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Metal contamination disturbs biochemical and microbial properties of calcareous agricultural soils of the Mediterranean area

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Abstract

Climate characteristics and carbonate content are key factors governing heavy metal accumulation in calcareous agricultural soils of the Mediterranean area. The low organic matter content of these soils may compromise the ability of microbial populations to cope with stress resulting from metal contamination. We studied the effects of metal contamination on a combination of biological parameters in soils having these characteristics. With this aim, soils were spiked with a mixture of Cd, Cu, Pb, and Zn, at the two limit values proposed by Current European legislation, and incubated for up to 12 months. Then, we measured biochemical (phosphatase, urease, β -galactosidase, arylsulfatase, and dehydrogenase activities) and microbial (fungal and bacterial DNA concentration by quantitative PCR) parameters. All the enzyme activities were strongly affected by metal contamination, showing the inhibition sequence: phosphatase (30-64%) < arylsulfatase (38-97%) \leq urease (1-100%) \leq β -galactosidase (30-100%) < dehydrogenase (69-100%). The high variability among soils was attributed to the different proportion of fine mineral fraction, organic matter, crystalline Fe oxides and divalent cations in soil solution. The decrease of fungal DNA concentration in metal-spiked soils was negligible, while the decrease of bacterial DNA was about 1-54% at the lowest level and 2-69% at the highest level of contamination. The lowest bacterial

DNA decrease occurred in soils with the highest organic matter, clay and carbonate contents.

Finally, regarding the strong inhibition of the biological parameters measured, and the alteration of the fungal/bacterial DNA ratio, we provide strong evidence that a disturbance on the system could alter key soil processes, even within the limiting values of contamination proposed by the current European Directive.

Keywords:

enzyme activity; quantitative PCR; biological parameters; heavy metals; calcareous soils

1. Introduction

Heavy metal accumulation in agricultural areas has increased in recent decades as a result of agricultural, industrial and urban activities (Bai et al., 2010; Roca-Perez et al., 2010). This metal accumulation in agricultural soils may result in adverse environmental and human health impacts, including metal contamination of groundwater and accumulation in food crops. The alteration of microbial communities has often been proposed as an easy and sensitive indicator of anthropogenic effects on soil ecology (Renella et al., 2005). However, while most of the studies on microbial disturbance by metals mainly refer to Northern European countries, there is less information in Southern Europe, particularly in the Mediterranean area. The nature and degree of inhibition of soil microorganisms by metals are strongly related to soil properties and components (Speir et al., 1992). In this sense, Mediterranean soils usually show relatively high pH and carbonate content (Toribio and Romanyà, 2006) and are typically low in organic matter content (Fernández et al., 2009; Lal, 2004). Low rainfall and high evapotranspiration, characteristic of the Mediterranean climate, limit metal mobility. In addition, metal retention capacity in soils is increased due to carbonate content, which results in an increased metal accumulation in the first few centimetres of soil. However, it has been reported that there can be a significant metal mobility and availability

even in calcareous soils of the Mediterranean area with significant implications for the risk of contamination (Martínez and Motto, 2000; Plassard et al., 2000; Rajaie et al., 2006; Sayyad et al., 2010). As organic matter plays a key role as a precursor for enzyme synthesis and in physical stabilization (Tabatabai, 1994), the microbial activity of these soils with low organic matter content is often very low (Bastida et al., 2006). This could compromise the response of soils to metal contamination and prevent microbial populations from coping with the stress resulting from heavy metal contamination (Moreno et al., 2009). Therefore, we highlight the necessity of paying particular attention to microbial disruption by metal contamination in calcareous agricultural soils of the Mediterranean area with low organic matter contents.

Biochemical parameters such as extracellular enzyme activities are good sensors of the long-term contamination of soils by heavy metals (Kızılkaya et al., 2004) and are recognized as a more sensitive bio-indicator than plants and animals of natural and anthropogenic disturbances (Hinojosa et al., 2004). Extracellular enzymes are usually dependent on the state of the organism (active, inactive or dead), with a temporal lag between cell death and the decrease in enzyme activity (Brookes, 1995). By contrast, dehydrogenase activity, which depends on the parent microbial cell, is considered as a good indicator of overall microbial activity (Obbard, 2001). In addition to biochemical parameters, several techniques have been used to quantify the soil microbial biomass, such as real-time quantitative PCR (qPCR). Molecular biological techniques involving PCR are not limited by the culturability of the microorganisms. Since the natural variability of soil properties could be a decisive impediment to the use of simple biological parameters alone (Nannipieri et al., 1990), using a combination of these parameters for diagnostic purposes could provide a better picture of the status of soil processes and functioning (Acosta-Martínez et al., 2003).

In the present work, we focus on the study of the disruption of a combination of biological parameters by metal contamination in calcareous agricultural soils of the Mediterranean area with low amounts of organic matter. With this aim, soils with these characteristics were metal-spiked and incubated for up to 12 months and some biochemical (phosphatase, urease, β -galactosidase,

arylsulfatase, and dehydrogenase activities) and microbial (total, fungal and bacterial DNA concentration) parameters of soils were monitored. Special attention was made to soil properties and components affecting patterns of biological inhibition.

2. Materials and methods

2.1. Soil characteristics and sampling

The soil samples of the present study come from different plots at “El Encín” Agricultural Research Station, in Alcalá de Henares (Madrid, Spain). The area is located on medium level alluvial terraces by the Henares River on quaternary sediments, mainly medium and coarse sands (IGME, 1990). These alluvial sediments have caused an ancient calcaric Fluvisol (Moreno Merino, 1998), to currently present Anthrosol characteristics (FAO, 2006) mainly as a result of agricultural use. The site is typical of a Mediterranean pluviseasonal-oceanic bioclimate on an upper meso-Mediterranean low dry bioclimatic belt (Rivas-Martínez, 1987).

We selected ten unpolluted agricultural soil samples with a natural gradient in organic matter content (ranging from 10 to 32 g kg⁻¹), with a wide range of variation in its carbonate content (from 9 to 190 g kg⁻¹) and in its particle-size distribution (sandy-loam and sandy-clay loam textural classes). These samples included a mixture of 30-40 kg of the soil surface horizons (0-30 cm) that have not been differentiated because of the agricultural activity. Soil samples were air-dried and passed through a 2 mm sieve.

2.2. Experimental design

Metal accumulation in agricultural soils was simulated by spiking soils with a mixture of Cd, Cu, Pb, and Zn in order to mimic a multi-element contaminated soil (Clemente et al., 2008; Maas et al., 2010). Three containers (40 cm wide x 59 cm long x 21 cm high) were set for each sample, 10 kg each, one for uncontaminated sample with addition of distilled water and the other two were spiked at two different concentration levels using nitrate salts of heavy metals in aqueous solution: low

level (Tt1) (3 mg kg⁻¹ of Cd + 140 mg kg⁻¹ of Cu + 300 mg kg⁻¹ of Pb + 300 mg kg⁻¹ of Zn) or high level (Tt2) (20 mg kg⁻¹ of Cd + 875 mg kg⁻¹ of Cu + 600 mg kg⁻¹ of Pb + 2000 mg kg⁻¹ of Zn) in line with the limits proposed by current European legislation (Directive 86/278/EEC). Each soil sample and the corresponding solution was mixed and left to equilibrate for a period of 12 months at room temperature without cover or drainage. During this equilibration period, the soils were air-dried, mixed and rewetted with distilled water in cycles of about 2 weeks in order to favour metal redistribution into the soil matrix (McLaughlin, 2001). At the end of equilibration period, duplicates were randomly taken out from each uncontaminated and metal-spiked soil samples to determine biochemical and microbial parameters. We selected the following soil enzyme activities because of their wide use in the assessment of biochemical disruption by metals: phosphatase (PHOS), urease (URE), β -galactosidase (BGAL), arylsulfatase (ARYS), and dehydrogenase (DHA) activities. In order to evaluate the microbial biomass, a real-time quantitative PCR (qPCR) was carried out for quantification of DNA from fungi and bacteria in the soil samples.

2.3. Analytical methods

2.3.1. Soil physicochemical analyses

Soil pH was analyzed in a 1:2.5 soil to water ratio (method 4-1, ISRIC, 2002). Total organic C was quantified by wet oxidation according to the Walkley and Black method (1934). We used the two-step acid hydrolysis procedure with H₂SO₄ to determine recalcitrant (RP) pool of organic matter (Rovira and Vallejo, 2000). Equivalent CaCO₃ (ECC) was determined according to the acid neutralization method (method 7-1, ISRIC, 2002). Particle-size distribution was determined by the Robinson's pipette method, following dispersion with sodium hexametaphosphate (method 3, ISRIC, 2002). The crystalline and amorphous Fe and Mn oxide contents were determined by dithionite-citrate extraction followed by acid oxalate extraction (methods 12-1.1 and 12-2, ISRIC, 2002). The cation exchange capacity (CEC) was determined by the ammonium acetate method (method 9, ISRIC, 2002). The major cations in soil solution were extracted in a 1:5 soil to water

ratio (method 13-1, ISRIC, 2002). Total Cd, Cu, Pb, and Zn contents of the soil samples were determined after wet digestion with a mixture of nitro-perchloric-hydrofluoric acids under high-pressure conditions (SISS, 1985). DTPA-extractable metals were determined by shaking 20 g of soil with 40 ml of 0.005 M DTPA + 0.01 M CaCl₂ + 0.01 M triethanolamine (TEA) for 2 h (Lindsay and Norwell, 1978). Elements in the corresponding extracts were quantified by atomic absorption spectroscopy for Fe, Mn, Ca, Mg, Cd, Cu, Pb, and Zn, by flame emission spectrometry for Na and K and by inductively coupled plasma-atomic emission spectroscopy for P. All analyses were performed in duplicate.

2.3.2. Biochemical analyses

Soil samples were incubated at 60% water holding capacity and 25 °C in the dark for 7 days. Moisture levels were maintained gravimetrically every 2 days using distilled water. The determination of PHOS, BGAL, ARYS, and URE activities were performed, respectively, according to the methods of Tabatabai and Bremner (1970), Dick et al. (1996), and Sinsabaugh et al. (2000). Briefly, soil solutions were prepared in triplicate with distilled water (1:6.25 w:v), stirring at 10250 rpm, and incubated with substrates (3 pseudo-replicates): 4-nitrophenylphosphate (0.05 M, 90 min at 37 °C for PHOS, Sigma), 4-nitrophenylgalactopyranoside (0.02 M, 3 h at 37 °C for BGAL, Sigma), 4-nitrophenylsulfate (25 mM, 4 h at 37 °C for ARYS, Sigma), and urea (0.4 M, 3 h at 25 °C for URE). For PHOS, BGAL, and ARYS activities the reaction was stopped with 0.5 M CaCl₂ and 0.1 M Tris pH 12 and the para-nitrophenol (PNP) released was measured at 405 nm with a Xenius microplate luminometer (SAFAS, Monaco). For URE activity, NH₄⁺ released was determined with a HACH reagent (610 nm). The concentration of product released was compared to para-nitrophenol standards (Sigma) for ARYS, BGAL and PHOS activities or to NH₄Cl standards (Sigma) for the URE activity. Activities were expressed in mU g⁻¹ dry soil. One mU of activity was defined as the amount of enzyme that catalyzed 1 μmol substrate in 1 min.

Soil DHA activity was assayed using the method of Schaefer (1963). Soil triplicates were mixed with distilled water (1:1 w:v) and 0.12 M 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) and incubated 16 h at 37 °C. The reaction was stopped with ethanol (5 vol.), incubated for 2 h in the dark at 37 °C and the tubes centrifuged 5 min at 6000 rpm. Absorbance of supernatants was measured at 485 nm (KONTRON Spectrophotometer). The concentration of product released was compared with 1,3,5-triphenyl formazan (Sigma) standards. DHA activity was expressed in mU g⁻¹ dry soil. One mU of activity was defined as the amount of enzyme that catalyzed 1 µmol substrate in 1 min.

2.3.3. Microbial analyses

Total genomic DNA was extracted in duplicate from 0.5 g of soil using Fast DNA kit for soil (MP Biomedical). rDNA was then amplified according to Gangneux et al. (2011) in 10 µl final qPCR mixture containing the following total amounts: 5 ng of total soil DNA, 12.5 pmoles of each primer, 5 µl of iq-SYBR®Green super Mix (Biorad). For bacterial 16S-rDNA, the forward primer used was S-BCT 5'-CAG GCC TAA CAC ATG CAA GTC-3' (63f, Marchesi et al., 1998) and the reverse primer was AS-BCT1 5'-CTG CTG CCT CCC GTA GG-3' (341F, Muyzer et al., 1993). For fungal 18S-rDNA, the forward primer used was S-FUN1 5'-GGA AAC TCA CCA GGT CCA GA-3' (nu-SSU-1196, Borneman and Martin, 2000) and reverse primer was AS-FUN1 5'-ATT GCA ATG CYC TAT CCC CA -3' (nu-SSU-1536, Borneman and Martin, 2000). All cycles were programmed on a CFX apparatus (Biorad) to perform 35 cycles consisting of: 95 °C for 40 s, 64 °C for 45 s and 72 °C for 30 s with bacterial rDNA and 95 °C for 10 s, 62 °C for 30 s and 72 °C for 30 s with fungal rDNA. Total DNA concentration was measured with Smart Spec3000 at 260 nm (Biorad). The quality of the DNA extracted was checked by the ratios 260/280.

3. Results and discussion

3.1. Soil physicochemical parameters

Relevant results of the soil physicochemical analyses of uncontaminated soils are shown in Table 1.

All soils showed pH values above 8, ranging from basic to slightly alkaline. The organic matter (OM) content ranged from high (H1, H2, and H3 soils) to moderate (M1, M2, M3, and M4 soils) and low (L1, L2, and L3 soils). The recalcitrant pool of organic matter was higher in H1, M1, M2, and L2 soils. The equivalent CaCO_3 (ECC) content ranged from moderate (H2, H3, M3, M4, and L3 soils) to low (H1 and L2 soils) and very low (M1, M2, and L1 soils). The textural classes of soils were sandy-loam (M1, M2, M3, and L1 soils) and sandy-clay loam (L2 and VL1 soils).

Crystalline Fe oxide content was low. Crystalline Mn and amorphous Fe-Mn oxides content was very low ($<1 \text{ g kg}^{-1}$). Ca and Mg concentration in soil solution was higher in H2 and M2 soils, without relation to carbonate content or to CEC values. The total Cu, Pb, and Zn content values were similar to those obtained by other authors for agricultural soils of the Mediterranean area (Acosta et al., 2011; Jiménez Ballesta et al., 2010; Micó et al., 2006; Peris et al., 2007; Ramos-Miras et al., 2011) and in no case exceeded the levels set by the European Union (Directive 86/278/EEC). The total Cd content was lower than the quantification limit ($<0.2 \text{ mg L}^{-1}$). The percentage values of metal DTPA-extractions are shown in Fig. 1. In all cases, the DTPA extraction % in metal-spiked soils were higher at Tt1 level than at Tt2 (Prokop et al., 2003).

3.2. Biochemical and microbial parameters

3.2.1. Biochemical parameters

Enzyme activities in uncontaminated soils were in general favoured in soils with higher recalcitrant OM content and higher proportion of fine mineral fractions (Fig. 2). Thus, high values of PHOS, URE, and BGAL activities in uncontaminated soils were obtained in M1 soil (71% recalcitrant fraction, 328 g kg^{-1} clay) and of ARYS activity in H2 soil (66% recalcitrant fraction, 215 g kg^{-1} silt). Accordingly, the lowest enzyme activities were often measured in soils with lower proportions

of fine mineral fractions (M4 and L3 soils). The protective role exerted by the humus-hydrolases and/or clay-humus-hydrolases complexes against proteases (Burns et al., 1972) could explain the relevance of these constituents in preventing the inhibition of enzyme activity.

As expected, all the enzyme activities decreased in metal-spiked soils, although we observed different patterns (Fig. 2). In general, the sequence of inhibition % of enzyme activities, from the lowest to the highest values, was: PHOS (30-60%) < URE (1-98) ≤ ARYS (38-90%) ≤ BGAL (30-94%) < DHA (69-100%) at Tt1 level and PHOS (41-64%) < ARYS (83-97%) < URE (85-100%) < BGAL (89-100%) < DHA (84-100%) at Tt2 level. These different inhibition patterns could be explained by the possible development of metal resistant microorganisms (Kunito et al., 1999), which release enzymes poorly affected by high concentrations of metals in soil solution (Kunito et al., 1998). In any case, these results highlight that, on one hand, not all the enzyme activities were affected in the same way, and, on the other hand, there was great variability among the different soils in the present study.

PHOS activity and the total P in uncontaminated soil samples showed an inverse relationship. Thus, the lowest PHOS activity was in M4 soil, with the highest total P content, with the highest PHOS activity being found in H1 soil, with the lowest total P content. This pattern could be attributed to regulation mechanisms of enzyme production by means of feed-back processes (Olander and Vitousek, 2000). In metal-spiked soil samples, PHOS activity was the least inhibited, suggesting that this enzyme, or the organisms that produce it, are less sensitive to the mixture of metals employed in this work, than for the other activities studied. The inhibition % of this enzyme was quite similar at both contamination levels, although it was slightly lower in soils with the highest OM content, favouring the persistence of total and immobilized PHOS activity (Pascual et al., 2002).

The strong inhibition observed on URE, BGAL, and ARYS activities would indicate a disruption of key biological processes such as N mineralization/transformation, cellulose degradation, and organic S turnover. This is consistent with the results obtained by other authors for soils with

slightly alkaline pH, who reported that URE, BGAL, and ARYS activities are sensitive bio-indicators of soil degradation caused by metal contamination (Belyaeva et al., 2005; Hinojosa et al., 2004; Martínez-Iñigo et al., 2009). Regarding soil properties affecting the range of inhibition of these enzyme activities, we must highlight the great variability found among different soils, mainly in the case of URE activity at Tt1 level (Fig. 2). In general, the inhibition % of URE, BGAL and ARYS activities at Tt1 level was higher in M3, M4, and L1 soils. These soils present a group of common characteristics that could enhance the greater inhibition of these enzymes. Firstly, these soils have the highest coarse sand content and lower contents of recalcitrant OM, which could limit the physical stabilization of the enzymes in the medium. The high inhibition in these soils indicates that these soil constituents, and their properties, are essential for the enzyme protection to metal contamination in these calcareous soils of the Mediterranean area, maybe through the formation of the complexes mentioned above. Secondly, we observed that these M3, M4 and L1 soils had lower content of crystalline Fe oxide which could favour the formation of these complexes (Gianfreda et al., 1995). Similarly, the possible formation of metal-organic complexes could increase metal retention (Renella et al., 2004) and mitigate enzyme inhibition by metals, and/or the organisms that produce them. In this sense, crystalline Fe oxides could bind and retain metals, making them less available to microorganisms (Doelman and Haanstra, 1984).

It should be noted that the lowest inhibition % of URE activity at Tt1 level was produced in H2 and M2 soils, being about 90 fold less than in L1 soil, with the highest inhibition. This behaviour could be explained by the higher content of divalent cations, Ca^{2+} and Mg^{2+} , in soil solution in both H2 and M2 soils. The presence of CaCO_3 in soils has been shown to lead to biological stabilization of both particulate organic carbon and humus (Duchaufour, 1977), probably because Ca^{2+} provides bridges between negatively charged organic functional groups and negatively charged clay surfaces (Krull et al., 2001). This fact could explain the positive relationship observed between the presence of divalent cations in soil solution and the higher persistence of URE activity.

Hydrolase activities in many cases reached values close to 100% inhibition, mainly in M3, M4 and L1 soils, which could suggest that the active centre of the enzymes may be inactivated by the metals, preventing them from carrying out their hydrolytic action. In this sense, M3, M4 and L1 soils showed the lowest recalcitrant pool of OM. At the range of pH of soils of the present study, metals could complex with OM, being immobilized but still able to interact with the enzymes maybe with enzyme sulphhydryl groups (Marzadori et al., 2000).

In the case of metal-spiked soil samples at Tt2 level, the inhibition percentage of URE, BGAL, and ARYS activities was so similar among soils that establishing a clear discrimination among soil properties was not possible. Nevertheless, the very high inhibition % obtained at this Tt2 level, from 41 to 64% for PHOS activity and from 83 to 100% for the other enzymes measured should be noted.

Our results showed a strong inhibition of DHA activity at both levels, suggesting a drop in soil microbial numbers. However, because metals such as Cu inhibit formazan colour development (Chander and Brookes, 1991), we calculated Pearson's correlations between total DNA concentration and DHA activity values (data not shown). A relationship was only established in the uncontaminated soil samples, making our results inconclusive.

Regarding DTPA extractions (fig. 1), we observed that soils with higher proportions of coarse mineral fractions (L2 soil) or with minor proportions of fine fractions (H1 soil) showed the highest metal extractability. The ECC content could also play a relevant role in affecting metal extractability, mainly in the case of Cd, since soils with higher ECC contents (L3, M4, M3 and H2 soils) showed lower percentage values of extractable Cd in metal-spiked soil samples. Although we did not observe the same patterns between DTPA-extractable metals and enzyme activities, it appears that the same soil components may be involved in both metal sorption and protection of enzyme activity. Since the toxicity occurs by the effect of the combination of various metals (Khan et al., 2007; Oorts et al., 2006; Renella et al., 2003), defining which metals employed had the

greatest affect on the different enzyme activities is not feasible under our experimental conditions and is not the subject of this work.

3.2.2. Microbial parameters

Total DNA concentration decreased in metal-spiked soil samples compared to uncontaminated ones (Fig. 3), with values of about 11-49% at Tt1 level and about 26-55% at Tt2 level. This high decrease reveals a disturbance of the microbial community, the main source of enzymes, which plays a crucial role in maintaining the main soil nutrient cycles. As applications of heavy metal concentrations to European standard levels (Directive 86/278/EEC) resulted in significant negative impacts on the microbial community (Abaye et al., 2005; Brookes, 1995), these reference values should be established according to soil characteristics (Moreno et al., 2009).

Fungal-DNA represented about 9%, ranging from 3 to 17% of the total DNA quantified in the different uncontaminated soil samples. Although a high decrease occurred in some metal-spiked soil samples, we observed the same proportion of fungal-DNA amount in metal-spiked soils as in uncontaminated soils (8-9% of the total DNA). Similar results were obtained by Anderson et al. (2008) when studying fungal community composition after addition of sewage sludge containing heavy metals in a concentration below the maximum permissible limits in current United Kingdom legislation.

Bacterial-DNA in uncontaminated soil samples was about 82% of the total DNA quantified, ranging from 62 to 100% for the different soil samples (Fig. 3). Bacterial biomass decreased strongly in metal-spiked soil samples, suggesting a higher sensitivity of the bacterial community to metals than the fungal community (Vig et al., 2003). On the one hand, the soil fungal community shows higher adaptation capacity to stress conditions (Chander and Dyckmans, 2001), probably due to a variety of intrinsic and inducible properties of fungi that can ensure survival (Gadd, 2007). These processes of adaptation to metal stress may be accompanied by the exclusion of metal-sensitive fungal strains (Baldrian, 2003), finding different fungal populations but not necessarily different total fungal

population size. On the other hand, the soil pH decreased by one unit in metal-spiked soil samples at Tt2 level as a result of metal salts added (data not shown). Since fungi generally exhibit wider pH ranges for optimal growth, it is possible that competitive interactions between fungi and bacteria could stimulate the fungal community, inhibiting the bacteria (Rousk et al., 2010). The decrease in the amount of bacterial DNA was about 20% at Tt1 level (1-54%), and about 50% at Tt2 level (2-69%), showing high variability among soil samples. The smallest bacterial DNA decrease was produced in soils with the highest OM (H1, H2, and H3 soils), clay (M2 soil) and carbonate content (L3 soil). As in the case of enzyme activities, these constituents play a key role in preventing the decrease of bacterial biomass, through physical stabilization and/or reduction of metal availability (Martínez-Iñigo et al., 2009; Moreno et al., 2009). However, no relationship was observed between patterns of hydrolytic enzyme activities and bacterial biomass, which has been previously reported (Piotrowska et al., 2006). Trasar-Cepeda et al. (2008) attributed this lack of relation to the fact that most of the enzyme activities in cropped soils are not associated with active microorganisms and must therefore be stabilized by soil components, such as OM. In any case, our results showed that the soil properties and constituents that favour survival and persistence of microbial communities and enzyme activities to metal contamination are similar in these calcareous agricultural soils. The fungal/bacterial ratio (Fig. 4) increased in some metal-spiked soils, mainly at Tt2 level, therefore involving a disturbance on the system. This disturbance could alter key soil processes such as the fungal decomposition of recalcitrant OM, in which fungi and bacteria are involved through complex interactions, both competitive and mutualistic (de Boer et al., 2005). Finally, metal contamination may modify microbial diversity due to the extinction of species that lack sufficient tolerance to stress and the increase of the population of resistant species (Atlas, 1984; Brandt et al., 2010; Ellis et al., 2003). The emergence of opportunistic organisms could be our case, since the decrease of bacterial biomass was much higher than that reached by total and fungal biomass.

Conclusions

We conclude that the mixture of metals added at levels consistent with European regulations to calcareous agricultural soils of the Mediterranean area resulted in a large inhibition of the biochemical and microbial parameters studied, under our experimental conditions. These strong adverse effects, as well as the alteration of the fungal/bacterial ratio, could be decisive for microbial function and soil processes. Mineral fraction, crystalline Fe oxides, carbonate, and organic matter were the main soil components affecting the persistence of enzyme activities and survival of bacterial and fungal populations to metal contamination in soils with these characteristics. Since our results provide evidence that these soils could not withstand an environmental impact from metal accumulation and that the inhibition of the biochemical and microbial parameters are strongly affected by soil components, we believe that the limiting values proposed by the current European legislation should be established according to soil characteristics. Nevertheless, further research work is necessary.

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Table 1. Physicochemical parameters of uncontaminated soil samples.

Soil sample	pH	OM	RP	ECC	CS	FS	Silt	Clay	cry-Fe	Total P	CEC	Soil solution		Total content		
		g kg ⁻¹	% of TOC	g kg ⁻¹						cmol _c kg ⁻¹		Ca	Mg	Cu	Pb	Zn
H1	8.2	32	72	106	159	590	78	172	13	0.6	6.9	249	44	12.0	25.5	52.9
H2	8.1	31	66	125	23	592	215	170	12	0.8	10.6	718	151	10.0	23.8	63.3
H3	8.4	26	54	118	95	605	146	154	11	1.0	8.9	243	46	10.8	24.4	62.0
M1	8.1	21	71	32	45	459	168	328	11	0.4	19.1	489	63	10.1	24.0	62.1
M2	8.2	21	74	27	99	386	172	344	12	0.6	20.4	610	96	13.2	21.1	70.7
M3	8.1	20	48	148	114	569	124	193	9	1.4	8.7	341	26	12.1	55.8	62.7
M4	8.7	17	57	117	166	567	129	139	8	2.1	5.6	308	31	15.0	23.7	74.9
L1	8.2	14	52	9	245	560	70	124	7	0.5	10.0	402	29	7.0	25.4	45.2
L2	8.1	13	75	100	112	462	167	259	10	0.8	13.3	338	35	8.5	21.5	55.1
L3	8.4	10	69	190	111	603	126	161	8	0.7	7.1	338	35	8.0	14.0	54.7

OM =organic matter; RP = recalcitrant pool of total organic carbon (TOC); ECC = equivalent

CaCO₃; CS = coarse sand; FS = fine sand; cry-Fe = crystalline Fe oxides; CEC = cation exchange capacity.

Fig. 1. Relative metal extractability (%) (DTPA-extractable metal vs. total metal content) in uncontaminated and metal-spiked soil samples. Tt1 = low metal amount, Tt2 = high metal amount.

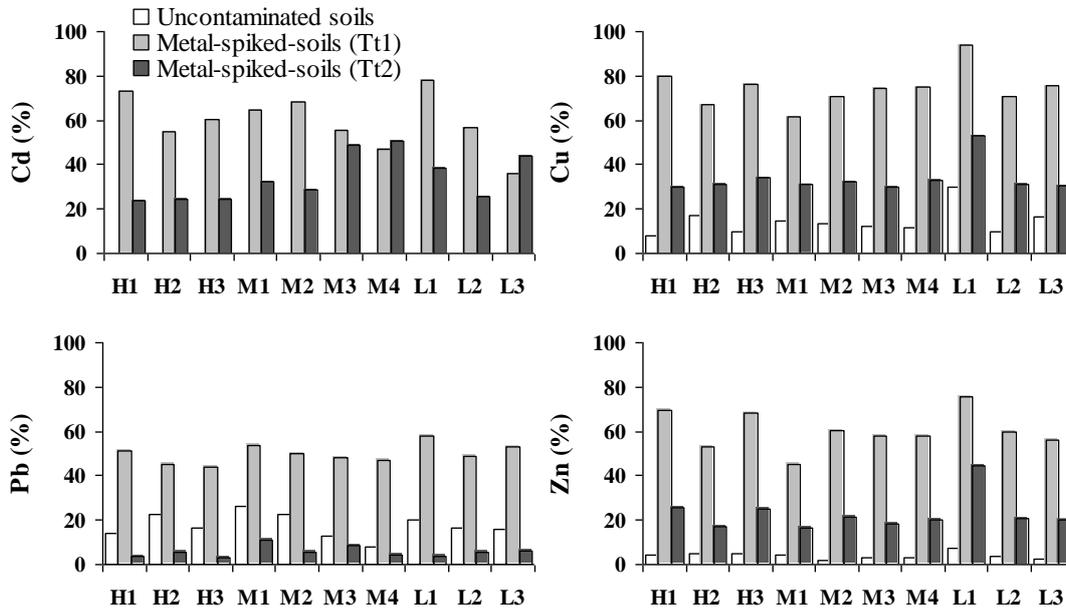


Fig. 2. Phosphatase (PHOS), urease (URE), β -galactosidase (BGAL), arylsulfatase (ARYS), and dehydrogenase (DHA) activity values in uncontaminated and metal-spiked soil samples, and inhibition % (metal-spiked vs. uncontaminated soil samples). Tt1 = low metal amount, Tt2 = high metal amount. Vertical bars are Standard Errors.

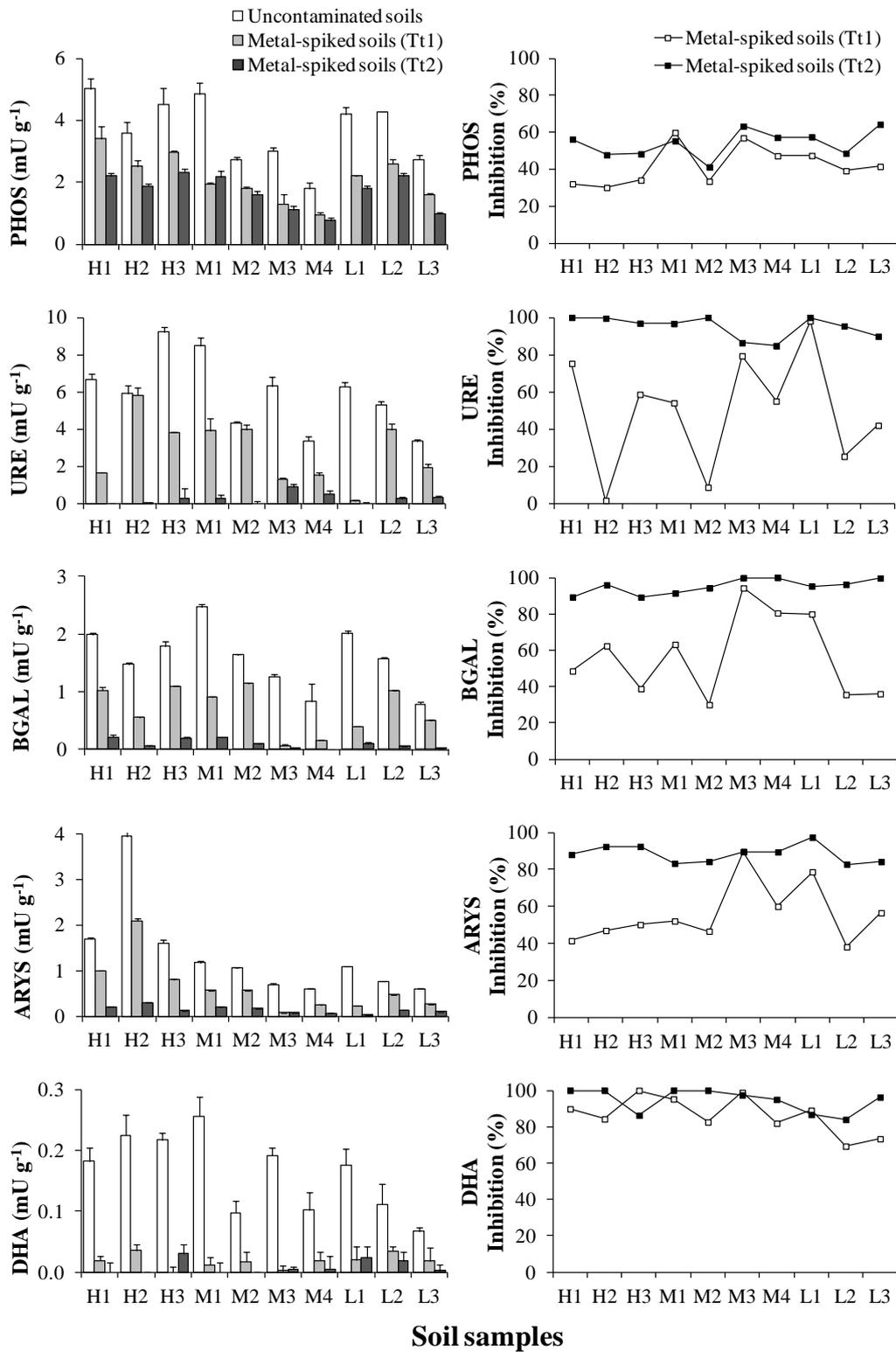


Fig. 3. Total, fungal and bacterial DNA amounts in uncontaminated and metal-spiked soil samples and decrease % (metal-spiked vs. uncontaminated soil samples). Tt1 = low metal amount, Tt2 = high metal amount. Vertical bars are Standard Errors.

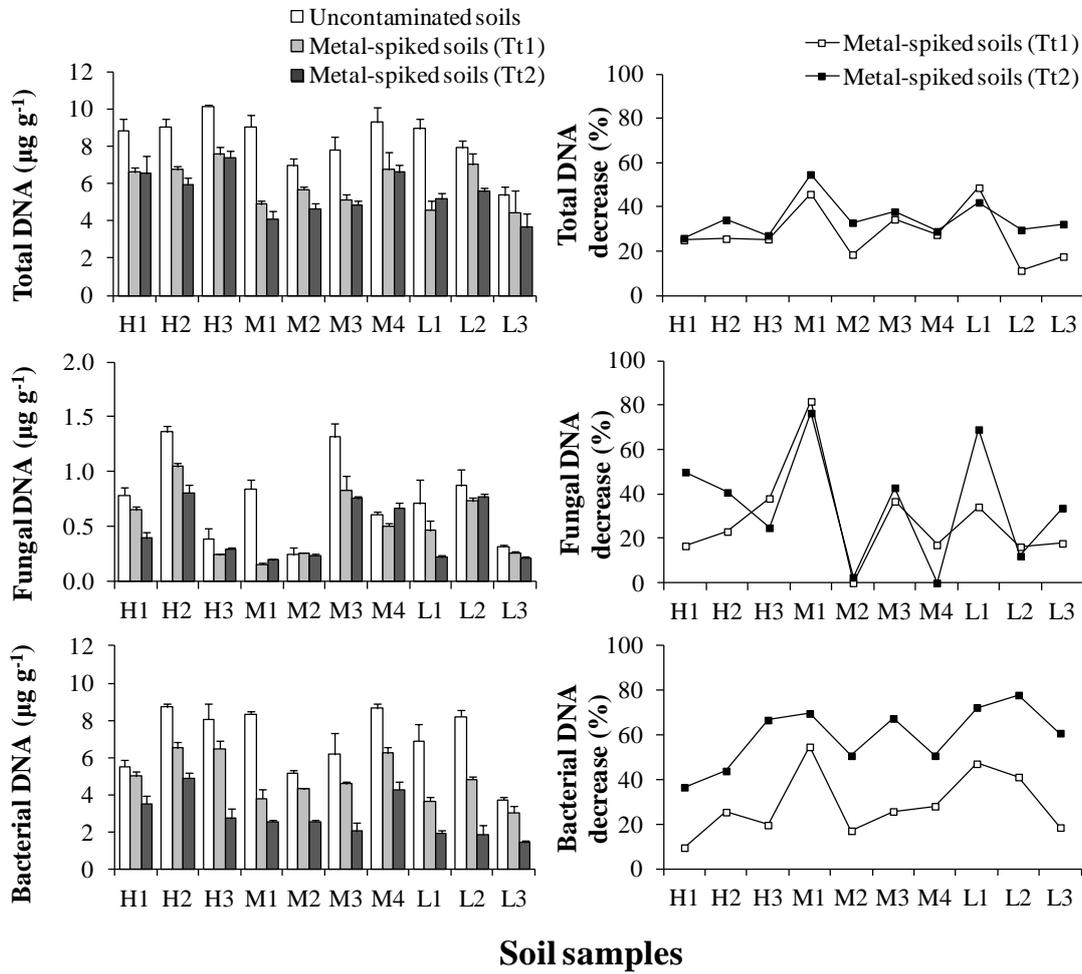


Fig. 4. Fungal/Bacterial DNA ratios in uncontaminated and metal-spiked soil samples. Tt1 = low metal amount, Tt2 = high metal amount.

