sole metal added. Thus, ED_{50} values were not calculated and the asymptote value obtained by the model represented about the 80% of the initial activity (Table 5). On the other hand, ED_{50} values could be calculated for the other metal treatments (Table 5) and were ranked in the following way: Cd + Zn > Cd + Cu > Cd + Cu + Zn. The alkaline phosphatase activity was more sensitive to heavy metals,

Table 3
NH_4NO_3-extractable Cd, Cu and Zn in the Romola soil incubated for 4 h, 7 days and 28 days

(Cd) (mg kg^{-1})	Metal treatments											
	(Cd)			(Cd + Cu)			(Cd + Zn)			(Cd + Cu + Zn)		
	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn
4 h extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.25	0.04	0.02	0.01	0.21	0.01	0.19	0.05	0.91	0.05	0.19	3.10
3	0.36	0.04	0.02	0.11	0.18	0.01	0.44	0.04	0.92	0.86	0.24	0.75
10	0.50	0.04	0.02	0.85	0.23	0.02	0.53	0.03	1.24	0.97	0.24	0.84
50	2.63	0.04	0.02	2.31	0.19	0.01	3.24	0.03	0.43	3.90	0.22	0.89
300	37.55	0.03	0.03	50.91	0.21	0.03	43.54	0.02	9.18	45.74	0.16	4.25
1000	617.68	0.03	0.04	588.22	0.23	0.04	853.64	0.03	13.33	64.65	0.25	2.79
5000	2255.31	0.03	0.09	2051.96	0.26	0.10	4836.3	0.17	21.82	3844.58	0.42	2.41
7 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.16	0.04	0.02	0.54	0.34	0.02	0.10	0.05	0.45	0.02	0.28	1.51
3	0.25	0.04	0.02	0.23	0.37	0.01	0.35	0.04	0.61	0.33	0.32	0.84
10	0.40	0.04	0.02	0.41	0.32	0.02	0.47	0.03	0.53	0.63	0.31	0.85
50	2.45	0.04	0.02	2.19	0.28	0.02	1.82	0.03	6.83	3.80	0.23	1.23
300	21.65	0.03	0.03	22.15	0.18	0.03	27.53	0.02	2.18	43.06	0.18	4.97
1000	156.71	0.03	0.05	87.01	0.22	0.04	212.85	0.03	7.02	319.04	0.26	32.89
5000	1349.91	0.03	0.09	1887.17	0.62	0.13	2730.03	0.21	4.12	1994.23	0.65	27.46
28 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.21	0.04	0.02	0.09	0.1	0.02	0.07	0.05	0.20	0.33	0.09	0.12
3	0.38	0.04	0.01	0.22	0.11	0.01	0.16	0.04	0.23	0.31	0.12	0.23
10	0.32	0.04	0.02	0.29	0.13	0.02	0.36	0.03	0.21	0.52	0.10	0.21
50	1.77	0.04	0.02	0.30	0.11	0.02	2.86	0.02	0.36	2.98	0.12	0.51
300	39.83	0.02	0.03	18.84	0.08	0.03	24.59	0.02	1.22	39.21	0.05	1.47
1000	167.62	0.03	0.02	164.55	0.09	0.05	114.56	0.03	3.46	170.03	0.08	5.90
5000	1991.26	0.03	0.08	3277.82	0.14	0.13	1651.86	0.21	10.48	1657.53	0.19	36.83

*Symbols indicate values significantly different ($P < 0.05$) in the same line.

with the ED_{50} values lower than those of the respective ED_{50} values of acid phosphatase activity. However, Cd was significantly less inhibitory than other heavy metal treatments when added as the sole heavy metal (Table 5). Reductions in the ED_{50} values of both enzyme activities were observed during the 28 day incubation (Table 5). At the end of the incubation period the toxicity for both acid and alkaline phosphatase activities was the highest for the Cu treatments (Table 5).

In the Romola neutral sandy soil both acid and alkaline phosphatase activities were sensitive to heavy metals. At time zero the alkaline phosphatase activity was much more sensitive than the acid phosphatase activity as it was inhibited by all metal treaments approximately the same extent (Table 5). The ED_{50} values of the acid phosphatase activity of the Romola soil markedly decreased during the incubation whereas the ED_{50} values of the alkaline activity only slightly decreased (Table 5), probably because they were low at time zero. At all incubation times Cd, as the sole heavy metal added to soil, was significantly less inhibitory for the acid phosphatase activity than the other heavy metal additions (Table 5).

Contrarily to what was observed in the Vallombrosa and Romola soils, alkaline phosphatase activity of the Vicarello alkaline clay soil was less sensitive than acid phosphatase activity to heavy metal treatments (Table 5). At time zero alkaline phosphatase activity was not reduced to the 50% of the initial value when Cd was the sole heavy metal added; thus the ED_{50} value could not be calculated for this treatment by the model and the calculated asymptote value represented about the 60% of the initial enzyme activity (Table 5). At time zero, acid phosphatase activity was sensitive to all metal treatments at approximately the same extent with ED_{50} values lower than those of alkaline phosphatase activity (Table 5). Also in this soil, ED_{50} values of both acid and alkaline phosphatase activities decreased on prolonging the incubation time (Table 5). At day 7, multiple-metal treatments inhibited the acid phosphatase activity more than the Cd treatment. At the same incubation time the ED_{50} values of alkaline phosphatase activity for Cd and Cd + Zn were of the same order of magnitude and significantly higher than those for Cd + Cu and Cd + Cu + Zn (Table 5). After 28 days both acid and alkaline phosphatase activities were more inhibited by the Cd + Cu and Cd + Cu + Zn treatments than the Cd and Cd + Zn treaments (Table 5).

4. Discussion

Generally, both Cu and Zn increased Cd toxicity to acid and alkaline phosphatase activities and ATP content of soil.

Table 4
NH_4NO_3-extractable Cd, Cu and Zn in the Vicarello soil incubated for 4 h, 7 days and 28 days

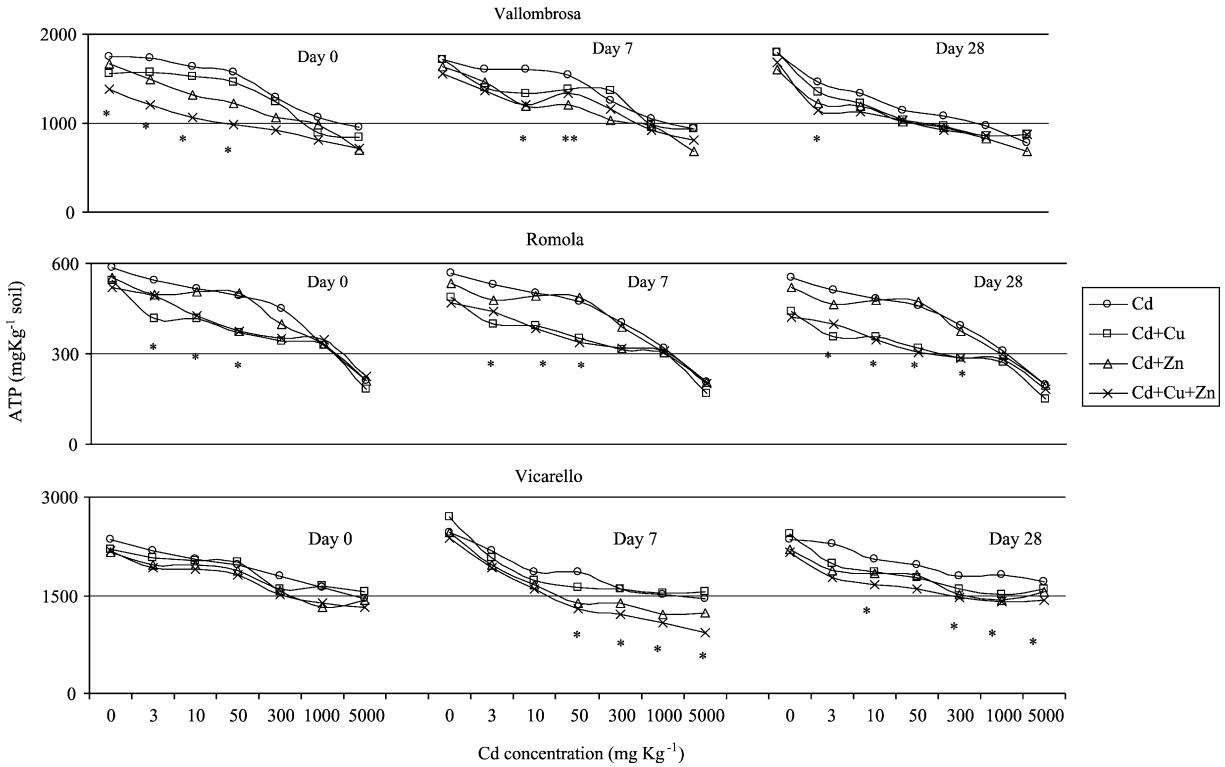
(Cd) (mg kg^{-1})	Metal treatments											
	(Cd)			(Cd + Cu)			(Cd + Zn)			(Cd + Cu + Zn)		
	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn	Cd	Cu	Zn
4 h extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.16	0.02	0.02	0.01	0.07	0.01	0.12	0.02	0.69	0.03	0.07	2.39
3	0.25	0.02	0.01	0.06	0.07	0.01	0.31	0.02	0.74	0.64	0.09	0.63
10	0.34	0.02	0.01	0.62	0.08	0.01	0.38	0.01	1.00	0.70	0.09	0.68
50	1.76	0.01	0.02	1.66	0.07	0.01	2.34	0.01	0.35	2.85	0.08	0.73
300	25.62	0.01	0.02	35.19	0.07	0.03	31.49	0.01	7.44	33.16	0.06	2.02
1000	453.51	0.01	0.04	426.51	0.08	0.03	550.37	0.01	10.66	454.19	0.09	2.63
5000	1589.61	0.01	0.08	1471.48	0.09	0.13	2585.90	0.06	17.58	3190.26	0.15	3.16
7 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.13	0.02	0.02	0.15	0.18	0.02	0.09	0.03	0.39	0.02	0.16	1.25
3	0.22	0.03	0.02	0.44	0.22	0.02	0.29	0.03	0.48	0.28	0.19	0.77
10	0.34	0.02	0.02	0.37	0.21	0.02	0.41	0.02	0.55	0.53	0.18	0.76
50	1.9	0.02	0.02	1.83	0.17	0.02	1.54	0.02	2.97	3.20	0.14	1.10
300	24.39	0.01	0.02	18.97	0.11	0.02	23.43	0.02	3.80	36.50	0.11	4.46
1000	132.61	0.02	0.03	153.06	0.14	0.04	182.04	0.02	3.73	269.30	0.15	7.85
5000	1143.17	0.02	0.08	1480.41	0.38	0.12	1738.16	0.12	6.40	2309.47	0.40	20.10
28 days extractable metals (mg Me^{2+} kg^{-1} soil)												
0	0.13	0.04	0.02	0.08	0.09	0.02	0.07	0.04	0.17	0.29	0.08	0.1
3	0.34	0.04	0.02	0.18	0.1	0.02	0.16	0.04	0.21	0.28	0.1	0.21
10	0.35	0.04	0.02	0.25	0.11	0.02	0.32	0.03	0.18	0.46	0.09	0.19
50	1.56	0.03	0.02	0.26	0.1	0.02	2.41	0.02	0.34	2.65	0.11	0.46
300	22.24	0.02	0.02	16.82	0.07	0.02	22.07	0.02	1.08	33.38	0.05	1.28
1000	122.25	0.02	0.02	147.04	0.08	0.04	95.56	0.02	3.09	154.49	0.07	5.37
5000	1013.94	0.03	0.07	1114.12	0.13	0.12	1438.98	0.19	9.36	1458.33	0.17	32.89

*Symbols indicate values significantly different ($P < 0.05$) in the same line.

The increased toxicity of multiple-metal treatments could not be explained by the amount of available heavy metal, as determined by the NH_4NO_3 extraction data, because greater toxic effects were generally observed after pre-treatment with Cu which did not increase Cd availability (Tables 2–4). Moreover, the ED_{50} values decreased (i.e. toxicity increased) with time whereas heavy metal availability decreased (Tables 2–5).

The extractable fraction of heavy metals has been considered to be the bioavailable fraction of heavy metals in soils (Swift and McLaren, 1991). A higher amount of available Cd was observed with Zn than with Cu in all soils, possibly due to competition between Cd and Zn for the adsorption sites. Competition between Cd and Zn added to soils in ionic form has been already described (Christensen, 1987). Probably Cu behaves differently from Cd and Zn in soil because it has has more affinity for organic ligands than both Cd and Zn (Inskeep and Baham, 1983; McBride, 1989), and it forms organic complexes in soil solution while Cd and Zn exist more likely as free hydrated ions (Elzinga et al., 1999). Microorganisms also have an ion exchange capacity (Ledin et al., 1999) and as soils were incubated under optimal conditions for microbial activity it can not be excluded that biochemical processes mediated by soil microorganisms contributed to change the chemical status of the added metals. In fact, microorganisms interacting with heavy metals do release organic chelating agents, melanins and exopolymers which can precipitate metals or reduce their activity at the cell surface (Charmugathas and Bollag, 1987; Hughes and Poole, 1989; Kurek et al., 1991; Gadd, 1993). The NH_4NO_3-extractable fraction of heavy metals is usually correlated with the plant uptake of heavy metals (Preuss, 1998) but the effects of microbial activity on this heavy metal fraction in soil are poorly known and need to be investigated.

Moreno et al. (2001) reported that both kinetic and sigmoidal dose-response models for calculating ED_{50} values fitted with soil total metal concentrations but not with water-soluble or DTPA-extractable Cd data. We also observed this lack of relation (data not shown). Generally, Cd solubility was greater in Romola than in Vicarello and Vallombrosa soils (Tables 2–4) possibly due to the coarser texture and lower organic matter content of the former than the latter soils. The most inhibitory effects were also observed in the Romola soil, where Cu and Zn generally increased Cd toxicity to the same extent. Moreover, in the Romola soil ATP content was more reduced by metal treatments than in the other two soils and no temporal trends could be described (Fig. 1). By using the ecological dose approach, Doelman and Haanstra (1989) and Moreno et al. (2001)



* symbols indicate significant differences ($P < 0.05$) of the means ($n = 3$).

Fig. 1. ATP content of the Vallombrosa, Romola and Vicarello soils treated with different heavy metals and incubated for 0, 7 and 28 days.

reported that inhibition of enzyme activity by heavy metals was greater in sandy than in finer textured soils, thus confirming the protective role of the clay fraction on soil enzyme activity (Burns, 1982; Nannipieri, 1995).

Generally, pre-treatments with Cu increased Cd toxicity more than Zn although total concentration of the latter metal was double than that of the former metal. Inhibition of soil phosphatases by Cu ions occurs through the Cu interaction with −SH groups of aminoacids of the active site of the enzyme (Acosta-Martinez and Tabatabai, 2001). Huang and Shindo (2000) reported that Cu^{2+} ions decreased both K_m and V_{max} of acid phosphatase adsorbed onto different soil constituents. Copper can bind not only the enzyme molecule but also the enzyme–substrate complex leading to a decrease in the enzyme activity (Huang and Shindo, 2000). Marzadori et al. (2000) demonstrated that some metal cations like Hg^{+2} and Cu^{+2} can inhibit some enzyme activities even if present as metal–organic complexes. Membrane and pericellular phosphatases are supposed to be indirectly involved in the decrease of heavy metal toxicity in bacteria and fungi by precipitating heavy metals as phosphates; in this way the heavy metal cellular uptake is prevented (Macaskie et al., 1992). We suggest that Cu might have increased the toxic effect of Cd in soil by reducing phosphatase activities and thus the protective mechanism of these enzymes toward microbial cells.

The lower impact of Zn than Cu on phosphatases might also be explained by the fact that microbial phosphatases are generally metallo-enzymes in which the metal is Zn or Mg (Nomoto et al., 1988; Coleman, 1992). Huang and Shindo (2000) reported that an acid phosphatase adsorbed onto Fe-oxides was not inhibited by Zn^{+2}. A possible mechanism explaining Cd toxicity can be the substitution of Zn and Mg by Cd due to the similar ionic properties of these ions.

Generally, ED_{50} values for both phosphatase activities decreased during the incubation. Similar temporal trends have been reported in earlier studies (Doelman and Haanstra, 1986; Haanstra and Doelman, 1991; Speir et al., 1995; Marzadori et al., 1996; Moreno et al., 2001) but the underlying mechanisms are poorly understood. Microorganisms can be affected by heavy metals in soil leading to a lower enzyme synthesis. Indeed, Landi et al. (2000) reported a decrease in the enzyme activity-to-microbial biomass ratio in the presence of available Cd. The death of heavy metal-sensitve microorganisms may occur with changes in the composition of microflora.

Geiger et al. (1998) reported a stronger inhibition of enzyme activity by heavy metals at alkaline than acidic pH values presumably due to higher reactivity of deprotonated amino acids. We have observed that in the Vallombrosa acidic soil alkaline phosphatase activity was more sensitive than acid phosphatase activity whereas in the Vicarello alkaline soil the opposite behaviour was observed. In the Romola neutral soil both the phosphatase activites were sensitive to heavy metals with the alkaline phosphatase

Table 5
The ED_{50} values of both phosphatase activities in different soils, different treatments and at various incubation times

Soil	Acid phosphatase				Alkaline phosphatase			
	(Cd)	(Cd + Cu)	(Cd + Zn)	(Cd + Cu + Zn)	(Cd)	(Cd + Cu)	(Cd + Zn)	(Cd + Cu + Zn)
$T = 0$								
Vallombrosa								
ED_{50}[a]	–	20.6	189.7	98.7	73.4	17.5	50.0	29.5
R^2[b]	–	0.90	0.91	0.93	0.92	0.94	0.94	0.97
Asymptote[c]	–	27.3	48.31	39.65	38.86	25.52	30.01	26.6
Romola								
ED_{50}	2461.9	58.1	2237.5	28.8	6.2	4.4	5.8	4.2
R^2	0.97	0.99	0.92	0.97	0.99	0.99	0.98	0.99
Asymptote	17.75	11.21	16.62	12.12	9.68	4.92	8.01	7.14
Vicarello								
ED_{50}	73.1	59.9	85.2	55.0	–	1545.7	555.0	667.9
R^2	0.97	0.99	0.96	0.99	–	0.98	0.95	0.97
Asymptote	32.41	22.45	27.87	19.99	–	46.67	40.02	44.53
$T = 7$								
Vallombrosa								
ED_{50}	2936.5	3310.3	286.8	68.7	35.1	11.4	34.9	19.1
R^2	0.94	0.95	0.95	0.98	0.94	0.99	0.99	0.99
Asymptote	45.84	48.60	41.43	26.89	16.58	9.71	19.54	12.31
Romola								
ED_{50}	45.7	9.5	11.5	5.8	4.5	2.9	3.8	3.3
R^2	0.98	0.97	0.98	0.98	0.97	0.99	0.97	0.99
Asymptote	12.10	8.22	10.03	7.63	4.41	2.09	3.34	3.16
Vicarello								
ED_{50}	63.6	23.8	38.5	22.4	543.9	67.1	420.8	61.3
R^2	0.98	0.97	0.99	0.96	0.92	0.94	0.91	0.94
Asymptote	28.34	18.21	20.12	18.43	39.33	18.43	31.09	18.10
$T = 28$								
Vallombrosa								
ED_{50}	558.2	46.1	260.7	159.9	15.1	9.7	16.1	7.9
R^2	0.93	0.94	0.97	0.98	0.95	0.94	0.99	0.98
Asymptote	27.35	17.67	21.8	9.51	5.84	5.39	11.41	5.16
Romola								
ED_{50}	14.3	3.9	3.5	3.1	4.1	2.9	3.1	2.6
R^2	0.97	0.97	0.96	0.97	0.98	0.98	0.95	0.99
Asymptote	7.18	6.71	6.12	5.58	4.66	3.13	3.28	2.88
Vicarello								
ED_{50}	30.0	15.1	28.1	10.7	25.5	15.9	17.1	14.4
R^2	0.98	0.97	0.97	0.94	0.95	0.94	0.95	0.96
Asymptote	15.99	12.86	19.33	9.09	13.65	11.54	18.47	10.20

ND, not determined.
[a] ED_{50} values are expressed in mg Cd kg^{-1} soil.
[b] R^2 is the correlation value.
[c] The asymptote is expressed in percentage of the uninhibited enzyme activity.

activity being more sensitive than the acid phosphatase activity.

Soil ATP content is an estimate of soil microbial biomass (Jenkinson, 1988) and it depends on synthesis and degradation processes, and the relative enzyme activities can be directly or indirectly inhibited by heavy metals. Thus, it is not erroneous to calculate ED_{50} values for ATP by the kinetic models as pointed out by Moreno et al. (2001). Indeed, valid interpolations of ATP by the kinetic model were obtained in our study (data not shown) indicating that heavy metals added to soils were active within microbial cells being the ATP content the result of synthesis and degradation processes.

In conclusion we have demonstrated the occurrence of additive effects of Cu, and Zn on Cd toxicity to acid and alkaline phosphatase activities and ATP content in soil; we have also observed that Cu increased the Cd toxicity more than Zn. The additive effects on soil phosphatases could be quantified by the ED_{50} values. As heavy metal polluted soils usually contain elevated concentrations of several heavy metals, calculation of ED_{50} values in the presence of two or more heavy metal might be helpful for

quantifying heavy metals effects on enzyme activities and interpreting data from long-term field experiments, where there is a gradual increase in the concentration of different heavy metals.

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