Environ Sci Pollut Res (2014) 21:6176–6187
DOI 10.1007/s11356-014-2561-0

RESEARCH ARTICLE

Biological activity in metal-contaminated calcareous agricultural soils: the role of the organic matter composition and the particle size distribution

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Received: 23 July 2013 / Accepted: 16 January 2014 / Published online: 30 January 2014

Abstract Organic matter (OM) plays a key role in microbial response to soil metal contamination, yet little is known about how the composition of the OM affects this response in Mediterranean calcareous agricultural soils. A set of Mediterranean soils, with different contents and compositions of OM and carbonate and fine mineral fractions, was spiked with a mixture of Cd, Cu, Pb, and Zn and incubated for 12 months for aging. Microbial (Biolog Ecoplates) and enzyme activities (dehydrogenase, DHA; β-galactosidase, BGAL; phosphatase, PHOS; and urease, URE) were assessed and related to metal availability and soil physicochemical parameters. All enzyme activities decreased significantly with metal contamination: 36–68 % (DHA), 24–85 % (BGAL), 22–72 % (PHOS), and 14–84 % (URE) inhibitions. Similarly, catabolic activity was negatively affected, especially phenol catabolism (~86 % compared to 25–55 % inhibition for the rest of the substrates). Catabolic and DHA activities were negatively correlated with ethylenediaminetetraacetic acid (EDTA)-extractable Cd and Pb, but positively with $CaCl_2$, $NaNO_3$, and DTPA-extractable Cu and Zn. Soluble OM (water- and hot-water-soluble organic C) was positively related to enzyme and catabolic activities. Recalcitrant OM and fine mineral fractions were positively related to BGAL and PHOS. Conversely, catabolic activity was negatively related to clay and positively to silt and labile OM. Results indicate that the microbial response to metal contamination is highly affected by texture and OM composition.

Keywords Microbial activity · Enzyme activity · Calcareous soils · Organic matter composition · Heavy metals · Metal availability

Responsible editor: Zhihong Xu

Electronic supplementary material The online version of this article (doi:10.1007/s11356-014-2561-0) contains supplementary material, which is available to authorized users.

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Introduction

Heavy metal content has increased in Mediterranean periurban agricultural soils in recent decades due to agricultural, industrial, and urban activity (Roca-Perez et al. 2010). Soil microorganisms are the most sensitive part of the soil system and rapidly respond to any disturbance in the ecosystem, thus leading to their consideration as good indicators of soil quality. Hence, the analysis of enzyme activity and the activity and diversity of microbial communities is frequently used to monitor the effect of any disruption on the soil system (Kızılkaya 2004). The ability of microbial populations to resist and recover from perturbations is highly affected by some soil constituents, which could provide physical protection for microbes, nutrients, and enzymes (Kızılkaya et al. 2004; Turner et al. 2002). It is therefore necessary to consider the intrinsic soil characteristics of each contaminated site in order to

properly monitor the microbial response to metal stress. Mediterranean soils often show relatively high values of pH and carbonate content which confers a high metal sorption capacity and therefore a low risk of metal toxicity. Nevertheless, previous works have shown that there is a significant fraction of potentially available metals in calcareous Mediterranean soils (de Santiago-Martín et al. 2013a, b). These authors also reported that the metal availability patterns in calcareous soils cannot be explained by the pH value nor the carbonate content alone, but must be viewed in combination with the content and composition of organic matter (OM) and fine mineral fraction, thus highlighting the need for further study of these soil fractions and their role in the metal (bio) availability. Interestingly, although soils under Mediterranean environmental conditions commonly have a low content in OM, the content and composition of soil OM have been shown to play a key role in governing metal (bio) availability, which directly affects soil microbial populations. Nevertheless, there is very little information on the implication of the composition of indigenous OM in the microbial response to soil metal contamination in calcareous soils in the Mediterranean area.

In order to assess the composition of OM, it must be noted that soil OM is constituted by the sum of an infinite number of pools of different qualities that define the ability of OM to be used by soil microorganisms. For practical purposes, this pool number should be reduced to a finite number which provides a quick and easy assessment of the biochemical quality of the soil OM. Ghani et al. (2003) proposed a two-step procedure for extracting water-soluble C (WSC) and hot-water-extractable C. These authors showed that the C extracted by the hot water method is highly available to soil microorganisms and a good indicator of soil quality. The quality of soil OM can also be assumed to depend on its distribution among labile pool (LP) and recalcitrant pool (RP) and the quality of each of these pools. With this perspective, Rovira and Vallejo (2000) proposed a procedure based on a two-step hydrolysis with H_2SO_4 to quantify the abundance of labile and recalcitrant pools.

In the present work, we have selected a group of Mediterranean peri-urban agricultural calcareous soils with different contents and compositions of OM, carbonate, and fine mineral fractions. Soils were spiked with a mixture of Cd, Cu, Pb, and Zn. After 12 months of incubation for aging, we evaluated (1) the microbial response to metal contamination (enzyme activity, functional diversity, and microbial catabolic activity); (2) the relationship between microbial response and metal availability (estimated with chemical extractants of different strengths); and (3) the role played by soil constituents in the microbial response, with a particular focus on the content and composition of the OM using the procedures proposed by Ghani et al. (2003) and Rovira and Vallejo (2000).

Materials and methods

Study area, soil sampling, and soil characteristics

The study area is located in central Spain (Alcalá de Henares, Madrid), within the European Mediterranean region, as described elsewhere (de Santiago-Martín et al. 2013a). We selected an agricultural area which has undergone an expansion in its industrial–residential use in recent decades, in common with numerous other European metropolitan areas. We took a set of nine unpolluted calcareous agricultural soil samples, with a narrow range of pH values (8.1–8.7), which have a potential risk of metal contamination due to their location. Three groups of soil samples were established according to their content in equivalent $CaCO_3$ (ECC): soils M1, M2, M3, M4, and M5 (>100 g kg^{-1} ECC), soils L1 and L2 (~100 g kg^{-1} ECC), and soils VL1 and VL2 (<100 g kg^{-1} ECC). Each group differed in the content and composition of the organic matter fraction and in its particle size distribution (Table 1). Soil physicochemical parameters were determined according to International Soil Reference and Information Center (ISRIC 2002) methods. In order to characterize the composition of the organic matter for the present work, we quantified the total N, the WSC and hot-water-soluble C (HWC), and the labile I and II (LPI and LPII) and recalcitrant pools (RP) of organic matter, as detailed below. At each sampling point, ~40 kg of soil surface horizons (0–30 cm) was taken, homogenized, air-dried, and passed through a 2-mm sieve prior to analysis.

Experimental design

In order to mimic a potential scenario of metallic multipollution, the selected nine soil samples were spiked with a multi-elemental salt solution of Cd, Cu, Pb, and Zn as nitrate salt. Briefly, 10 kg of each soil sample was placed in plastic containers, and the metal mixture was added at the rates of 3 mg kg^{-1} of Cd, 140 mg kg^{-1} of Cu, 300 mg kg^{-1} of Pb, and 300 mg kg^{-1} of Zn within the limits proposed by current European legislation (Directive 86/278/EEC). Soil samples without metal spiking were prepared as the control. Each soil sample was incubated for a period of 12 months (at room temperature without cover or drainage) during which they were air-dried, mixed, and rewetted with deionized water in cycles of about 2 weeks, in order to favour metal redistribution processes into the soil matrix. The appropriate amount of deionized water was added to bring the soil samples to each estimated field capacity. The incubation period of 12 months was chosen in accordance with the previous studies where it was observed that although the $NaNO_3$-extractable metal concentration values reached equilibrium within the first 6 months of incubation, this time was extended to 12 months for metals extracted with diethylene triamine pentaacetic acid (DTPA) (de Santiago-Martín et al. 2013b). Indeed, a closer relationship

Table 1 Physicochemical parameters of the un-spiked soil samples

Soil sample	pH	Carbonate fraction	Organic fraction								Oxide fraction		Particle size distribution			
		ECC[a] g kg^{-1}	TOC[a] g kg^{-1}	OM[a]	N	WSC	HWC	LPI % of TOC	LPII	RP[a]	Cry-Fe[a] g kg^{-1}	Am-Fe	CS[a] g kg^{-1}	FS[a]	Silt[a]	Clay[a]
M1	8.4	190	6	10	0.7	0.1	0.3	17	14	69	8.1	0.2	111	603	126	161
M2	8.1	148	12	20	1.0	0.2	0.3	40	12	48	8.5	0.2	114	569	124	193
M3	8.1	125	18	31	1.7	0.5	0.5	10	24	66	11.7	0.5	23	592	215	170
M4	8.4	118	15	26	1.4	0.3	0.5	20	25	54	11.1	0.6	95	605	146	154
M5	8.7	117	10	17	0.7	0.2	0.4	18	24	57	8.0	0.4	166	567	129	139
L1	8.2	106	18	32	1.7	0.3	0.6	10	18	72	13.4	0.8	159	590	78	172
L2	8.1	100	8	13	0.8	0.2	0.4	14	11	75	10.3	0.3	112	462	167	259
VL1	8.1	32	12	21	1.1	0.4	0.5	13	15	71	11.4	0.7	45	459	168	328
VL2	8.2	27	12	21	1.1	0.2	0.3	9	17	74	12.2	0.7	99	386	172	344

ECC equivalent $CaCO_3$, *TOC* total organic C, *OM* organic matter, *WSC* water soluble C, *HWC* hot water-soluble C, *LPI* labile pool I, *LPII* labile pool II, *RP* recalcitrant pool, *Cry-Fe* crystalline Fe oxides, *Am-Fe* amorphous Fe oxides, *CS* coarse sand, *FS* fine sand

[a] de Santiago-Martín et al. (2013a)

between the DTPA-metal desorption patterns and the persistence of microbial activity to metal pollution in calcareous soils was reported, with the same soil components being involved (de Santiago-Martín et al. 2013c). After the equilibration period, duplicates were randomly removed from each of the 18 soil samples (nine un-spiked and nine metal-spiked duplicates) to assess enzyme activity (dehydrogenase (DHA), β-galactosidase (BGAL), phosphatase (PHOS), and urease (URE)) and microbial activity and functional microbial diversity (by means of substrate utilization patterns), as well as the mobile (neutral salts) and potentially mobile (chelating agents) fractions of metals.

Analytical methods

All chemicals were obtained from analytic grade reagents from Merck (Germany) and Sigma-Aldrich (St. Louis, MO, USA). All glassware used was pre-washed with an aqueous solution of HNO_3 0.1 % for 24 h and rinsed with deionized type I water (Water Purification System, Younglin, Aqua MAX-Basic 360 series).

Soil organic matter fractionation

The WSC and HWC fractions were determined according to the procedure in Ghani et al. (2003). The first step involves removal of readily soluble C from the soils that may have come from recent liming of the soil or from animal excreta and soluble plant residues, and the second step involves extraction of labile components of soil carbon at 80 °C for 16 h. Briefly, soil samples (3 g) were extracted with 30 mL of deionized water into centrifuge tubes for 30 min on an end-over-end shaker at 30 rpm. The supernatant was centrifuged at 3,500 rpm for 20 min and then filtered (0.45 μm cellulose nitrate membrane filter) to organic C analysis (WSC fraction). A further 30 mL of deionized water was added to the sediments in the same tubes. The tubes were left for 16 h in a hot-water bath at 80 °C and then were centrifuged at 3,500 rpm for 20 min and filtered (0.45-μm cellulose nitrate membrane filters) to organic C analysis (HWC fraction). The LPI, LPII, and RP of organic matter were determined according to the two-step acid hydrolysis procedure proposed by Rovira and Vallejo (2000). The LPI fraction basically corresponds to sugars, amino acids, and fatty acids with low molecular weight; the labile fraction LPII contains compounds with a greater degree of polymerization; and the RP fraction contains compounds with a high molecular weight (polymers of lipidic nature, fats, waxes, resins, suberins, and lignin). Briefly, soil samples (0.5 g) were hydrolyzed with 20 mL of 5 N H_2SO_4 in centrifuge tubes for 30 min at 105 °C. The hydrolysate (LPI) was recovered by centrifugation at 3,500 rpm for 15 min. The residue was washed with water and dried. Then, 2 mL of 26 N H_2SO_4 was added, and the tubes were shaken overnight in a vibrator agitator (Vibromatic, Selecta) at 400 oscillations per minute (opm). After diluting the acid with water to 2 N, the residue was hydrolyzed for 3 h at 105 °C. The hydrolysate (LPII) was also recovered by centrifugation. The residue (RP) was washed again, dried at 60 °C, and weighed. The content of organic C was determined on a Shimadzu total organic carbon (TOC) analyser model 5000A or by wet oxidation according to the method of Walkley and Black (ISRIC 2002).

One-step metal extraction methods

In order to estimate the immediately mobile (neutral salt extraction techniques) and potentially mobile fractions

(chelating agent solutions) (Gupta et al. 1996) of metals in both un-spiked and metal-spiked soil samples, four one-step extraction methods were selected as described by de Santiago-Martín et al. (2013a). The mobile fraction was estimated with 0.01 M $CaCl_2$ and 0.1 M $NaNO_3$ methods, and the potentially mobile fraction was estimated with 5 mM DTPA and 0.05 M ethylenediaminetetraacetic acid (EDTA) methods. In brief, 0.01 mol L^{-1} $CaCl_2$ solution, 1:5 *w/v* for 2 h (Van Ranst et al. 1999); 0.1 molL^{-1} $NaNO_3$ solution, 1:2.5 *w/v* for 2 h (Gupta and Aten 1993); 0.005 mol L^{-1} DTPA solution in 0.01 mol L^{-1} $CaCl_2$ solution; 0.01 mol L^{-1} triethanolamine, 1:2 *w/v* for 2 h (Lindsay and Norvell 1978); and 0.05 mol L^{-1} EDTA solution, 1:10 *w/v* for 1 h (Quevauviller et al. 1996). In all cases, the samples and extraction solution were shaken in a vibrator agitator (Vibromatic, Selecta) at 400 opm. The supernatant of each extraction was centrifuged at 3,500 rpm for 15 min and then filtered (low-ash filters, 5–7 μm). Cadmium, Cu, Pb, and Zn concentration in the extracts was quantified by flame atomic absorption spectroscopy (Analytikjena NovAA 300). All samples were extracted and analysed in duplicate. Quantification limits in milligrams per litre were the following: Cd=0.2, Cu=0.2, Pb=0.5, and Zn=0.1. Values of Cd, Cu, Pb, and Zn concentration obtained in single chemical extractions in the un-spiked and metal-spiked soil samples are shown in Electronic Supplementary Material 1, as a percentage of total metal.

Enzyme analyses

Soils were activated 48 h before the enzyme assessments with the addition of deionized water to bring them to 60 % of field capacity. The determination of DHA activity was performed according to the method of Von Mersi and Schinner (1991) modified by Carmiña et al. (1998). The method is based on the colorimetric estimation of iodotetrazolium formazan (INTF), which is formed when the soil is incubated with the substrate 2-*p*-iodophenyl-3-*p*-phenyltetrazolium (INT). The specific DHA activity was expressed in micromoles of INTF per gram dry soil per hour. The methods used for the assessment of BGAL (Eivazi and Tabatabai 1988) and PHOS (Tabatabai and Bremner 1969) activities are based on the colorimetric measurement of *p*-nitrophenol (PNP) produced when the soil is incubated with the synthetic substrates *p*-nitrophenyl galactopyranoside and *p*-nitrophenyl phosphate, respectively. The specific BGAL and PHOS activities were expressed in micromoles PNP per gram dry soil per hour. The method of Tabatabai and Bremner (1977) modified by Nannipieri et al. (1978) was used for the assessment of URE activity, which involves determining the ammonium released when soil is incubated with urea. The ammonium produced was assessed by an ammonium-selective electrode (781 pH/Ion Meter, Metrohm). The specific URE activity was expressed in micromoles ammonium per gram dry soil per hour.

Community-level physiological profile

The community-level physiological profile (CLPP) was determined using Biolog Ecoplates (BIOLOG Inc., Hayward, CS) (Garland and Mills 1991; Grayston et al. 2001). Bacterial suspension and metabolic profiling was performed according to Garcia-Villaraco Velasco et al. (2009). Briefly, 2 g of soil samples was homogenized in 20 mL of deionized water for 2 min in a homogenizer Omni Mixer Sorvall and then centrifuged at 2,500 rpm for 10 min and filtered through glass wool to obtain the bacterial suspension. Biolog Eco-microplates were inoculated with 150-μL aliquots of the bacterial suspension (diluted to 10^{-1}) and incubated in darkness at 26°C. Absorbance values at 595 nm were measured at time 0 and every 24 h until 168 h (from 72 h) with a BIO-RAD Model 550 Microplate reader. Average well colour development (AWCD) was calculated according to $AWCD=\sum(C-R)/N$, where C is the colour production of each well (optical density measurement at 595 nm), R is the absorbance value of the plate's blank well, and N is the number of substrates (Ecoplates, $N=31$). Three replicates were made per sample. Kinetics of AWCD was performed to fix the speed and development level of the bacterial communities using the 31 substrates provided. The absorbance data at 595 nm obtained at 168 h were used to calculate the functional diversity using the Shannon functional diversity index: $H=-\sum$ (Pi* log Pi), where Pi is the ratio of the blanked absorbance (absorbance value of the wells minus the absorbance value of the control well) to the sum of absorbance values of all wells.

Statistical analysis

The significance of differences in means ($n=9$), enzyme activity, microbial activity, and functional microbial diversity between un-spiked and metal-spiked soil samples was investigated using Student's *t* test. Pearson's correlation coefficients ($n=9$) were calculated to relate enzyme activity, microbial activity, and functional microbial diversity to soil physicochemical parameters and extractable metals. The analytical data of metal extractions used in the present work (Electronic Supplementary Material 1) were obtained from data published previously (de Santiago-Martín et al. 2013a). Analyses were conducted using Statistical Package for the Social Sciences (SPSS) v. 17 (SPSS, Inc.) software.

We used multivariate canonical ordination methods to investigate enzyme activity, microbial activity, and functional microbial diversity patterns in both un-spiked ($n=9$) and metal-spiked ($n=9$) soil samples, as a function of the soil physicochemical parameters measured using the procedure known as redundancy analysis (RDA) (Rao 1964). This methodology allows the explanation of one dataset (response variables) from another dataset (explanatory variables). In the linear method used, the canonical axes obtained are

constrained into being a linear combination of the explanatory variables (Ter Braak 1994). In our analyses, the response variables are the enzyme activity (DHA, BGAL, PHOS, and URE), in one case, and the activity of the different functional groups in the other (AWCD). The explanatory variables in both cases were the soil physicochemical parameters (12 variables). The multivariate analyses were performed with CANOCO 4.5 (Ter Braak and Smilauer 2002) software. Since the response variables are in different units, it was necessary to use the centering and standardizing procedure. These variables were logarithmically transformed to approximate them to a normal distribution. The explanatory variables also underwent a previous standardization in order to make them comparable. The software allows the individual importance of each explanatory variable to be ranked, quantifying its contribution to the model and assessing its significance with the Monte Carlo permutation test. We selected correlation biplots for the graphic representation. The biplots synthesize the relationships between the two datasets and directly display the correlations between explanatory and response variables (Ter Braak 1994). The biplots were prepared with the CanoDraw (Ter Braak and Smilauer 2002) software.

Results and discussion

Biological activity

Enzyme activity

The stress caused by heavy metals affects the growth, morphology, and metabolism of soil microorganisms. DHA activity reflects the respiratory activity of soil microorganisms and is widely used to estimate the overall microbiological activity in soils. A decrease in DHA involves the alteration of the microbial community, the main source of enzymes. Soil hydrolase activities play a key role in controlling the availability of nutrients in the soil. For the present work, we selected the activities of key enzymes involved in soil biogeochemical cycles (BGAL, C cycle; alkaline PHOS, P cycle; and URE, N cycle). Decreases in these activities involve a disruption in essential biological processes, such as the cellulose degradation, the turnover of organic P, and the mineralization/nitrogen transformation.

All enzyme activity (DHA, BGAL, PHOS, and URE) decreased significantly with the addition of metals (Table 2 and Fig. 1a), as previously reported by other authors (Gúlser and Erdogan 2008; Kızılkaya 2004; Martinez-Iñigo et al. 2009; Sadar et al. 2007). In all cases, the inhibition percentage (metal-spiked vs. un-spiked soil samples) was very high and showed a wide range of variation: 36–68 % (DHA), 24–85 % (BGAL), 22–72 % (PHOS), and 14–84 % (URE) (Fig. 1b). This higher percentage of inhibition, as compared to that obtained by other authors when metals are applied individually (Nourbakhsh and Monreal 2004; Zeng et al. 2007), could be attributed to the possible synergistic effect of metals when applied together, as in the present study (Moreno et al. 2009; Yang et al. 2006). It is worth noting the wide inhibition range observed in enzymes that could be considered extracellular (BGAL, PHOS, and URE), as opposed to the narrower inhibition range observed for the DHA activity that is exclusive to living cells (Fig. 1b). The intrinsic variability of our soil samples may be responsible for the wide range of inhibition of BGAL, PHOS, and URE activities to metal contamination, highlighting the importance of the soil characteristics that differentiate our samples (i.e., textural class and organic matter) in determining the persistence of these enzymes, as discussed below. In the case of inhibition of DHA activity, the uniformity of the values suggests that the most important factor was the contamination level, regardless of the soil components.

Community-level physiological profile

Microbial physiological profile has been recommended as a biological indicator of heavy metal stress. The Biolog technique is widely used to produce a catabolic profile of microorganisms. This technique provides a meaningful assay of community structure because it measures the utilization of different sources of carbon, the major nutrient that regulates microbial growth and community structure in soil.

Overall, microbial catabolic activity (ALL), estimated by AWCD, decreased significantly due to the effect of metal contamination (Table 2 and Fig. 2), as has been widely described (Yao et al. 2003). The decrease of microbial activity affects enzyme production by soil microorganisms and could be one of the causes of the decline in enzyme activity observed. In order to determine whether the shifts observed in overall substrate utilization could be attributed to changes in the ability of the microbial population for degrading some particular carbon source, we analysed the ability of each soil to oxidize different guilds of carbon sources included in Ecoplate (polymers, carboxylic acids, carbohydrates, amino acids, amines, and phenols) (Table 2 and Fig. 2). The analysis of substrate utilization patterns indicated that the amine and phenol utilization capacity was much lower than that of the other substrates, signaling that the microorganisms that catabolised these substrates (amines and phenols) may represent a less active fraction in these soils (Fig. 2a). Metal contamination was also observed to drastically decrease the ability to use the Ecoplate substrates, especially phenols with ~86 % inhibition compared to values ranging from 25 to 55 % inhibition for the rest of the substrates (Fig. 2b). These results demonstrated that microbial populations using phenols were the most sensitive to metal contamination. The different sensitivities of soil microbial communities to metal

Table 2 Average well colour development (AWCD) of microbial communities for all substrates (ALL), polymers (POLYM), carboxylic (CARBX), carbohydrates (CARBH), amino acids (AA), amines (AMINE), phenols (PHENOL), Shannon index (SI), dehydrogenase (DHA), β-galactosidase (BGAL), alkaline phosphatase (PHOS), urease (URE) activity values in un-spiked and metal-spiked soil samples

Level	Soil sample	ALL	POLYM	CARBX	CARBH	AA	AMINE	PHENOL	SI	DHA	BGAL	PHOS	URE
		AWCD								Specific activity			
Un-spiked	M1	1.04	1.32	1.06	0.91	1.29	0.98	0.30	4.66	0.25	0.08	1.63	1.69
	M2	1.20	1.54	1.23	1.07	1.54	0.69	0.48	4.69	0.31	0.10	1.77	4.47
	M3	1.35	1.73	1.38	1.38	1.62	0.88	0.42	4.72	0.54	0.18	2.95	2.84
	M4	1.33	1.82	1.39	1.19	1.60	0.87	0.52	4.68	0.44	0.11	2.44	4.16
	M5	1.28	1.63	1.39	1.02	1.71	1.07	0.40	4.71	0.21	0.04	1.07	2.38
	L1	1.21	1.71	1.31	1.02	1.47	0.68	0.66	4.70	0.66	0.19	2.82	4.39
	L2	0.85	1.23	0.88	0.76	0.95	0.60	0.38	4.66	0.33	0.14	2.18	2.71
	VL1	0.80	1.04	0.80	0.69	0.98	0.59	0.47	4.64	0.27	0.13	2.12	3.48
	VL2	0.93	1.14	0.94	0.91	1.25	0.56	0.29	4.69	0.27	0.17	2.2	1.73
Metal-spiked	M1	0.58	0.76	0.76	0.51	0.67	0.19	0.06	4.30	0.08	0.05	0.65	1.25
	M2	0.61	0.77	0.69	0.64	0.64	0.36	0.05	3.99	0.11	0.02	0.49	0.73
	M3	0.67	0.76	0.89	0.60	0.81	0.28	0.07	4.45	0.21	0.11	2.26	2.45
	M4	0.88	1.28	1.00	0.86	0.93	0.35	0.14	4.52	0.20	0.04	1.14	2.38
	M5	0.72	1.02	0.82	0.68	0.81	0.34	0.10	4.45	0.14	0.02	0.69	1.01
	L1	1.01	1.50	1.02	0.89	1.33	0.53	0.09	4.52	0.30	0.09	1.34	1.92
	L2	0.95	1.03	1.24	0.82	1.12	0.64	0.24	4.54	0.18	0.05	1.16	1.02
	VL1	0.69	0.85	0.89	0.70	0.68	0.29	0.06	4.42	0.13	0.09	1.66	1.33
	VL2	0.58	0.70	0.84	0.41	0.76	0.31	0.06	4.31	0.12	0.13	1.45	1.19

contamination may affect the soil microbial structure due to the disturbance of the system. Müller et al. (2001) described an adaptation by soil microbial communities to exposure to inorganic and organic contaminants consisting of selecting specific groups of bacteria adapted to environmental stresses. This adaptation could account for the decrease observed in the Shannon index for metal-spiked soils in the present work (Fig. 2). In this scenario, key biological processes taking place in the soil such as nutrient recycling could be affected. In fact, in a parallel work, we observed a slight decrease in the TOC from un-spiked to metal-spiked calcareous agricultural soils (from 12.3±1.4 to 10.7±1.2 g kg^{-1} TOC) and, within this fraction, an appreciable decrease in the RP (from 65.2±3.2 to 53.7±4.4 % RP of TOC) and the corresponding increase in the LP (from 34.8±3.2 to 46.3±4.4 % LPI+LPII of TOC).

Biological activity related to metal mobility patterns

A high inhibition of soil biological activity was observed demonstrating that there is a significant fraction of bioavailable metals in these soils, despite their high metal sorption capacity (de Santiago-Martín et al. 2013a). We, therefore, decided to evaluate the possible relationship between metal mobility (estimated with some extractants of different strengths) and enzyme and microbial catabolic activity, using Pearson's correlation analysis. As shown in Table 3, a high number of significant or nearly significant correlations were obtained among the extractable metals with different types of extractants and microbial activity (AWCD for all substrates and for each of the substrates and DHA activity). Liao and Xie (2007) also observed that data obtained from Biolog show a great potential as a sensitive and effective indicator of the impact of soil metal contamination on the microbial community and functional diversity. Nevertheless, and in contrast to the results obtained by other authors (Lee et al. 2009), no significant correlations were obtained in the present work with the rest of the enzyme activity measured (BGAL, PHOS, and URE). This could be due to the fact that in multi-elemental contamination systems, the inhibition of enzyme activity may take place through interactions occurring between metals (Moreno et al. 2009; Renella et al. 2003), highlighting the limitation of using extractable metals to explain the inhibition patterns of exocellular enzyme activity. The lack of significant correlations between this activity and extractable metals indicates that soil characteristics—especially those that differentiate our soils (OM and textural class)—may play an essential role in determining the persistence of the exocellular enzymes measured, as described above. It is important to note that the pattern of inhibition of microbial activity (AWCD and DHA) could be partially explained by the extractable metals. Thus, numerous positive correlations were obtained with extractable Cu and Zn with weak extractants ($CaCl_2$ and $NaNO_3$), which mainly simulate the soluble and exchangeable fractions of these metals, and with DTPA-based extraction solution,

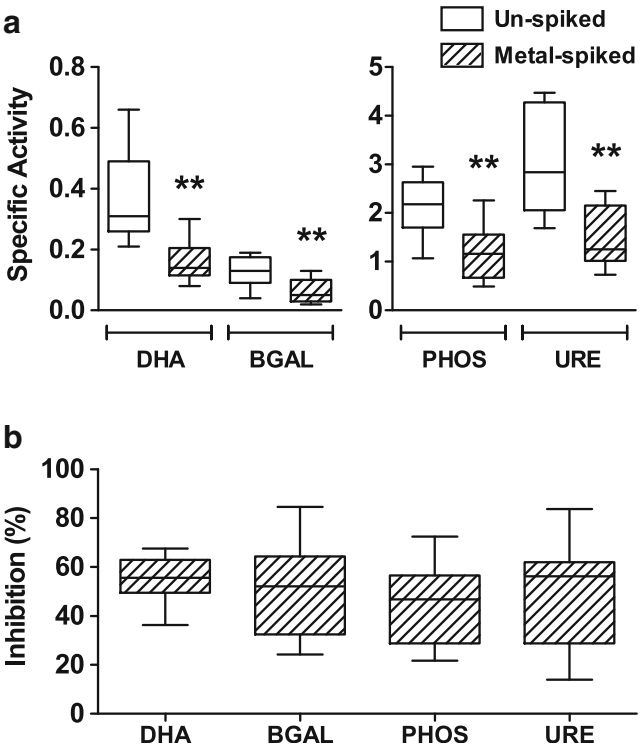

Fig. 1 Dehydrogenase (DHA), β-galactosidase (BGAL), alkaline phosphatase (PHOS), and urease (URE) activity values (**a**) and inhibition percentages (**b**) in un-spiked and metal-spiked soil samples. The *boxplots* show the lower, median, and upper quartiles, with *whiskers* extending to the most extreme data point (n=9). *, **, and *** indicate significant differences between un-spiked and metal-spiked samples for each activity at $p<0.05$, $p<0.01$, and $p<0.001$ after Student's t test

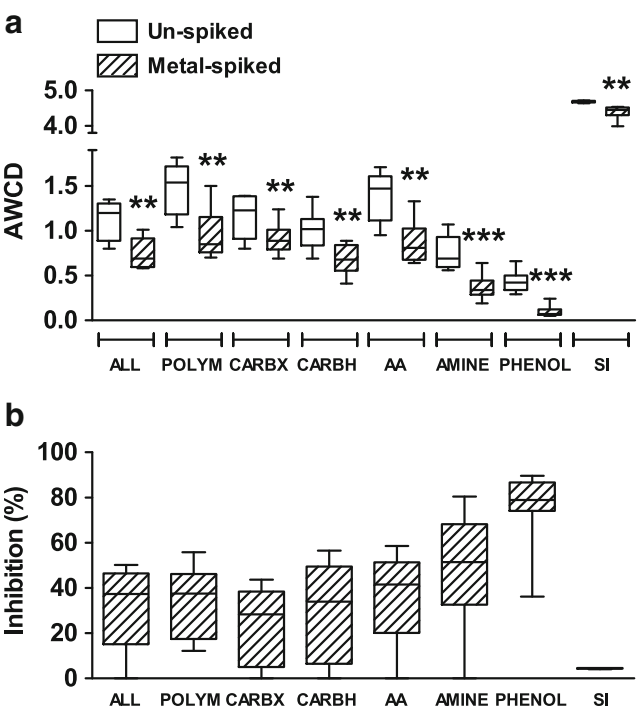

Fig. 2 Average well colour development (AWCD) (**a**) and inhibition percentages (**b**) at 168 h of incubation of microbial communities originating from un-spiked and metal-spiked soil samples for all substrates (*ALL*), polymers (*POLYM*), carboxylic (*CARBX*), carbohydrates (*CARBH*), amino acids (*AA*), amines (*AMINE*), and phenols (*PHENOL*), and for the Shannon index (*SI*). The *boxplots* show the lower, median, and upper quartiles, with *whiskers* extending to the most extreme data point (n=9). *, **, and *** indicate significant differences between un-spiked and metal-spiked samples for each substrate at $p<0.05$, $p<0.01$, and $p<0.001$ after Student's t test

buffered at pH 7.3, which exclude effects involving carbonate dissolution (Table 3). The positive correlations obtained have been attributed to the fact that both Cu and Zn in low available concentrations (soluble and/or exchangeable) could act as activators or stimulators (Renella et al. 2003). Conversely, in the case of Cd and Pb, metals without any known biological function and with high toxicity, the most significant correlations were obtained with EDTA, a stronger extractant that would remove Cd and Pb from other soil fractions such as the carbonate and organic fractions. As previously reported (de Santiago-Martín et al. 2013a, b), Cd and Pb sorption–desorption processes are mainly governed by different soil constituents related to the carbonate fraction (ECC, active lime, total Ca, calcite, and/or dolomite), but also by the organic fraction. Thus, these results could indicate that the Cd and Pb sorbed to these fractions may be partially available to affect the activity of microbial populations. Although this work is conducted in a multi-elemental contamination system, it could be inferred from the results that individual levels of Cd and Pb are primarily responsible for the inhibitory effect on microbial activity. Nevertheless, no such inhibition can be attributed to the available levels of Cu and Zn, since the numerous positive correlations obtained indicate a stimulatory effect (Belyaeva et al. 2005; Zeng et al. 2007). The selection of Cu-resistant and Zn-resistant microorganisms by prolonging the contact time (12 months in our case) could have occurred, as observed by other authors for Cd (Kandeler et al. 2000; Liao et al. 2005) or Cu and Zn (Rajapaksha et al. 2004). These latter authors also observed that the recovery of microbial activity is higher in metal-contaminated soils with a neutral or alkaline pH value than in acidic soils.

Biological activity related to soil physicochemical parameters

In order to further study the biological activity patterns, RDA was performed using, as response variables, the enzyme activity (Fig. 3) or the microbial catabolic activity (Fig. 4) and, as environmental variables, the soil physicochemical parameters that are the object of study. These analyses were complemented by Pearson correlations. In general, results indicated that the fine mineral fraction and different fractions of OM were the most important explanatory variables in ordering the patterns of variation.

With regard to enzyme activity, the first ordination axis takes into account most of the variability (eigenvalues of 0.60

Table 3 Correlation coefficients (*r*) among microbial catabolic activity (Shannon index and AWCD for the different groups of carbon compounds in Biolog Ecoplates) and biochemical parameters (enzyme activities) and extractable metals in metal-spiked soil samples

Extractant	Metal	ALL	POLYM	CARBX	CARBH	AA	AMINE	PHENOL	SI	DHA	BGAL	PHOS	URE
$CaCl_2$	Cd	a	a	a	a	a	a	a	a	a	a	a	a
	Cu	0.690[a]	0.862[b]	0.340	0.617	0.720[a]	0.413	0.253	0.440	0.772[a]	−0.144	−0.027	0.536
	Pb	0.407	0.411	0.494	0.093	0.572	0.197	0.289	0.804[b]	0.478	0.492	0.405	0.461
	Zn	0.685[a]	0.911[b]	0.279	0.650	0.693[a]	0.359	0.141	0.349	0.720[a]	−0.145	−0.120	0.478
$NaNO_3$	Cd	0.257	0.039	0.470	0.046	0.360	0.344	0.382	0.219	0.079	0.264	0.053	−0.070
	Cu	0.627	0.846[b]	0.151	0.668[a]	0.623	0.346	0.104	0.246	0.705[a]	−0.359	−0.212	0.395
	Pb	−0.113	−0.405	0.361	−0.241	−0.129	−0.002	0.283	0.298	−0.305	0.438	0.372	−0.140
	Zn	0.550	0.798[a]	0.205	0.414	0.638	0.319	0.114	0.299	0.554	−0.078	−0.229	0.287
DTPA	Cd	0.444	0.422	0.379	0.330	0.489	0.447	0.031	0.222	0.582	0.530	0.477	0.261
	Cu	0.859[b]	0.857[b]	0.624	0.853[b]	0.737[a]	0.706[a]	0.669[a]	0.824[b]	0.493	−0.49	−0.357	−0.004
	Pb	−0.177	−0.175	−0.126	−0.214	−0.112	−0.094	−0.253	−0.154	−0.302	0.218	−0.126	−0.472
	Zn	0.812[b]	0.907[b]	0.514	0.760[a]	0.754[a]	0.618	0.514	0.742[a]	0.628	-0.346	−0.265	0.265
EDTA	Cd	−0.701[a]	−0.483	−0.725[a]	−0.716[a]	−0.526	−0.707[a]	−0.796[a]	−0.774[a]	−0.473	0.289	−0.071	−0.248
	Cu	−0.423	−0.412	−0.296	−0.303	−0.511	−0.428	−0.208	−0.011	−0.587	−0.118	-0.129	−0.449
	Pb	−0.719[a]	−0.6	0.636	−0.720[a]	−0.584	−0.62	−0.794[a]	−0.768[a]	−0.427	0.516	0.253	−0.239
	Zn	−0.468	−0.537	−0.160	−0.331	−0.635	−0.531	−0.149	0.050	−0.592	0.132	0.223	−0.121

ALL all substrate, *POLYM* polymers, *CARBX* carboxylic acids, *CARBH* carbohydrates, *AA* amino acids, *AMINE* amines, *PHENOL* phenols, *SI* Shannon index, *DHA* dehydrogenase, *BGAL* β-galactosidase, *PHOS* phosphomonoesterase, *URE* urease activities, *a* cannot be calculated because at least one variable is constant

[a] At 5 % significance level

[b] At 1 % significance level

[c] At 0.1 % significance level

and 0.56 in un-spiked and metal-spiked soils, respectively), whereas the second axis has lower values (0.30 and 0.22, respectively). These ordinations were statistically significant, and the canonical axes created from the environmental variables explained a very high proportion of the variance in the response variables (90 % and 79 % in un-spiked and metal-spiked soils, respectively). As shown in the ordination diagrams, for both un-spiked and metal-spiked soil samples (Fig. 3a,b), BGAL and PHOS activities showed similar patterns, with vectors in the same direction as the fine mineral fraction (silt and/or clay) and to the recalcitrant OM (RP). Fine mineral and RP fractions could play an essential role as physical support favouring the persistence of exocellular enzymes in the soil through the formation of clay-humus-hydrolase complexes (Burns et al. 1972), as well as acting as a metal sink, thereby reducing metal bioavailability. Pearson's correlation analysis supported these results (Table 4), BGAL and PHOS being negatively correlated to CS (significant at both levels) and positively to RP (significant in metal-spiked soils). Similarly, the positive and significant correlations obtained between these activities and the content of crystalline Fe oxide could favour the formation of these complexes and thus this hypothesis. Furthermore, Pearson's correlation analysis indicated that soluble organic fractions (WSC and HWC) were positively correlated to URE, BGAL, PHOS, and DHA activities (Table 4). The abundance of readily available organic substrates contributes to the increase in microbial biomass (related to DHA activity) and, therefore, to the production of exocellular enzymes (BGAL, PHOS, and URE activities), as stated by other authors (Ghani et al. 2003). It is also interesting to note that HWC, enriched pectin fraction, is responsible for the formation of soil microaggregation, which also favours enzyme protection.

In the case of the microbial catabolic activity, the first and second ordination axes have a fairly similar weight in the ordination, with eigenvalues of 0.38 and 0.28 in un-spiked and 0.36 and 0.18 in metal-spiked soils. These ordinations were statistically significant, and the canonical axes created explained a very high proportion of variance (66 % and 55 % for un-spiked and metal-spiked soils, respectively). As shown in the ordination diagrams, although different patterns were observed between un-spiked (Fig. 4a) and metal-spiked soils (Fig. 4b); in both cases, silt, labile OM, and soluble OM were the main explanatory variables. In un-spiked soils, both the RDA and the Pearson's correlation analysis indicated a similar pattern for the microbial group's catabolising polymers (POLYM), carbohydrates (CARBH), carboxylic acids (CARBX), and amino acids (AA), with vectors in the same direction as LPII and silt (Fig. 4a) and significant and positive correlations with LPI (Table 4). These soil constituents

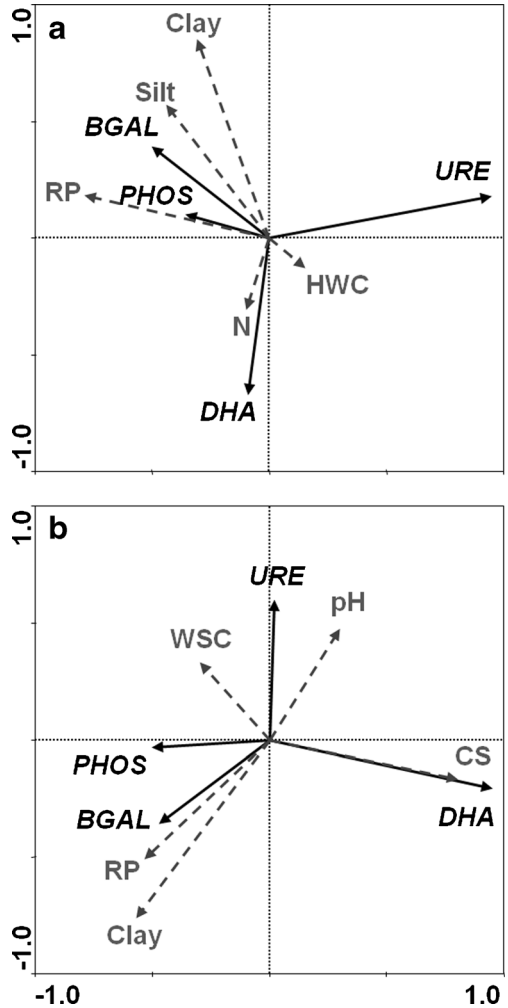

Fig. 3 Correlation biplot based on the redundancy analysis (RDA) performed between the enzyme activities (response dataset represented by *black vectors*) in un-spiked (**a**) and metal-spiked soil samples (**b**) and soil physicochemical parameters (explanatory variables represented by *grey dashed arrows*). The explanatory variables displayed are statistically significant and with the greatest contributions to the RDA variable selection procedure. *DHA* dehydrogenase, *BGAL* ß-galactosidase, *PHOS* alkaline phosphatase, and *URE* urease activities; *WSC* water soluble C, *HWC* hot water-soluble C, *CS* coarse sand, and *RP* recalcitrant pool of organic matter

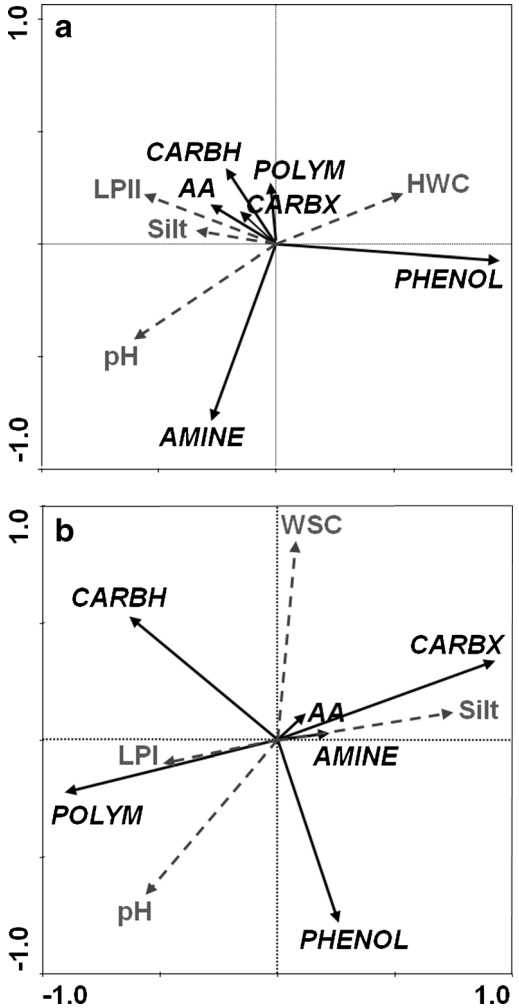

Fig. 4 Correlation biplot based on the redundancy analysis (RDA) between the activity of the different functional groups (response dataset represented by *black vectors*) in un-spiked (**a**) and metal-spiked soil samples (**b**) and soil physicochemical parameters (explanatory variables represented by *grey dashed arrows*). The explanatory variables displayed are statistically significant and with the greatest contributions to the RDA variable selection procedure. *POLYM* polymers, *CARBX* carboxylic, *CARBH* carbohydrates, *AA* amino acids, *AMINE* amines, *PHENOL* phenols, *WSC* water soluble C, *HWC* hot water-soluble C, *LPI* labile pool I of organic matter, and *LPII* labile pool II of organic matter

possibly create favourable conditions of substrate availability and aeration for these populations. The negative and significant correlations obtained with the clay content, with levels above 30 % in some of the soil samples analysed, could be due to the small pore size conferred by this mineral fraction. The protective effect of clay on soil microorganisms has been widely reported, but this effect is limited. Thus, Müller and Höper (2004) indicated that values higher than 25 % clay may reduce microbial growth by restricting gas exchange, producing water stress and decreasing nutrient availability. Furthermore, populations catabolising phenols were positively related to HWC (Fig. 4a and Table 4), attributed to the fact that this fraction is enriched with phenolic compounds. In metal-spiked soils, both RDA and Pearson's correlation analyses also showed that silt and readily available OM were the most important variables explaining the Biolog pattern of soil microbial communities. Nevertheless, unlike the results for un-spiked soils, we observed a greater dispersion of the microbial catabolic activity of the soil samples (Fig. 4b), and the correlations were not significant (Table 4). Generally, in metal-contaminated environments, there are a reduction in the microbial species diversity and a development of metal-resistant microbial populations (Yao et al. 2003). Accordingly, the Biolog method suggested that the appearance of qualitative differences in the soil microbial communities in metal-spiked soils may explain the sample dispersion in the ordination

Table 4 Correlation coefficients (*r*) among microbial catabolic activity (AWCD for the different groups of carbon compounds in Biolog Ecoplates) and biochemical parameters (enzyme activities) and soil physicochemical parameters in un-spiked and metal-spiked soil samples

Level	Parameter	ALL	POLYM	CARBX	CARBH	AA	AMINE	PHENOL	DHA	BGAL	PHOS	URE
Un-spiked	ECC	0.525	0.518	0.515	0.424	0.464	0.680[a]	−0.012	0.085	−0.469	−0.228	0.052
	OM	0.530	0.574	0.517	0.613	0.442	−0.144	0.696[a]	0.842[b]	0.666	0.771[a]	0.579
	WSC	0.308	0.328	0.281	0.452	0.184	−0.032	0.475	0.607	0.512	0.682[a]	0.372
	HWC	0.204	0.321	0.219	0.209	0.064	−0.169	0.782[a]	0.720[a]	0.513	0.678[a]	0.602
	LPI	0.245	0.201	0.221	0.135	0.297	0.117	0.115	−0.278	−0.509	−0.438	0.452
	LPII	0.748[a]	0.697[a]	0.750[a]	0.703[a]	0.731[a]	0.603	0.200	0.307	−0.067	0.156	0.058
	RP	−0.654	−0.582	−0.632	−0.522	−0.694[a]	−0.449	−0.223	0.100	0.532	0.340	−0.472
	N	0.458	0.526	0.432	0.596	0.324	−0.179	0.641	0.907[b]	0.760[a]	0.896[b]	0.523
	Cry-Fe	−0.076	0.030	−0.081	0.081	−0.175	−0.583	0.455	0.697[a]	0.912[b]	0.864[b]	0.281
	CS	0.162	0.226	0.255	−0.169	0.255	0.209	0.198	−0.057	−0.387	−0.473	0.063
	Silt	−0.167	−0.254	−0.242	0.159	−0.213	−0.118	−0.502	−0.143	0.273	0.271	−0.383
	Clay	−0.838[b]	−0.852[b]	−0.857[b]	−0.653	−0.773[a]	−0.821[b]	−0.296	−0.328	0.414	0.111	−0.236
Metal-spiked	ECC	−0.057	0.038	−0.262	0.061	−0.062	−0.153	−0.013	−0.066	−0.597	−0.506	0.039
	OM	0.333	0.438	0.108	0.327	0.392	0.105	−0.242	0.771[a]	0.443	0.625	0.738[a]
	WSC	0.186	0.166	0.149	0.274	0.137	−0.088	−0.187	0.560	0.460	0.831[b]	0.744[a]
	HWC	0.519	0.554	0.331	0.597	0.448	0.175	−0.057	0.750[a]	0.341	0.636	0.679[a]
	LPI	−0.263	−0.173	−0.479	0.022	−0.414	−0.110	−0.165	−0.392	−0.747[a]	−0.682[a]	−0.428
	LPII	0.105	0.295	−0.040	0.139	0.076	−0.262	−0.096	0.360	0.028	0.318	0.680[a]
	RP	0.198	0.004	0.488	−0.099	0.361	0.253	0.213	0.182	0.711[a]	0.487	0.040
	N	0.359	0.421	0.195	0.319	0.430	0.106	−0.182	0.788[a]	0.527	0.691[a]	0.828[b]
	Cry-Fe	0.441	0.396	0.462	0.256	0.544	0.311	0.022	0.696[a]	0.777[a]	0.753[a]	0.588
	CS	0.352	0.513	0.044	0.268	0.425	0.402	0.212	0.126	−0.505	−0.718[a]	−0.404
	Silt	−0.339	−0.594	0.106	−0.374	−0.369	−0.236	0.071	−0.203	0.458	0.664	0.239
	Clay	−0.204	−0.390	0.140	−0.323	−0.179	0.064	−0.019	−0.286	0.569	0.337	−0.332

ALL all substrate, *POLYM* polymers, *CARBX* carboxylic acids, *CARBH* carbohydrates, *AA* amino acids, *AMINE* amines, *PHENOL* phenols, *DHA* dehydrogenase, *BGAL* β-galactosidase, *PHOS* phosphomonoesterase, *URE* urease activities, *ECC* equivalent $CaCO_3$, *OM* organic matter, *WSC* water soluble C, *HWC* hot water-soluble C, *LPI* labile pool I, *LPII* labile pool II, *RP* recalcitrant pool, *Cry-Fe* crystalline Fe oxides, *CS* coarse sand, *a* cannot be calculated because at least one variable is constant

[a] At 5 % significance level

[b] At 1 % significance level

[c] At 0.1 % significance level

diagrams. Thus, despite the usual difficulty of finding significant correlations in calcareous soils (Nourbakhsh and Monreal 2004), results suggest that both particle size distribution and the composition of the OM are mediating the imbalance in the microbial population structure of metal-contaminated calcareous soils.

Conclusions

In this study, soil enzyme and microbial catabolic activities were negatively affected by the combination of heavy metals in calcareous agricultural soils despite their high metal sorption capacity. Microbial populations catabolising phenols were the least active in these soils and the most sensitive to metal contamination. The pattern of inhibition of microbial activity was partially explained by the extractable metals. The Pearson correlation analysis indicated that individual available levels of Cd and Pb could be responsible for the inhibition of microbial activity observed, while Cu and Zn appear to have a stimulatory effect. In general, readily available organic matter (soluble and/or labile) favoured both enzyme activity and microbial catabolic activity. Likewise, the recalcitrant organic matter, together with the fine mineral fraction, contributed to the persistence in the soil of enzymes responsible for β-galactosidase and alkaline phosphatase activities in both un-spiked and metal-spiked soil samples. Conversely, an excessive content of clay (~30 %) may reduce microbial growth decreasing microbial catabolic activity. The results suggest that the structure of microbial populations has been altered in the contaminated soils and that this alteration could be mediated by the particle size distribution and the composition

of the organic matter. In summary, our results highlight the importance of the composition of organic matter on the soil microbial response to metal contamination. Hence, further work is required on the relationship between the composition of organic matter and the structure of the microbial community in metal-contaminated soils in order to provide a greater understanding of the results.

Acknowledgments This work was supported by the Complutense University in Madrid and the Madrid Autonomous Region through Grant GR58/08, Research Team 950605, and Network CARESOIL, Ref. P2009/AMB-1648. We especially wish to thank both English correction experts, Ms Pru Brooke-Turner and Mr David Bruhn, for the revision of the English version of the manuscript as well as the comments and suggestions of the editor and the anonymous referees.

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