



Effectiveness of ecotoxicological tests in relation to physicochemical properties of Zn and Cu polluted Mediterranean soils



M. García-Carmona^{a,1}, A. Romero-Freire^{b,1}, M. Sierra Aragón^a, F.J. Martín Peinado^{a,*}

^a Departamento de Edafología y Química Agrícola, Universidad de Granada, Granada, Spain

^b Departamento de Bioquímica Mariña, Instituto de Investigacións Mariñas (IIM-CSIC), Vigo, Spain

ARTICLE INFO

Handling Editor: Edward A Nater

Keywords:

Heavy metals
Soil pollution
Toxicity tests
EC50

ABSTRACT

Zinc (Zn) and copper (Cu) are essential elements for living organisms, but they are also among the most frequent soils pollutants. Toxicity of these elements is difficult to assess and often shows contradictory results among authors due to the great complexity of the interactions with soil properties and constituents. The study of the individual metal behaviour will help provide a complete overview of the metal patterns in field pollution processes. Here, the influence of different soil properties on the mobility of Zn and Cu is studied in laboratory spiked soil samples by assessing metal toxicity from three different toxicity tests: root elongation, heterotrophic soil respiration and Microtox® toxicity tests. The results indicate that toxicity is significantly correlated with soil properties, calcium carbonate and organic carbon being the variables with highest influence in the reduction of Zn and Cu toxicity. However, large differences among soil samples in the toxicity results are observed. Thus, endpoints measured differ strongly in each soil sample because of the different sensibility of the organisms used in the toxicity tests and the high variability in soil properties. In addition, the results indicate differences between the soil toxicity depending on whether total or bioavailable metal concentrations are taken into account. Therefore, the current threshold concentrations usually selected for determining soil toxicity are not realistic, and a new approach is necessary taking into account the soil properties and the metal availability as well as using different toxicity tests to assess the toxic risk of metal-polluted soils.

1. Introduction

Soil pollution by metal in industrialized countries has increased exponentially in recent decades to become a worldwide concern. Activities such as mining, industrial manufacturing, smelting, power generation, waste spills, and the use of fossil fuels are the main sources of heavy metals in the environment (Díez et al., 2009; Martín et al., 2013). In addition, the rising use of agrochemicals, manures, biosolids, and compost amendments in agricultural soils are also increasing the metal accumulation, posing a risk of toxicity by metal transfer into the food web (Martínez and Motto, 2000; Chary et al., 2008; Mirzaei et al., 2014). Metals usually strongly bound to soil components and are persistent in soils over the long term (Adriano et al., 2004; van Gestel, 2008). When metal concentrations in soils exceed a certain threshold, the reduction of primary production and the alteration of nutrient cycling (Smolders et al., 2009) leads to the degradation of the soil functions (Burgos et al., 2008), and can generate toxicological risk for wildlife and the ecosystem (Song et al., 2009; Gebrekidan et al., 2013;

Li et al., 2014). In general terms, a soil is considered polluted when one or more substances exceed the critical load and hence can alter the natural soil environment. In the case of heavy metals, soil properties and components will determine the soil buffering capacity (Simón et al., 2010). The potential risk of metals in soils strongly depends less on the total concentrations than on their bioavailability, which represents the fraction of metal that is available for uptake by organisms and consequently may cause potential toxicity in the food chain (van Gestel, 2008; Favas et al., 2011). The determination of these potential toxic effects in the organisms is required for the protection of ecosystems (García-Gómez et al., 2014). Metal bioavailability depends on soil properties such as pH, Eh, organic matter, oxy-hydroxides, clays and carbonates (Alloway, 1995). Therefore, the interaction between soil properties and metals is crucial in the proposal of soil guideline values and in environmental-risk assessment (ERA) (Sołek-Podwika et al., 2016).

Zinc and copper are involved in different biochemical and physiological functions that make them essential elements for living

* Corresponding author.

E-mail address: fjmartin@ugr.es (F.J. Martín Peinado).

¹ These authors have contributed equal as first authors.

organisms (Li et al., 2014; Cherfi et al., 2014). However, they are also among the most frequent pollutants into the heavy-metal group (Barna et al., 2007), both in agricultural soils (Nicholson et al., 2003; Kabata-Pendias, 2011) and in freshwater (Barry, 2011). Although their toxicity occurs mostly in combination with other elements (ATSDR, 2004), the study of the individual metal behaviour in soils is key to gain a complete overview of the potential metal patterns in polluted areas. Zn is an essential trace element for living organisms and is relatively harmless, but the Zn accumulation in soils is because human activity can rise Zn to toxic levels at certain sites. High Zn concentrations can inhibit microbial activity in soils, causing phytotoxicity; meanwhile, in humans long-term exposure can interfere with Cu uptake, causing toxicity due to Cu deficiency (Jacquat et al., 2008; Donner et al., 2010; Plum et al., 2010). Although Cu is an essential trace element for plants, animals, and microorganisms, as in the case of Zn, it is toxic above a certain threshold (Shaheen et al., 2009). In humans, Cu toxicity causes brain, skin, pancreas, and heart diseases (Veli and Alyüz, 2007). The accumulation of Cu in soils is also due to human activity, in particular by persistent application of copper salts in agriculture. The two metals have different physicochemical properties, and therefore their chemical behaviour in soils differs. The bioavailability of Zn in soils is strongly related to soil pH, reaching more mobility under acidic conditions, while its adsorption is more related to clay minerals and oxides (Alloway, 1995). Zn adsorption in soils is significant at around pH 5.0–6.5, while precipitation as insoluble (not bioavailable) forms appears to become substantial at pH 6.0 to 7.0 (Rieuwerts et al., 1998). Copper is strongly bound to soil, is less mobile, and is less sensitive to soil pH changes than is Zn, with relative adsorption at pH values below 5.0 (Rieuwerts et al., 1998). For this metal, organic matter and oxide contents are the most significant parameters governing its adsorption processes in soils (Alloway, 1995).

Ecotoxicological studies with metal-spiked soils are required to generate toxicity information and establish safety levels for the environment and humans. However, the definition of soil-toxicity criteria is not easy because of the variability of the pollutant behaviour in soils (Romero-Freire, 2016). Therefore, multiple toxicity tests must be conducted with representative species of different taxonomic groups to assess different routes of exposure, as well as to evaluate toxic effects over the short, medium, and long term. Previous studies have demonstrated that, under controlled conditions, a specific pollutant will act in a completely different way depending on soil properties, even showing no toxic effects and total inhibition for the same concentration (Romero-Freire et al., 2016). It is essential to understand how soil properties may affect the toxicity of metals and whether the influence of soil properties is consistent across different bioassays. However, the influence of soil properties on metal bioavailability is often ignored in the setting where guideline values are determined (Song et al., 2006), without reflecting their effects on regulatory standards.

The present study deals with two of the potentially most toxic metals in soils, Zn and Cu, and seeks to assess the effectiveness of different ecotoxicity tests for being applied to soils with different physicochemical properties. For this objective, samples were collected from seven typical Mediterranean soils having different physicochemical characteristics and were laboratory spiked with concentrations below and above threshold values for Cu and Zn defined in specific regulations. Toxicity assessments were made according to selected toxicity tests using organisms based on their environmental relevance for the soil structure and functionality. The ultimate goal of the work is to offer valuable information needed to validate risk limits for Zn and Cu according to soil properties.

2. Material and methods

2.1. Selected samples

Seven soils (Table 1) were selected to represent major soil groups

(IUSS, 2006) in southern Spain. From each soil, one horizon with a more relevant *qualifier* was selected: a *Mollic* surface horizon from a *Calcaric cambisol* (CC-Ah); a *Cambic* subsurface horizon from a *Calcaric cambisol* (CC-Bw); a *Calcic* subsurface horizon from a *Calcaric Kastanozem* (CK-Ck); a *Cambic* subsurface horizon from a *Leptic cambisol* (LC-Bw); an *Ochric* surface horizon from a *Leptic Regosol* (LR-Ah); a *Haplic* subsurface horizon from a *Leptic Regosol* (LR-C1); and a *Cutanic* subsurface horizon from a *Cutanic Luvisol* (CL-Bt). The main properties and constituents were analysed: soil pH (measured using a soil:water ratio of 1:2.5), calcium carbonate content (CaCO₃), organic carbon (OC) content, texture, water retention at field capacity (FC), cation-exchange capacity (CEC), and base saturation (V). These properties were determined following official analysis methods (MAPA, 1994). Moreover, iron (Fe_d), aluminum (Al_d), and manganese (Mn_d) free oxides were analysed according to Holmgren (1967). Total Zn and Cu concentrations were determined after acid digestion of the soil samples (HNO₃ + HF), and measured by Inductively Coupled Plasma-Mass Spectrometry using an ICP-MS NEXION 300D spectrometer, and considered as background total concentrations (Zn_B and Cu_B, respectively). The accuracy of the method was corroborated by analyses of standard reference material SRM 2711 (n = 6); with a recovery of 94 ± 7% for Zn and 96 ± 8% for Cu.

2.2. Sample spiking

Soil samples were individually spiked in the laboratory using chemical species with the most abundant oxidation states of these elements in soils: Zn(II) and Cu(II). The samples were spiked with aqueous solutions from [ZnCl₂] and [Cu(NO₃)₂·3H₂O] salts. Pollution levels were established by increasing the average background soil concentrations based on a literature review of reference thresholds for different countries (Aguilar et al., 1999) and adding two more levels of extreme pollution. In total, five contamination levels were defined for each pollutant: 600, 1000, 3000, 6000, and 9000 mg Zn kg⁻¹ soil; and 300, 500, 1000, 2000, and 4000 mg Cu kg⁻¹ soil. In addition, uncontaminated soil samples were used as control (T0), making a total of 6 treatment levels (T0, T600, T1000, T3000, T6000, and T9000 for Zn; and T0, T300, T500, T1000, T2000, and T4000 for Cu) for each soil horizon studied.

Samples of 150 g were individually spiked with the selected concentrations, moistened to 60% of their field capacity, and divided into 3 replicates. Spiked samples were incubated for 4 weeks at 25 ± 1 °C and 60% air humidity, with a light/dark cycle of 10/14 h. Sample moisture content was checked periodically by weighing and adjusted when necessary. Total metal concentrations (Zn_T and Cu_T) in samples after incubation were checked by analysing by XRF with a NITON XLt 792 instrument. In all cases, the concentrations measured differed < 10% from the total estimated concentrations (background + added metal concentrations). After incubation, in each of the six treatments with Zn and Cu, a soil aqueous extract (SAE) from a soil:water ratio 1:1 was taken after 24 h of shaking followed by extraction with 10 cm Rhizon MOM soil moisture samplers. In the SAE, pH (pH_W), electrical conductivity (EC_W), and water-extractable Zn and Cu concentrations (Zn_W, Cu_W) were measured by ICP-MS. Ionic strength was calculated from the EC_W data according to Simón and García (1999). All measurements were performed within 48 h after taking the extracts.

2.3. Ecotoxicological tests

Three toxicity tests were selected for this study and run in triplicate:

1. Seed germination/root elongation toxicity test (Ls) was applied according to USEPA (1996) recommendations. This test, which assesses the phytotoxic effects on seed germination and initial seedling growth (Torres, 2003), was performed using the SAE. In Petri dishes 15 seeds of *Lactuca sativa* L. and 5 ml of SAE were incubated at

Table 1
Mean values of the main properties of selected soil samples for the study of zinc and copper toxicity.

Soil type (WRB, 2006)	Calcaric cambisol (humic)	Calcaric cambisol (humic)	Calcaric kastanozem (antric)	Leptic cambisol (eutric)	Leptic regosol (eutric)	Leptic regosol (distic)	Cutanic luvisol (chromic)
Sample name ^a	CC-Ah	CC-Bw	CK-Ck	LC-Bw	LR-Ah	LR-C1	CL-Bt
Sample upper-lower limit (cm)	0–21	30–52	60–85	10–38	0–9	62–87	40–65
pH	7.96	8.67	8.79	6.74	7.20	5.87	7.03
CaCO ₃	%	37.1	72.4	92.3	nd	nd	0.92
OC	%	5.43	0.42	0.38	0.61	8.22	0.49
Clay	%	23.6	11.8	7.7	19.1	23.8	8.3
Sand	%	34.1	41.4	28.3	56.7	43.0	70.5
CEC	cmol _c kg ⁻¹	21.4	9.83	2.94	9.91	25.9	3.83
V	%	100	100	100	59	50	31
Al _d	g kg ⁻¹	0.24	0.11	0.06	0.19	0.19	0.09
Fe _d	g kg ⁻¹	1.90	0.87	0.33	1.79	1.94	0.78
Mn _d	g kg ⁻¹	0.05	0.01	0.00	0.03	0.09	0.02
Zn _B	mg kg ⁻¹	128.6	61.4	27.2	22.2	50.1	12.8
Cu _B	mg kg ⁻¹	14.4	7.2	4.0	19.1	19.5	4.1

^a Sample name include acronym of soil type followed by the soil horizon selected; nd: not detected; CaCO₃: calcium carbonate content; OC: organic carbon content; CEC: cation exchange capacity; V: base saturation; Al_d/Fe_d/Mn_d: free aluminum, iron and manganese forms; Zn_B: zinc total background concentration; Cu_B: copper total background concentration.

25 ± 1 °C for 120 h. The number of seeds germinated and the length of the developing roots were measured after this period. The endpoint calculated was root-elongation reduction (LsR) compared to the control.

- The Microtox[®] test (ASTM, 2004), based on the reduction of the luminescence emitted by the marine bacterium *Aliivibrio fischeri* (Af) (Ribo and Kaiser, 1987), was performed in SAE in a Microtox 500 analyser from Microbics Corporation, according to a modification of Microtox Basic Test for Aqueous Extracts Protocol (AZUR, 1998; Martín et al., 2010). The luminescence was measured at time 0 and 15 min after mixing the bacteria with SAE. The results were expressed as the luminescence reduction (Afr) in the samples compared to the control.
- Heterotrophic soil respiration (Sr) was measured by determining the CO₂ flux from samples studied with a microbiological analyser-Trac 4200 SY-LAB model, performed directly in soil samples (ISO 17155, 2002). The soil-moisture content was fixed at field capacity and samples were incubated at a constant temperature (30 °C) for 96 h. CO₂ production was determined by absorption in vials with a solution of potash (KOH 0.2%) and related to the mass of sample used to determine a normalized measure of respiration. The results were expressed as the respiration reduction (SrR) compared to control.

The Ls test, highly recommended for studying soil pollution in a wide range of metal concentrations, is also a good estimator of soil toxicity in natural and artificial spiked soils (Escoto et al., 2007; Bagur et al., 2010; Romero-Freire et al., 2015). The Af toxicity test is widely used due to its easy interpretation and, according to Costa et al. (2015), the results usually correlate well with toxicity tests performed with higher organisms, such as the test carried out with the plant *L. sativa*. However, both tests are performed in the water-extractable fraction of soils and, as reported by Leitgib et al. (2007), the use of tests in the water extracts of soil may not reproduce exactly what happens in the contaminated soil matrix, and could be poorly predictive when the whole soil is considered as an environmental element. However, this contradiction can be solved with the use of living organisms belonging to different taxonomic groups, which is highly recommended in ecotoxicological risk assessment. The soil respiration test, by contrast, evaluates the toxic effect for a diversity of soil communities directly in soil. Microbial communities respond quickly under changing conditions (Nannipieri et al., 2003) but results to date with this test have shown striking differences, suggesting that soil microbial communities can

develop resistance and resilience to specific pollutants (Hänsch and Emmerling, 2010).

2.4. Data analyses

For a better interpretation of the availability and toxicity, soil-water partition coefficients (K_d) were calculated as the ratio of the total metal concentration in sample (ZnT or CuT, in mg kg⁻¹ dry soil) and water-extractable metal content (ZnW or CuW, in mg L⁻¹), as a normalization of water-soluble concentration data (Blaser et al., 2000).

The normal distribution of the data was verified by a Kolmogorov-Smirnov test, significant differences were determined by ANOVA, and for multiple comparisons Tukey's test was applied (p < 0.05) to assess differences among samples and treatments. For the analysis of the influence of soil properties, Spearman's correlations were performed, whereas factor analyses were applied from a rotated component analysis by the method of Varimax normalization including total and water-soluble metal concentrations, the main soil properties (CaCO₃; EC_w; CEC; OC; Al_d/Fe_d/Mn_d forms) and the three toxicity endpoints (LsR, SrR and Afr). All these analyses were performed with a confidence level of 95% by using SPSS v.20.0 (SPSS Inc., Chicago, USA). The effective concentrations causing 50% reduction compared to the control (EC50) and the corresponding 95% confidence intervals were reached by fitting a log-logistic model to the data (Doelman and Haanstra, 1989), using the method of Marquardt (proc NLIN, SAS 9.1, SAS Institute, Cary, NC, USA). In samples where possible subtoxic stimulus appeared (hormesis effect), endpoint calculations were performed according to Vanewijk and Hoekstra (1993).

3. Results

3.1. Zn and Cu water solubility and effect of soil properties

ZnW varied significantly among soil samples, while treatments showed a significant increase with the rise of total metal content (Table 2). Significant differences appeared from T3000 in all samples, except in CC-Bw (this appearing from T6000) and sample LR-C1 (differing from T1000). The percentage of water-soluble forms in relation to the total concentration in carbonate samples (CC-Ah, CC-Bw and CK-Ck) registered values lower than 11%, while non-carbonate samples (LC-Bw, LR-Ah, LR-C1 and CL-Bt) had values higher than 21%.

Partition coefficients (K_d) were calculated to normalize the

Table 2

Water-extractable Zinc concentrations (ZnW) and partition coefficients (Kd) for the 7 soil samples in the control (T0) and in the 5 spiked treatments of zinc (T600; T1000; T3000; T6000; T9000; numbers indicate added concentration in mg Zn kg⁻¹ soil). Lowercase letters represent significant differences between treatments (Tukey's test, p < 0.05).

Treatment	CC-Ah	CC-Bw	CK-Ck	LC-Bw	LR-Ah	LR-C1	CL-Bt
ZnW (mg kg⁻¹)							
T0	0.23a	0.28a	0.17a	1.47a	1.19a	1.31a	0.11a
T600	0.33a	0.30a	5.13a	53.84a	1.50a	173.83ab	14.68a
T1000	0.77a	0.34a	16.28a	196.93a	3.76a	470.08b	44.76a
T3000	28.03b	5.64a	104.89b	1813.41b	131.46b	2103.66c	698.65b
T6000	284.03b	77.31b	174.46c	4388.30c	822.03c	4592.48d	2216.27c
T9000	946.75c	129.91c	345.70d	7124.98d	1935.22d	7601.88e	4200.92d
KdZn (L kg⁻¹)							
T0	564b	241a	183c	15.1d	44.7a	9.8d	271b
T600	2223d	2265b	124bc	11.7c	437c	3.5c	44a
T1000	1506c	3165c	65.3ab	5.2b	282b	2.2b	24a
T3000	112a	565a	29.3a	1.7a	23.6a	1.4ab	4.4a
T6000	21.8a	78.7a	34.7ab	1.4a	7.4a	1.3a	2.7a
T9000	9.6a	70.3a	26.7a	1.3a	4.7a	1.2a	2.2a

availability of the metals for the different treatments in relation to the total concentration (Table 2). In general, Kd values decreased with the increase of Zn added to samples, with the lowest Kd found at T9000 of non-carbonate samples (LR-C1 and LC-Bw) and the highest Kd in carbonate samples. However, in the different samples, the decreases were not inversely related to the increase in total concentration added in all cases; CC-Ah and LR-Ah showed their highest Kd at T600, and CC-Bw presented the highest Kd at T1000.

CuW also showed a significant increase with the rise of total Cu concentration added (Table 3). Significant differences appeared from T1000 or T2000, with the exception of sample CL-Bt, in which significant differences appeared only in the treatment with the highest concentration (T4000). The percentage of water-soluble forms in relation to the total concentration presented for carbonate samples (CC-Ah, CC-Bw and CK-Ck) and LR-Ah had values lower than 0.1%. The clayey iron-rich sample (CL-Bt) had values of around 0.4%, and the rest of non-carbonate samples (LC-Bw and LR-C1) reached values of between 3 and 8%.

Partition coefficients (Kd) for Cu increased with the total Cu added to samples in CC-Ah, CC-Bw, CK-Ck and LR-Ah, with the lowest Kd in the control treatment (T0) and the highest at T4000 for CC-Ah and CC-Bw, and at T1000 for CK-Ck and LR-Ah. Soil samples LC-Bw and LR-C1 presented the inverse trend, Kd values decreasing with the increase of total concentrations added. Sample CL-Bt differed, with the maximum Kd values reached at T500.

Multiple stepwise regressions were made in order to study the

Table 3

Water-extractable copper concentrations (CuW) and partition coefficients (Kd) for the 7 soil samples in the control (T0) and in the 5 spiked treatments of copper (T300; T500; T1000; T2000; T4000; numbers indicate added concentration in mg Cu kg⁻¹ soil). Lowercase letters represent significant difference between treatments (Tukey's test, p < 0.05).

Treatment	CC-Ah	CC-Bw	CK-Ck	LC-Bw	LR-Ah	LR-C1	CL-Bt
CuW (mg kg⁻¹)							
T0	0.044a	0.0017a	0.0027a	0.0028a	0.0242a	0.0108a	0.0042a
T300	0.054ab	0.0118ab	0.094ab	0.016a	0.242ab	0.6259a	0.014a
T500	0.058ab	0.0115ab	0.096ab	0.145a	0.485ab	2.15ab	0.0064a
T1000	0.105b	0.0319ab	0.133b	1.74a	0.447ab	12.91b	0.095a
T2000	0.164c	0.047bc	0.280c	17.80b	0.933b	88.51c	0.967a
T4000	0.222d	0.079c	0.552d	130.85c	2.14c	313.34d	15.58b
KdCu (L kg⁻¹)							
T0	3325a	4535a	1483a	9705ab	937a	601b	8159ab
T300	5808ab	36423ab	3238b	24049b	1334ab	510ab	39522b
T500	9541bc	48096b	5264c	3836a	1194ab	239ab	84684c
T1000	9769bc	32494ab	7609d	623a	2428b	77.8ab	11034ab
T2000	12367c	42512b	7204d	114a	2194ab	22.7a	2724ab
T4000	18522d	56667b	7364d	30.8a	2019ab	12.8a	273a

Table 4

Multiple stepwise regression for Zn and Cu water-soluble forms, from the variables obtaining significant coefficients (p < 0.05) in Spearman's correlations.

	coef	
ZnW		
pH _W	-307.76	r ² = 0.676; p < 0.001
Sand	42.51	
ZnT	0.31	
CuW		
pH _W	-7.70	r ² = 0.430; p < 0.001
Sand	0.99	
CuT	0.02	

influence of soil properties on the water solubility of the two metals studied (Table 4). Both ZnW and CuW showed a significant correlation (p < 0.01) with pH, sand content, and the total concentrations added to the samples. In both cases, the pH of the water extract (pH_W), correlated negatively, while the sand fraction and the total concentration in samples correlated positively with ZnW and CuW. Values of pH and EC for all treatments and samples were included as supporting information (Tables SI.1 and SI.2).

3.2. Zinc and copper ecotoxicity

Ecotoxicological results for the different tests were included as

Table 5

EC50 values, with confidence intervals (CI95%), obtained from the total (ZnT) and water-extractable (ZnW) zinc concentrations in the three toxicity tests (Ls: *Lactuca sativa*, Af: *Aliivibrio fischeri* and Sr: Soil respiration) applied to the 7 different soil samples.

		Ls			Af			Sr		
		EC50	CI95%		EC50	CI95%		EC50	CI95%	
		mg Zn kg ⁻¹ soil			mg Zn kg ⁻¹ soil			mg Zn kg ⁻¹ soil		
ZnT	CC-Ah	1259	889	1783	3162	1980	5050	> 9000		
	CC-Bw	1000	813	1230	6026	4556	7969	> 9000		
	CK-Ck	316	146	385	> 9000			> 9000		
	LC-Bw	316	130	770	501	347	724	1995	1629	2444
	LR-Ah	501	295	852	1585	1265	1986	> 9000		
	LR-C1	398	202	784	200	102	391	2512	2062	3060
	CL-Bt	501	257	976	2512	1458	4327	6310	5188	7674
ZnW	CC-Ah	1.26	0.58	2.75	35.5	22.6	55.8	^a		
	CC-Bw	1.17	0.24	5.87	79.4	59.1	107	^a		
	CK-Ck	2.82	1.03	7.74	^a			^a		
	LC-Bw	31.6	10.7	93.6	50.1	28.6	87.8	794	547	1154
	LR-Ah	1.99	0.58	6.85	20.0	10.2	39.1	^a		
	LR-C1	100	23.1	433	31.6	9.06	110	1585	1103	2278
	CL-Bt	10.0	2.76	36.2	501	331	759	3162	1943	5146

Italics represent the minimum and maximum value of the 95% confidence interval.

^a EC50 was not reached in the range of the water-soluble metal concentrations measured.

Table 6

EC50 values, with confidence intervals (CI95%), obtained from the total (CuT) and water-extractable (CuW) copper concentrations in the three studied toxicity tests (Ls: *Lactuca sativa*, Af: *Aliivibrio fischeri* and Sr: Soil respiration) applied to the 7 different soil samples.

		Ls			Af			Sr		
		EC50	CI95%		EC50	CI95%		EC50	CI95%	
		mg Cu kg ⁻¹ soil			mg Cu kg ⁻¹ soil			mg Cu kg ⁻¹ soil		
CuT	CC-Ah	> 4000			> 4000			> 4000		
	CC-Bw	> 4000			> 4000			316	179	558
	CK-Ck	> 4000			> 4000			> 4000		
	LC-Bw	631	154	2577	1259	539	2940	891	660	1203
	LR-Ah	> 4000			> 4000			> 4000		
	LR-C1	1122	659	1911	562	462	685	251	148	427
	CL-Bt	447	304	656	2188	1477	3240	> 4000		
CuW	CC-Ah	^a			^a			^a		
	CC-Bw	^a			^a			0.02	0.01	0.03
	CK-Ck	^a			^a			^a		
	LC-Bw	1.58	0.08	32.55	2.00	0.29	13.7	1.58	0.34	7.49
	LR-Ah	^a			^a			^a		
	LR-C1	12.6	8.96	17.7	2.51	1.71	3.70	1.00	0.23	4.39
	CL-Bt	0.02	0.01	0.02	2.51	1.05	6.00	^a		

^a EC50 was not reached in the range of the water-soluble metal concentrations measured.

supporting information for Ls and Af tests (Fig. SI.1) and in Romero-Freire et al. (2016) for Sr test. In all cases, EC50 values were calculated both for ZnT and CuT as for ZnW and CuW (Tables 5 and 6). For all toxicity tests, the 95% confidence intervals (CI95%), calculated from the EC50, were used for discriminating the significant differences between soil horizons.

ZnT differed according to the toxicity tests applied. In the case of the Ls test, large variation among soil samples was detected according to EC50 values. Samples CC-Ah and CC-Bw, presented the highest EC50 values (1259 and 1000 mg kg⁻¹, respectively), while the other samples presented EC50 values ranging between 316 and 501 mg kg⁻¹. In the bioluminescence Af test, carbonate samples (CC-Ah, CC-Bw and CK-Ck) showed high EC50 values (> 3160 mg kg⁻¹ in all cases) and samples CL-Bt and LR-Ah presented lower EC50 values (2512 and 1585 mg kg⁻¹, respectively), whereas LC-Bw and LR-C1 presented higher toxicity with the lowest EC50 values (501 and 200 mg kg⁻¹, respectively). In the case of Sr test, no EC50 values were reached at the maximum concentration for spiked Zn (9000 mg kg⁻¹) for carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah samples; CL-Bt presented high EC50 value (6310 mg kg⁻¹), and the lowest EC50 values were found in

LR-C1 and LC-Bw samples (2512 and 1995 mg kg⁻¹, respectively; Table 5).

ZnW also differed according to the toxicity test. In the Ls test, large variation among soil samples was detected according to EC50 values. Samples LR-C1 and LC-Bw, had the highest EC50 values (100 and 31.6 mg kg⁻¹, respectively), in relation to the other samples, which presented EC50 values equal to or lower than 10 mg kg⁻¹. In Af, sample CL-Bt showed the highest values of EC50 (501 mg kg⁻¹). CK-Ck did not reach EC50 value in the range of the water-soluble concentrations measured for this soil horizon, while there were no significant differences in EC50 values among the other samples, with values ranging from 20.0 and 79.4 mg kg⁻¹. In the case of Sr, no EC50 values were reached for carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah soil horizons; CL-Bt presented high EC50 value (3162 mg kg⁻¹), and the lowest EC50 values were found in LR-C1 and LC-Bw (1585 and 794 mg kg⁻¹, respectively).

In the case of CuT, the Ls test presented marked variation among soil samples according to EC50 values. No EC50 values were reached for carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah samples; LR-C1 presented a high EC50 value (1122 mg kg⁻¹), while the lowest EC50

values were found in LC-Bw and CL-Bt (631 and 447 mg kg^{-1} , respectively). In Af, no EC50 values were reached at the maximum concentration for spiked Cu (4000 mg kg^{-1}) for carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah samples; CL-Bt and LC-Bw samples presented high EC50 values (2188 and 1259 mg kg^{-1} , respectively), while LR-C1 presented low EC50 values (562 mg kg^{-1}). In Sr, no EC50 values were reached at the maximum concentration for spiked Cu (4000 mg kg^{-1}) for CC-Ah, CK-Ck, LR-Ah and CL-Bt samples; LC-Bw presented high EC50 value (891 mg kg^{-1}), and the lowest EC50 values were found in CC-Bw and LR-C1 samples (316 and 251 mg kg^{-1} , respectively; Table 6).

CuW also presented differences among the toxicity tests applied. In Ls test, strong variations among soil samples were detected in terms of EC50 values. Carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah soil horizons did not reach EC50 values within the range of the water-soluble concentrations measured; LR-C1 presented high EC50 value (12.6 mg kg^{-1}), while LC-Bw and CL-Bt soil horizons registered the lowest EC50 value (< 1.6 mg kg^{-1}). For Af test, carbonate (CC-Ah, CC-Bw and CK-Ck) and LR-Ah samples did not reach EC50 and there were no significant differences in EC50 among the other samples, with values equal or lower than 2.5 mg kg^{-1} . In the case of Sr test, no EC50 values were reached for CC-Ah, CK-Ck, LR-Ah and CL-Bt samples; LC-Bw and LR-C1 presented low EC50 value (1.6 and 1.0 mg kg^{-1} , respectively), and the lowest EC50 values were found in CC-Bw sample (0.02 mg kg^{-1}).

3.3. Effect of soil properties on Zn and Cu toxicity

The range of responses to Zn pollution found for the three tests used showed direct correlations (correlation coefficient, p value): LsR-SrR (0.493 , $p < 0.01$), LsR-AfR (0.670 , $p < 0.01$), and SrR-AfR (0.611 , $p < 0.01$). For toxicity data recorded in the Cu-polluted soil horizons, the tests used significantly correlated: LsR-SrR (0.626 , $p < 0.01$), LsR-AfR (0.470 , $p < 0.01$), and SrR-AfR (0.414 , $p < 0.01$).

Principal component analysis (PCA) was applied for the response of the toxicity tests and the main soil properties in the soil horizons spiked with Zn (Fig. 1); samples which did not present toxic effects for the metal content used in the toxicity tests were eliminated from the analyses. PCA analysis for Zn showed that toxicity-test responses were

grouped together in the PC1 and PC2, explaining a 35.1% and 24.2% of the variance, respectively. LsR was directly related to ZnT as well as with electrical conductivity (EC_W) and ionic strength (I_W) of the soil solution. AfR and SrR were grouped together and closely with a direct relationship to ZnW and an inverse relationship to the pH of the water extracts (pH_W), soil pH (pH_H₂O) and CaCO₃ content.

Principal component analysis for the responses found in the toxicity tests and the main soil properties in the soil horizons spiked with Cu were also presented (Fig. 2). In this case, PCA analysis showed that toxicity-test responses were grouped together in two components (PC1 and PC2), explaining a 30.2% (PC1) and 24.6% (PC2) of the variance. The relationship between toxicity tests and soil properties for Cu toxicity showed differences compared to Zn. In this case, the three toxicity endpoints (LsR, AfR and SrR) were grouped in the second component (PC2), directly related to CuW and sand content in the samples, and inversely related to the pH of the water extracts (pH_W) and soil pH (pH_H₂O). Toxicity tests also showed a slight inverse influence with OC and CEC, while no relationship with CuT was detected.

4. Discussion

4.1. Water solubility of Zn

The present study confirmed that water solubility of metal is strongly controlled by soil properties, this significantly differing in selected soil horizons. Our results indicate that zinc mobility is directly controlled by soil properties and constituents. This agrees with Kraus and Wiegand (2006), Madejón et al. (2006), Clemente et al. (2008), and Romero-Freire et al. (2016), who reported in previous studies that Zn reaches the highest mobility under acidic conditions. LR-C1 and LC-Bw samples, acidic soil horizons with low content in OC, presented the highest concentrations of ZnW, while the lowest concentrations were detected in the calcareous soil horizons (CC-Ah, CC-Bw and CK-Ck). Therefore, the pH and CaCO₃ content appear to be the main properties controlling Zn water solubility, especially at high total Zn concentrations. Our results corroborate the negative correlations between ZnW with soil pH and CaCO₃ content. Soil carbonates may affect metal solubility in water through their surface interactions, providing specific adsorption or precipitation reactions, and indirectly through their pH

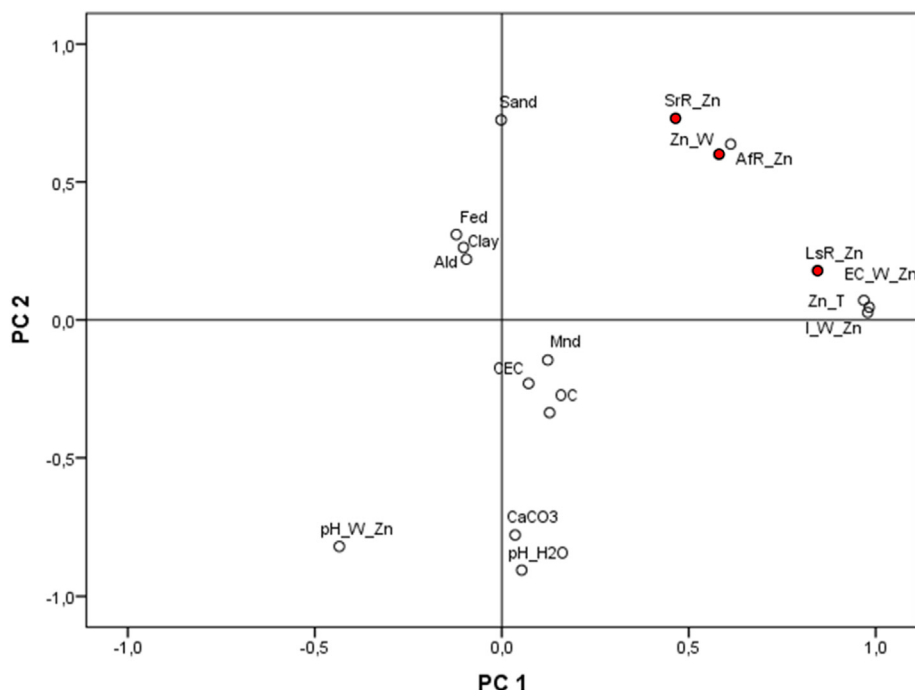


Fig. 1. Principal component analysis (PCA) for zinc after Varimax normalization method including total Zn content (ZnT), water-soluble zinc concentration (ZnW), the main soil properties (CaCO₃: calcium carbonate; EC_W: electrical conductivity; CEC: cation exchange capacity; OC: organic carbon content; Al_d/Fe_d/Mn_d: free aluminum, iron and manganese forms) and the three toxicity endpoints (red circles) (LsR: *Lactuca sativa* Reduction, SrR: Soil respiration Reduction and AfR: *Aliivibrio fischeri* Reduction). Percentage of variance explained: PC1 = 35.1%, PC2 = 24.2%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

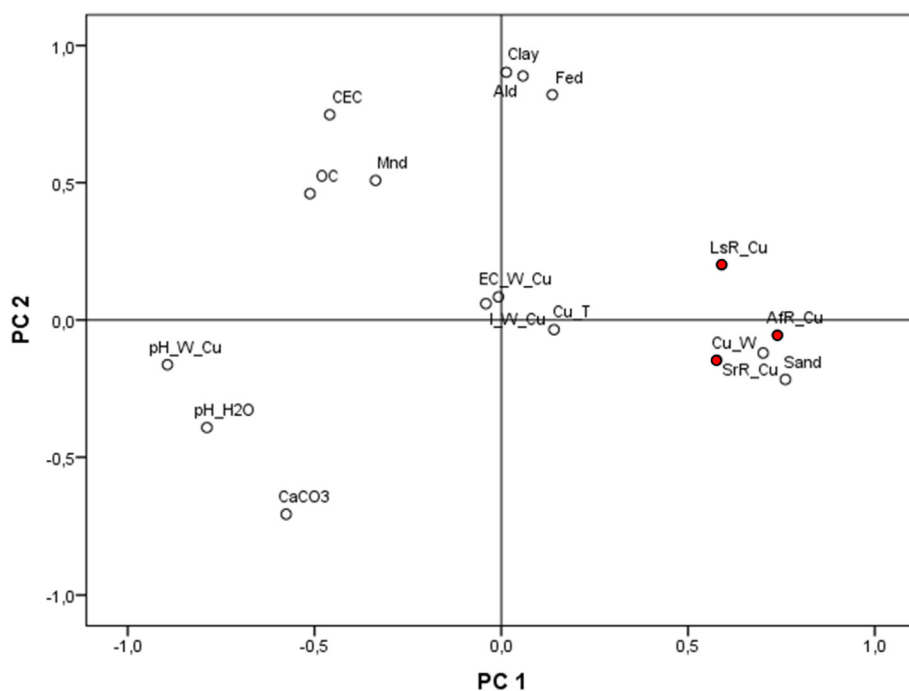


Fig. 2. Principal component analysis (PCA) for copper after Varimax normalization method including total Cu content (CuT), water-soluble Cu concentration (CuW), the main soil properties (CaCO₃: calcium carbonate; EC_W: electrical conductivity; CEC: cation exchange capacity; OC: organic carbon content; Al_d/Fe_d/Mn_d: free aluminum, iron and manganese forms) and the three toxicity endpoints (red circles) (LsR: *Lactuca sativa* Reduction, SrR: Soil respiration Reduction and AFR: *Aliivibrio fischeri* Reduction). Percentage of variance explained: PC1 = 30.2%, PC2 = 24.6%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect (Martínez and Motto, 2000; Simón et al., 2010). However, soil carbonates have a limited capacity of controlling Zn water solubilization and severe Zn soil pollution can exceed thresholds of toxicological concern even in these soil horizons (Jacquat et al., 2008; Díez et al., 2009).

Soils polluted with the lowest Zn concentrations (from 600 to 1000 mg kg⁻¹) revealed the importance of soil cation-exchange capacity (CEC) regarding to Zn water solubilization. In this sense, both carbonate (CC-Ah-CK-Ck) and non-carbonate (LC-Bw-CL-Bt) soil horizons, registered higher ZnW concentrations at lower CEC values. The cation-exchange complex depends heavily on the organic carbon and clay content, in addition to having an important role in Zn water solubilization, but when this mechanism is saturated, CaCO₃ and pH are the soil properties that control Zn water solubility. In this sense, Jacquat et al. (2008) reported that in neutral soils, organic matter, clay minerals, and carbonates become more influential than pH.

In non-carbonate soil horizons, sample LR-Ah (organic-rich) with neutral pH presented the lowest water solubility of Zn. This high capacity of the organic matter to retain Zn in soils has been previously reported (Song et al., 2009; Li et al., 2011; Asensio et al., 2013). In our samples, texture also correlated with Zn water solubility by a direct correlation with sand content; in general, small soil particles, and usually the clay content, are described as the optimum component in reducing Zn water solubility at pH < 7 (Veli and Alyüz, 2007). Only sample CL-Bt (neutral clay-soil horizon) contained significantly high clay content compared to the other soil horizons, and Zn water solubility differed from that of the other samples (carbonate and non-carbonate soil horizons). Thus, texture may also be a key factor determining Zn retention in non-carbonate soil horizons with low OC content. Soil horizons LC-Bw and LR-C1, which showed the highest water solubility also presented the worst soil properties for Zn retention, such as: high sand content, low content in calcium carbonates and low content in organic carbon, in agreement with previous studies (Zhang et al., 2006).

4.2. Water solubility of Cu

Copper showed lower water solubility than did Zn in all the soil horizons studied, in agreement with Zhang et al. (2006). The water

solubility of Cu also strongly depended on the soil properties, soil pH being the most important (Barna et al., 2007), as it could increase irreversible Cu sorption in soils with basic pH values (Vega et al., 2009). In our study, CuW and partitioning coefficient were statistically correlated with soil pH and calcium carbonates. Carbonate soil samples (CC-Ah, CC-Bw and CK-Ck) showed the lowest values of CuW, while non-carbonate samples LC-Bw and LR-C1 showed the highest water solubility. As in the case of Zn, the presence of carbonates reduced the water solubility of Cu. According to Shaheen et al. (2009), calcareous soils can retain higher amounts of Cu than can acidic soils due to the great adsorption capacity of carbonates, but they have a limited ability to buffer Cu and their retention capacity can be exceeded in highly contaminated sites (Díez et al., 2009). However, calcareous soil horizons polluted with Cu ranging from 300 to 4000 mg kg⁻¹ showed increasing K_d values and, therefore, soil pH and carbonate content appeared to be the soil properties that control Cu water solubility, reducing the proportion of CuW in relation to CuT concentrations.

Non-carbonate samples showed higher Cu water solubility than did calcareous ones. Within non-carbonate soil horizons, LR-Ah and CL-Bt were more efficient in controlling Cu water solubility than were LC-Bw and LR-C1. This may be due to the difference in soil properties and the mechanisms that influence Cu adsorption on soils. In non-carbonate soil horizons with pH ranging from neutrality to light acidity, the soil-exchange complex is the main property responsible of Cu fixation. Therefore, the LR-Ah and CL-Bt soil horizons, with high organic carbon and clay content, respectively, can significantly reduce the CuW concentration in relation to LC-Bw and LR-C1 soil horizons. The adsorption effectiveness of organic carbon and clay has been reported elsewhere (Yin et al., 2002; Barna et al., 2007; Veli and Alyüz, 2007; Clemente et al., 2008; Moreno et al., 2009; Fernández-Calviño et al., 2015). However, LR-Ah and CL-Bt soil horizons showed marked differences in Cu water solubilization, as reflected in the K_d data. Organic matter (LR-Ah) appeared to be more effective in Cu adsorption, reducing the proportion of CuW in relation to CuT concentrations, while the clayey soil horizon (CL-Bt) showed a reduction in K_d values with greater Cu pollution, even though CL-Bt had the highest Fe oxide content, which heavily influences Cu retention (Petrangeli Papini et al., 2004; Shaheen et al., 2009). On the other hand, LC-Bw and LR-C1 are the soil horizons most sensitive to Cu water solubility due to the low organic carbon

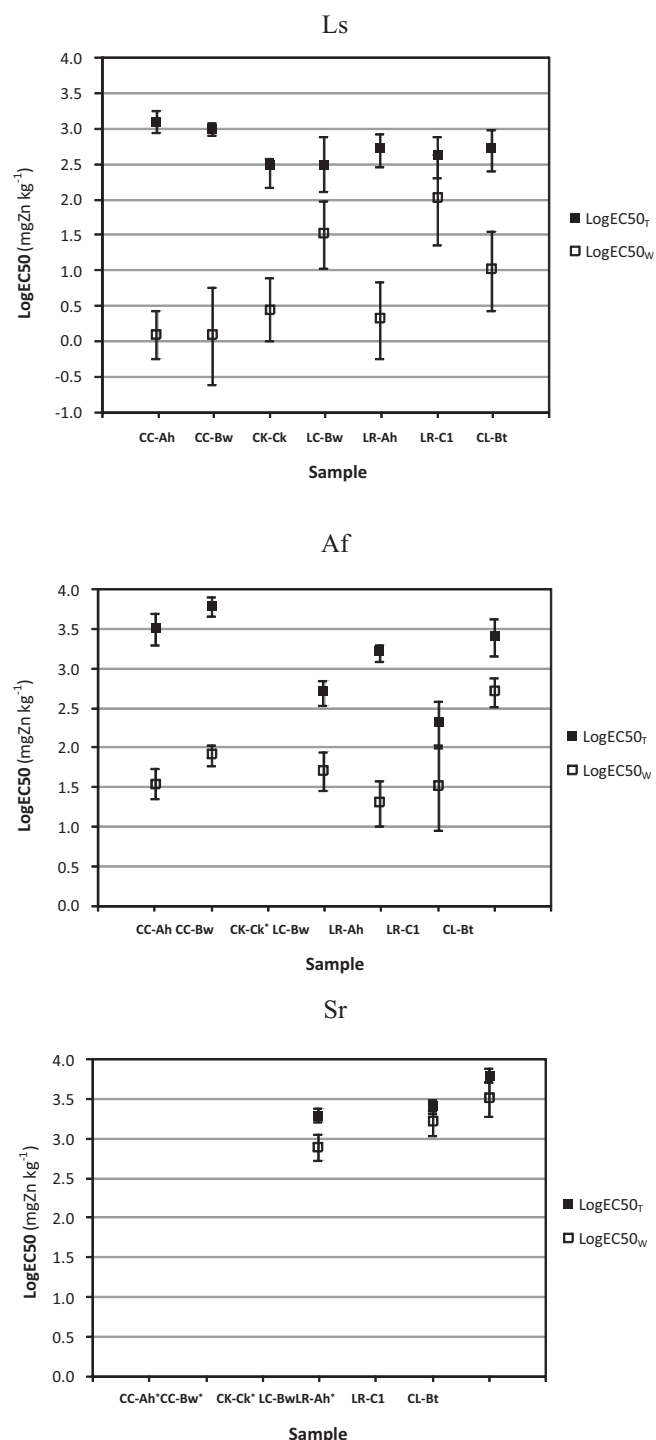


Fig. 3. Median effective concentrations (\pm 95% confidence interval; $n = 3$) calculated from total ($EC50_T$) and water-extractable metal content ($EC50_W$) for the different toxicity tests performed in 7 soil samples spiked with Zn. Samples with asterisk did not reach $EC50$ in the range of studied concentrations (Ls: *Lactuca sativa* test; Af: *Aliivibrio fischeri* test; Sr: Soil respiration test).

content and cation-exchange capacity, especially LR-C1 with a sandy texture (Favas et al., 2011).

4.3. Zinc-toxicity assessment

The range of toxicity thresholds of the toxicity tests is given in Fig. 3. A general correlation between $EC50$ values and the soil horizon was found among the different toxicity tests, with higher values in

carbonate soil horizons (CC-Ah, CC-Bw and CK-Ck) and lower values in non-carbonate soil horizons (LC-Bw, LR-Ah, LR-C1 and CL-Bt). However, significant differences appeared in the $EC50$ values depending on the degree of sensitivity of the organism tested. In carbonate samples, the Ls test rendered the highest sensitivity ($EC50$ ranging from 316 to 1259 $mg\ kg^{-1}$); the Af test showed moderate sensitivity ($EC50$ ranging from 3162 to $> 9000\ mg\ kg^{-1}$); and Sr was the least sensitive test, with thresholds exceeding the highest concentration tested ($> 9000\ mg\ kg^{-1}$). In non-carbonate samples, all tests were highly consistent, with samples LR-Ah and CL-Bt (soil horizons rich in OC and clay, respectively) presenting higher $EC50$ values (lower toxicity) than LC-Bw and LR-C1 samples (soil horizons with no significant properties to reduce Zn water solubility).

The differences in $EC50$ values in the Ls test in relation to the other tests could be related to the fact that accumulation in plants is better described by bioavailable rather than the total concentration (García-Gómez et al., 2014) and also, at low concentrations of Zn, as an essential element, can show uptake regulation (Heemsbergen et al., 2010). In addition, although the accumulation of Zn in plants depends on the type and nature of the plant, it also depends mainly on the soil texture (Gebrekidan et al., 2013) and clay content (Tiecher et al., 2016), the latter being detected in the non-carbonate soil horizons tested in this study.

In the Af test, our results suggest that in carbonate-rich soil horizons, *A. fischeri* can tolerate high total Zn concentrations without showing toxic effects. By contrast, toxic effects from low Zn concentrations were detected for the sandy and acidic samples LR-C1 and LC-Bw. Moreover, as in the case of Sr test, the toxicity of zinc in non-carbonate soil horizons was influenced by organic carbon content, as also reported in other studies (Tsiridis et al., 2006).

Soil respiration is strongly influenced by moisture, temperature and soil characteristics (Dinesh and Chaudhuri, 2013), the organic carbon content being a component that highly influences soil respiration response (Bahn et al., 2008). In our study, the sample with the highest OC content in non-carbonate soil horizons presented the lowest toxicity, indicating that soil horizons with low organic matter content are more susceptible to toxicity response in this test (Renella et al., 2007; Moreno et al., 2009) since microbial population in soils with high content in organic matter can withstand better the stress of heavy-metal contamination.

4.4. Copper-toxicity assessment

A general relationship between $EC50$ values and sample was observed among the different toxicity tests, with higher values (lower toxicity) in carbonate soil horizons (CC-Ah, CC-Bw and CK-Ck) and lower values (higher toxicity) in non-carbonate soil horizons (LC-Bw, LR-Ah, LR-C1 and CL-Bt). However, lower toxicity than Zn was found for the Cu-spiked samples in the three tests applied, with $EC50$ values exceeding the highest concentration tested ($> 4000\ mg\ kg^{-1}$) in most cases for carbonate and LR-Ah soil horizons.

The Ls test showed pronounced differences in $EC50$ values among soil horizons. Copper accumulation in plants is also mostly explained by the bioavailable metal concentrations, since plants uptake the nutrients from the soil solution (McLaughlin et al., 2000). However, Cu is an essential micronutrient for plants and therefore they closely regulate its uptake (Heemsbergen et al., 2010); this could be related to the minor reduction in elongation at lower concentrations of Cu or to the hormesis effects observed in LR-C1 soil horizon.

The Af test for Cu indicated great differences among treatments and samples, but low sensitivity to Cu toxicity was found, as in the other tests. The values found can be explained by the influence of Cu water solubility, and the influence of soil pH and $CaCO_3$ content. Also, the role of the organic matter to retain Cu is well known (Minkina et al., 2014), this occurring in our study in the non-carbonate soil horizons.

The Sr test for Cu registered differences among soil horizons,

presenting higher sensitivity than in the case of Zn. Organic matter has been reported as main sink for metals, and therefore the Cu effect can be counteracted by reducing its bioavailability, allowing microbial growth and boosting basal respiration (Nwachukwu and Pulford, 2011). Soil pH is directly related to CaCO₃ and reportedly has great regulatory capacity for sorption, exchangeability, and metal precipitation (Azarbad et al., 2013), in agreement with our results. Texture is another important parameter governing metal sorption (Ma and Uren, 1998), since sandy-textured soils have less capacity for cation sorption and therefore can show higher toxicity, as clearly reflected in our study.

4.5. Conclusion

Toxicity tests, like those performed in this study, can be used to recommend regulatory risk limits for potential pollution of Zn and Cu in soil. However, substantial differences in the values were found in this work, due not only to the high diversity of soil properties but also to the different behaviour of soil organisms. In the case of Zn and Cu, soil ecotoxicological assessment using water-extractable concentrations differs from the results using total concentrations. Regulatory frameworks frequently use only the total concentrations to declare a soil contaminated, but they should incorporate the main soil properties and metal mobile fraction (we recommend the use of water-extractable forms for their simplicity and good correlation with toxicity tests). Moreover, in the case of these two potentially toxic elements, the influence of soil properties like pH and organic carbon should be also considered for regulatory purposes; the establishment of different regulatory limits based on these soil properties is advisable. Otherwise, the use of different organisms of diverse taxonomic groups in toxicity bioassays is also recommended for a proper predictive risk assessment of soils contaminated by metals. The *Lactuca sativa* (Ls) and *Alivibrio fischeri* (Af) toxicity tests use the soil aqueous extract and correlate well, although according to the organism tested, the Ls (using a terrestrial plant), would be strongly recommended over the Af (using a marine bacterium) test. The soil-respiration test presented lower sensitivity to Zn and Cu toxicity in relation to the other tests, so its use in toxicity-risk assessment would be less advisable.

Acknowledgements

Authors thank the financial support of this work by the Project CGL2010-19902 and Grant FPI-MICINN BES-2011-045101. Also thanks to Mr. David Nesbitt for the English corrections and comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2018.12.016>.

References

Adriano, D.C., Wenzel, W.W., Vangronsveld, J., Bolan, N.S., 2004. Role of assisted natural remediation in environmental cleanup. *Geoderma* 122 (2), 121–142.

Agency for Toxic Substances and Disease Registry (ATSDR), 2004. Interaction Profile for Lead, Manganese, Zinc, Copper. U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA, pp. 140.

Aguiar, J., Gómez, J.L., Galán, E., 1999. Criterios y estándares para declarar un suelo contaminado en Andalucía y la metodología y técnicas de toma de muestra y análisis para su investigación. *Consejería de Medio Ambiente de la Junta de Andalucía*.

Alloway, B.J., 1995. The origins of heavy metals in soils. In: Alloway, B.J. (Ed.), *Heavy Metals in Soils*. Blackie Academic and Professional, Glasgow, UK.

American Society for Testing and Materials (ASTM), 2004. Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test With a Luminescent Marine Bacterium, USA. pp. D5660–D5696.

Asensio, V., Vega, F.A., Singh, B.R., Covelo, E.F., 2013. Effects of tree vegetation and waste amendments on the fractionation of Cr, Cu, Ni, Pb and Zn in polluted mine soils. *Sci. Total Environ.* 443, 446–453.

Azarbad, H., Niklinska, M., Van Gestel, C.A.M., Van Straalen, N.M., Roling, W.F.M., Laskowski, R., 2013. Microbial community structure and functioning along metal pollution gradients. *Environ. Toxicol. Chem.* 32, 1992–2002.

AZUR Environmental, 1998. The Microtox® Acute Basic, DIN, ISO and Wet Test Procedure. (Carlsbad, Calif, USA).

Bagur, M.G., Estepa, C., Martín, F., Morales, S., 2010. Toxicity assessment using *Lactuca sativa* L. bioassay of the metal(loid)s As, Cu, Mn, Pb and Zn in soluble-in-water saturated soil extracts from an abandoned mining site. *J. Soils Sediments* 11, 281–289.

Bahn, M., Rodeghiero, M., Anderson-Dun, M., Dore, S., Gimeno, C., Dröser, M., Williams, M., Ammann, C., Berninger, F., Flechard, C., Jones, S., Balzarolo, M., Kumar, S., Newesely, C., Privitzer, T., Raschi, A., Siegwolf, R., Susiluoto, S., Tenhunen, J., Wohlfahrt, G., Cernusca, A., 2008. Soil respiration in European grasslands in relation to climate and assimilate supply. *Ecosystems* 11 (8), 1352–1367.

Barna, R., Fernandez, A., Hlavackova, P., 2007. Assessment methodologies for copper and zinc mobility in a neutral synthetic soil: the influence of pH. *Colloids Surf. A Physicochem. Eng. Asp.* 306 (1), 56–67.

Barry, M.J., 2011. Effects of copper, zinc and dragonfly kairomone on growth rate and induced morphology of *Bufo arabicus* tadpoles. *Ecotoxicol. Environ. Saf.* 74 (4), 918–923.

Blaser, P., Zimmermann, S., Luster, J., Shoty, W., 2000. Critical examination of trace element enrichments and depletions in soils: As, Cr, Cu, Ni, Pb, and Zn in Swiss forest soils. *Sci. Total Environ.* 249 (1), 257–280.

Burgos, P., Madejón, E., Pérez-de-Mora, A., Cabrera, F., 2008. Horizontal and vertical variability of soil properties in a trace element contaminated area. *Int. J. Appl. Earth Obs. Geoinf.* 10 (1), 11–25.

Chary, N.S., Kamala, C.T., Raj, D.S.S., 2008. Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicol. Environ. Saf.* 69 (3), 513–524.

Cherfi, A., Abdoun, S., Gaci, O., 2014. Food survey: levels and potential health risks of chromium, lead, zinc and copper content in fruits and vegetables consumed in Algeria. *Food Chem. Toxicol.* 70, 48–53.

Clemente, R., Dickinson, N.M., Lepp, N.W., 2008. Mobility of metals and metalloids in a multi-element contaminated soil 20 years cessation of the pollution source activity. *Environ. Pollut.* 155, 254–261.

Costa, S.P., Pinto, P.C., Lapa, R.A., Saraiva, M.L.M., 2015. Toxicity assessment of ionic liquids with *Vibrio fischeri*: an alternative fully automated methodology. *J. Hazard. Mater.* 284, 136–142.

Diez, M., Simón, M., García, I., Martín, F., 2009. Assessment of the critical load of trace elements in soils polluted by pyrite tailings. A laboratory experiment. *Water Air Soil Pollut.* 199 (1–4), 381–387.

Dinesh, R., Chaudhuri, S.G., 2013. Soil biochemical/microbial indices as ecological indicators of land use change in mangrove forests. *Ecol. Indic.* 32, 253–258.

Doelman, P., Haanstra, L., 1989. Short-and long-term effects of heavy metals on phosphate activity in soils: an ecological dose-response model approach. *Biol. Fertil. Soils* 8 (3), 235–241.

Donner, E., Broos, K., Heemsbergen, D., Warne, M.S.J., McLaughlin, M.J., Hodson, M.E., Nortcliff, S., 2010. Biological and chemical assessments of zinc ageing in field soils. *Environ. Pollut.* 158 (1), 339–345.

Escoto, M., Fernández, J., Martín, F., 2007. Determination of phytotoxicity of soluble elements in soils, based on a bioassay with lettuce (*Lactuca sativa*). *Sci. Total Environ.* 378, 63–66.

Favas, P.J., Pratas, J., Gomes, M.E.P., Cala, V., 2011. Selective chemical extraction of heavy metals in tailings and soils contaminated by mining activity: environmental implications. *J. Geochem. Explor.* 111 (3), 160–171.

Fernández-Calviño, D., Rodríguez-Salgado, I., Pérez-Rodríguez, P., Nóvoa-Muñoz, J.C., Arias-Estévez, M., 2015. Time evolution of the general characteristics and Cu retention capacity in an acid soil amended with a bentonite winery waste. *J. Environ. Manag.* 150, 435–443.

García-Gómez, C., Esteban, E., Sánchez-Pardo, B., Fernández, M.D., 2014. Assessing the ecotoxicological effects of long-term contaminated mine soils on plants and earthworms: relevance of soil (total and available) and body concentrations. *Ecotoxicology* 23 (7), 1195–1209.

Gebrekidan, A., Weldegebriel, Y., Hadera, A., Van der Bruggen, B., 2013. Toxicological assessment of heavy metals accumulated in vegetables and fruits grown in Ginfel river near Sheba Tannery, Tigray, Northern Ethiopia. *Ecotoxicol. Environ. Saf.* 95, 171–178.

Hänsch, M., Emmerling, C., 2010. Effects of silver nanoparticles on the microbiota and enzyme activity in soil. *J. Plant Nutr. Soil Sci.* 173 (4), 554–558.

Heemsbergen, D.A., McLaughlin, M.J., Whatmuff, M., Warne, M.S.J., Broos, K., Bell, M., Nash, D., Barry, G., Pritchard, D., Penney, N., 2010. Bioavailability of zinc and copper in biosolids compared to their soluble salts. *Environ. Pollut.* 158 (5), 1907–1915.

Holmgren, G., 1967. A rapid citrate-dithionite extractable iron procedure. *Soil Sci. Soc. Am. Proc.* 38, 647–652.

ISO 17155, 2002. Soil Quality. Determination of Abundance and Activity of Soil Microflora Using Respiration Curves. pp. 12.

IUSS Working Group WRB, 2006. World reference base for soil resources 2006. In: *World Soil Resources Reports No. 103*. FAO, Rome.

Jacquet, O., Voegelin, A., Villard, A., Marcus, M.A., Kretschmar, R., 2008. Formation of Zn-rich phyllosilicate, Zn-layered double hydroxide and hydrozincite in contaminated calcareous soils. *Geochim. Cosmochim. Acta* 72 (20), 5037–5054.

Kabata-Pendias, A., 2011. *Trace Elements in Soils and Plants*, 4th edition. CRC, Boca Raton.

Kraus, U., Wiegand, J., 2006. Long-term effects of the Aznalcóllar mine spill-heavy metal content and mobility in soils and sediments of the Guadiamar river valley (SW Spain). *Sci. Total Environ.* 367, 855–871.

Leitig, L., Kálmán, J., Gruiz, K., 2007. Comparison of bioassays by testing whole soil and the water extract from contaminated sites. *Chemosphere* 66 (3), 428–434.

Li, T., Di, Z., Yang, X., Sparks, D.L., 2011. Effects of dissolved organic matter from the rhizosphere of the hyperaccumulator *Sedum alfredii* on sorption of zinc and cadmium

- by different soils. *J. Hazard. Mater.* 192 (3), 1616–1622.
- Li, Z., Ma, Z., van der Kuijp, T.J., Yuan, Z., Huang, L., 2014. A review of soil heavy metal pollution from mines in China: pollution and health risk assessment. *Sci. Total Environ.* 468, 843–853.
- Ma, Y.B., Uren, N.C., 1998. Transformations of heavy metals added to soil—application of a new sequential extraction procedure. *Geoderma* 84 (1), 157–168.
- Madejón, E., De Mora, A.P., Felipe, E., Burgos, P., Cabrera, F., 2006. Soil amendments reduce trace element solubility in a contaminated soil and allow regrowth of natural vegetation. *Environ. Pollut.* 139 (1), 40–52.
- MAPA (Ministerio de Agricultura, Pesca y Alimentación), 1994. Métodos Oficiales de Análisis. Tomo III Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación (MAPA), Madrid.
- Martín, F., Escoto, M., Fernández, J., Fernández, E., Arco, E., Sierra, M., Dorronsoro, C., 2010. Toxicity assessment of sediments with natural anomalous concentrations in heavy metals by the use of bioassay. *Int. J. Chem. Eng.* 1–6.
- Martín, J.R., Ramos-Miras, J.J., Boluda, R., Gil, C., 2013. Spatial relations of heavy metals in arable and greenhouse soils of a Mediterranean environment region (Spain). *Geoderma* 200, 180–188.
- Martínez, C.E., Motto, H.L., 2000. Solubility of lead, zinc and copper added to mineral soils. *Environ. Pollut.* 107 (1), 153–158.
- McLaughlin, M.J., Zarcinas, B.A., Stevens, D.P., Cook, N., 2000. Soil testing for heavy metals. *Commun. Soil Sci. Plant Anal.* 31 (11–14), 1661–1700.
- Minkina, T.M., Soldatov, A.V., Motuzova, G.V., Podkovyrina, Y.S., Nevidomskaya, D.G., 2014. Speciation of copper and zinc compounds in artificially contaminated chernozem by X-ray absorption spectroscopy and extractive fractionation. *J. Geochem. Explor.* 144, 306–311.
- Mirzaei, R., Ghorbani, H., Moghaddas, N.H., Martín, J.A.R., 2014. Ecological risk of heavy metal hotspots in topsoils in the Province of Golestan, Iran. *J. Geochem. Explor.* 147, 268–276.
- Moreno, E., Peñalosa, J.M., Manzano, R., Carpena-Ruiz, R.O., Gamarra, R., Esteban, E., 2009. Heavy metals distribution in soils surrounding an abandoned mine in NW Madrid (Spain) and their transference to wild flora. *J. Hazard. Mater.* 162 (2), 854–859.
- Nannipieri, P., Ascher, J., Ceccherini, M., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. *Eur. J. Soil Sci.* 54 (4), 655–670.
- Nicholson, F.A., Smith, S.R., Alloway, B.J., Carlton-Smith, C., Chambers, B.J., 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. *Sci. Total Environ.* 311 (1), 205–219.
- Nwachukwu, O.I., Pulford, I.D., 2011. Microbial respiration as an indication of metal toxicity in contaminated organic materials and soil. *J. Hazard. Mater.* 185, 1140–1147.
- Petrangeli Papini, M., Saurini, T., Bianchi, A., Majone, M., Beccari, M., 2004. Modeling the competitive adsorption of Pb, Cu, Cd, and Ni onto a natural heterogeneous sorbent material (Italian “red soil”). *Ind. Eng. Chem. Res.* 43 (17), 5032–5041.
- Plum, L.M., Rink, L., Haase, H., 2010. The essential toxin: impact of zinc on human health. *Int. J. Environ. Res. Public Health* 7 (4), 1342–1365.
- Renella, G., Chaudri, A.M., Falloon, C.M., Landi, L., Nannipieri, P., Brookes, P.C., 2007. Effects of Cd, Zn, or both on soil microbial biomass and activity in a clay loam soil. *Biol. Fertil. Soils* 43 (6), 751–758.
- Ribo, J.M., Kaiser, K.L., 1987. *Photobacterium phosphoreum* toxicity bioassay. I. Test procedures and applications. *Environ. Toxicol.* 2 (3), 305–323.
- Rieuwerts, J.S., Thornton, I., Farago, M.E., Ashmore, M.R., 1998. Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. *Chem. Speciat. Bioavailab.* 10 (2), 61–75.
- Romero-Freire, A., 2016. Safe levels for ecological risk assessment of soils polluted by metals: from normative to field (European Union Case). *Environ. Pollut. Clim. Chang.* 1, 101.
- Romero-Freire, A., Peinado, F.M., van Gestel, C.A.M., 2015. Effect of soil properties on the toxicity of Pb: assessment of the appropriateness of guideline values. *J. Hazard. Mater.* 289, 46–53.
- Romero-Freire, A., Sierra, M., Martínez, F.J., Martín, F., 2016. Is soil basal respiration a good indicator of soil pollution? *Geoderma* 263, 132–139.
- Shaheen, S.M., Tsadilas, C.D., Mitsibonas, T., Zouvalakas, M., 2009. Distribution coefficient of copper in different soils from Egypt and Greece. *Commun. Soil Sci. Plant Anal.* 40, 214–226.
- Simón, M., García, I., 1999. Physico-chemical properties of the soil-saturation extracts: estimation from electrical conductivity. *Geoderma* 90, 99–109.
- Simón, M., Díez, M., González, V., García, I., Martín, F., de Haro, S., 2010. Use of liming in the remediation of soils polluted by sulphide oxidation: a leaching-column study. *J. Hazard. Mater.* 180 (1), 241–246.
- Smolders, E., Oorts, K., van Sprang, P., Schoeters, I., Janssen, C.R., McGrath, S.P., McLaughlin, M.J., 2009. Toxicity of trace metals in soil as affected by soil type and aging after contamination: using calibrated bioavailability models to set ecological soil standards. *Environ. Toxicol. Chem.* 28 (8), 1633–1642.
- Sołek-Podwika, K., Ciarkowska, K., Kaleta, D., 2016. Assessment of the risk of pollution by sulfur compounds and heavy metals in soils located in the proximity of a disused for 20 years sulfur mine (SE Poland). *J. Environ. Manag.* 180, 450–458.
- Song, J., Zhao, F.J., McGrath, S.P., Luo, Y.M., 2006. Influence of soil properties and aging on arsenic phytotoxicity. *Environ. Toxicol. Chem.* 25 (6), 1663–1670.
- Song, J., Guo, Z.H., Xiao, X.Y., Miao, X.F., Wang, F.Y., 2009. Environmental availability and profile characteristics of arsenic, cadmium, lead and zinc in metal-contaminated vegetable soils. *Trans. Nonferrous Metals Soc. China* 19 (3), 765–772.
- Tiecher, T.L., Ceretta, C.A., Ferreira, P.A., Lourenzi, C.R., Tiecher, T., Giroto, E., Nicoloso, F.T., Soriani, H.H., De Conti, L., Mimmo, T., Cesco, S., Brunetto, G., 2016. The potential of *Zea mays* L. in remediating copper and zinc contaminated soils for grapevine production. *Geoderma* 262, 52–61.
- Torres, M., 2003. Empleo de los ensayos con plantas en el control de contaminantes tóxicos ambientales. *Rev. Cuba. Hig. Epidemiol.* 41, 2–3.
- Tsiridis, V., Petala, M., Samaras, P., Hadjispyrou, S., Sakellaropoulos, G., Kungolos, A., 2006. Interactive toxic effects of heavy metals and humic acids on *Vibrio fischeri*. *Ecotoxicol. Environ. Saf.* 63 (1), 158–167.
- US EPA, 1996. Ecological effects test guidelines. In: Seed Germination/Root Elongation Toxicity Test. US Environmental Protection Agency, Office of Prevention, Pesticide and Toxic Substances (OPPTS) 850.4200, Washington DC (EPA 712-C-96-154).
- Van Gestel, C.A., 2008. Physico-chemical and biological parameters determine metal bioavailability in soils. *Sci. Total Environ.* 406 (3), 385–395.
- Vanewijk, P.H., Hoekstra, J.A., 1993. Calculation of the EC50 and its confidence interval when subtoxic stimulus is present. *Ecotoxicol. Environ. Saf.* 25 (1), 25–32.
- Vega, F.A., Covelo, E.F., Andrade, M.L., 2009. Hysteresis in the individual and competitive sorption of cadmium, copper, and lead by various soil horizons. *J. Colloid Interface Sci.* 331 (2), 312–317.
- Veli, S., Alyüz, B., 2007. Adsorption of copper and zinc from aqueous solutions by using natural clay. *J. Hazard. Mater.* 149 (1), 226–233.
- Yin, Y., Impellitteri, C.A., You, S.J., Allen, H.E., 2002. The importance of organic matter distribution and extract soil: solution ratio on the desorption of heavy metals from soils. *Sci. Total Environ.* 287 (1), 107–119.
- Zhang, M.K., Zhen-Li, H.E., Calvert, D.V., Stoffella, P.J., 2006. Extractability and mobility of copper and zinc accumulated in sandy soils. *Pedosphere* 16 (1), 43–49.