Progress in Microbial Activity and Chemical Properties of a Trace Element Polluted Soil Under Assisted Natural Remediation

Alfredo Pérez-de-Mora, Pilar Burgos, Francisco Cabrera and Engracia Madejón

Abstract In this work, we studied the temporal dynamics of several microbiological properties in a trace element polluted soil under the influence of various amendments and/or a plant cover during a 30 month-period. The experiment was carried out in containers filled with ca. 150 kg of contaminated soil. Seven treatments were established: four organic (leonardite LEO, litter LIT, municipal waste compost MWC and biosolid compost BC) and one inorganic (sugarbeet lime SL), where the grass *Agrostis stolonifera* L. was sown, and two control treatments (with plant CTRP or without plant CTR). Soil was sampled four times during the experimental period. The microbiological properties studied were: microbial biomass C, microbial biomass C/total organic C, dehydrogenase, aryl-sulphatase, β-glucosidase, acid-phosphatase and protease enzyme activities. Dynamics of microbiological properties differed between treatments being results not only affected by soil pH or trace element concentrations, but also by changes derived from the different treatments in organic matter quality and quantity, as well as nutrient content in soil. While microbial biomass C, dehydrogenase, aryl-sulphatase and protease activities were highly correlated with soil pH and soluble trace element contents, changes in β-glucosidase activity were mainly influenced by water soluble C concentrations. It was also observed that enzymatic activities generally decreased over time after no more amendment additions occurred. Nonetheless, during the experiment microbial biomass and activities were generally higher in all treatments compared to the untreated control and thus remediation practices had a positive and significant effect on trace element stabilization and microbial activity in the contaminated soil.

1 Introduction

Mining activities can release significant amounts of trace elements such as As, Cd, Cu, Pb and Zn into the environment negatively affecting organisms and ecological processes within atmospheric, aquatic and terrestrial systems (Iskandar and Adriano 1997). In terrestrial systems, trace elements tend to accumulate in the surface of soils due to binding with its components (clay minerals, iron/manganese oxides, organic matter, etc.) in a variety of ways. Owing to its small size and crucial role in nutrient cycling, the soil microbiota is the first group of organisms that undergoes direct and indirect impact due to accumulation of trace elements in soil (Giller et al. 1998). Although high concentrations of trace elements may promote the development of tolerant microbial populations (Ellis et al. 2003), trace element polluted soils generally show less microbial biomass and altered microbial activity patterns compared to non-affected soils (Kandeler et al. 2000). Among the different methodologies to assess microbial activity in soil, enzymatic assays, either based on colorimetric or fluorescence reactions, have been widely used due to: (1) their specificity, (2) their simplicity and (3) the integrative nature of enzymatic activities, from the micro-up to the macroscale (Nannipieri et al. 2002). Soil enzymatic assays may be therefore used to evaluate not only anthropogenic disturbances such as those related to contaminant accumulation, but also restoration and remediation practices in affected systems. Since trace elements cannot be degraded, remediation of soils polluted with trace elements is based either on the extraction or the stabilization of the contaminants. Among stabilization techniques, assisted natural remediation has been proposed as a potential low-cost and environmentally friendly alternative to treat extensive areas moderately contaminated (Madejón et al. 2006). This technique is based on the use of amendments to accelerate those processes (sorption, precipitation and complexation reactions) that take place naturally in soils and reduce the mobility and bioavailability of toxic elements (Bolan and Duraisamy 2003). In addition to the incorporation of amendments, the development of a plant cover may prevent wind-blow of contaminated particles and reduce water pollution (Tordoff et al. 2000). There is, however, concern regarding the longevity of utilizing amendments to assist natural remediation. Reacidification of soil may reverse the action of amendments that make soils more alkaline. Mineralization of organic matter present in biosolids may also release trace elements in potentially bioavailable forms. Traditionally, repeated applications of amendments have been recommended to maintain trace element immobile, but more work is required to refine these procedures and understand the effects of such practices on soil dynamics in the mid-and long-term.

Stabilization techniques aim at immobilizing trace elements in the soil to reduce their availability to biological targets, run-off transport and leaching. However, reliance on abiotic properties is insufficient to assess the efficiency of remediation practices from an environmental perspective. Alternative parameters are required which can be used as bioindicators to monitor changes in soil ecological processes such as those related to anthropogenic and natural disturbances,

Table 1 Mean values ± standard deviation of some chemical characteristics of the soil

pH		3.32 ± 0.76
TOC[a]	g kg^{-1}	5.40 ± 0.07
Tot-As	mg kg^{-1}	120 ± 3
Tot-Cd	mg kg^{-1}	2.43 ± 0.04
Tot-Cu	mg kg^{-1}	78.3 ± 1.4
Tot-Mn	mg kg^{-1}	645 ± 25
Tot-Pb	mg kg^{-1}	201 ± 6
Tot-Zn	mg kg^{-1}	226 ± 3

[a] TOC total organic carbon

remediation practices or land management. Enzymatic activities are particularly attractive for this purpose due to their crucial role in soil organic matter transformations and their direct link to the soil microbiota.

The aim of this study was to evaluate the mid-term effects of various amendments and/or a plant cover on trace element stabilization and the different processes related to the cycling of nutrients (C, N, P and S) in a soil moderately contaminated with As, Cd, Cu, Pb and Zn. A 30 month experiment was conducted in containers to simulate potential field remediation practices under more controlled conditions. In addition to trace element availability, general soil physical and chemical characteristics as well as various microbiological and biochemical properties were investigated.

2 Materials and Methods

2.1 Soil Characteristics

Soil was sampled in an area affected by the Aznalcóllar mine accident named "El Vicario", where the only remediation work carried out by the authorities was the removal of the sludge layer together with the first 15 cm of topsoil. The soil was clayey loamy classified as Typic Xerofluvent (Soil Survey Staff 1996). Some relevant characteristics are presented in Table 1.

2.2 Experimental Design

The experiment was carried out in 28 containers (70 cm long × 60 cm wide × 40 cm deep) that were placed outdoors in the experimental farm "La Hampa" (IRNAS-CSIC) in Coria del Río (Southern Spain) (485 mm mean rainfall, average for 1971-2008; Mean annual daily temperature is around 17°C, with maximum and minimum temperatures in July of 33.5°C and in January of 5.2°C). The containers

were filled with the upper 20 cm of the soil (1.32 g cm^{-3} bulk density). Containers were arranged according to a complete randomized block design with seven treatments (four organic, one inorganic and two controls) and four replicates per treatment. The organic treatments were: leonardite (LEO), a low rank coal between peat and sub-bituminous, rich in humic acids from a coal mine (DAYMSA), litter (LIT) collected from a deciduous forest (*Castanea sativa* Miller.) in the Sierra of Aracena (Huelva, Southern Spain), municipal waste compost (MWC) from a city refuse treatment plant (Villarrasa, Southern Spain), and biosolid compost (BC) constituted from wastewater sludge from a water treatment plant and green waste from parks and gardens (EGMASA, Sevilla, Southern Spain). The inorganic treatment was sugar beet lime (SL), a residual material from the sugar manufacturing process with 70–80% of $CaCO_3$ (dry basis) (AZUCARERA EBRO, San José de la Rinconada, Southern Spain). Two control treatments without amendments were also established: control with plant (CTRP) and control without plant (CTR). These amendments were chosen because they constitute low-cost, representative materials for land treating extensive areas. The characteristics of the amendments are described in Pérez de Mora et al. (2005). Trace element contents of all amendments were below the limits established by the European Union (CEC 1986) for sewage sludge. The annual loads of trace elements of the products used were also in accordance with the same directive. The amendments were applied on a fresh basis (20–25% moisture content) and mixed with the topsoil (10 cm) in the containers. Within the 30 months of the study two doses of each amendment were applied: the first one at the beginning of the experiment (70–75 Mg dw ha^{-1} for leonardite and composts and 50–60 Mg dw ha^{-1} for sugarbeet lime and litter) and again after 12 months (February 2003) (35.0–37.5 Mg dw ha^{-1} for leonardite and composts and 25–30 Mg dw ha^{-1} for sugarbeet lime and litter). The grass *Agrostis stolonifera* L. was sown (167 kg ha^{-1}) in the containers and grown for 5 months (March–July) for 3 consecutive seasons (2002–2004). *A. stolonifera* L. was selected because it is known to show metal tolerance. The containers were routinely watered when necessary to maintain plant growth.

2.3 Soil Sampling and Chemical Analysis

Soil was sampled on four occasions: 1, 6, 18 and 30 months after the beginning of the experiment. In each case, 10 soil cores (2 cm diameter, 10 cm depth) regularly distributed were taken from each container to make a composite sample. Subsamples for chemical analysis were previously air-dried, crushed and sieved (2 mm). Soil and amendment aliquots for trace element determinations were additionally ground to 60 μm. Subsamples for microbial biomass and enzymatic activities were sieved (2 mm) and stored at 4°C until analysis (within two weeks after the sampling). Soil pH values were measured in a 1:2.5 sample:1 M KCl extract after shaking for one hour The 0.01 M $CaCl_2$-extractable trace element

concentrations in soils were determined in 1:10 soil sample (< 2 mm):0.01 M $CaCl_2$ extracts (Ure et al. 1993) via ICP-OES (Inductively coupled plasma-optical emission spectrometry). Total organic carbon (TOC) in soil was analysed by dichromate oxidation and titration with ferrous ammonium sulphate (Walkley and Black 1934).

2.4 Microbial Biomass and Enzyme Activities

Microbial biomass carbon (MBC) content was determined by the chloroform fumigation-extraction method modified by Gregorich et al. (1990). The concentration of C in the extract was measured as described by Jenkinson and Powlson (1976) using dichromate digestion. An extraction efficiency coefficient of 0.38 was used to convert the difference in soluble C between the fumigated and the unfumigated soil to MBC (Vance et al. 1987). Dehydrogenase activity (DH) was determined by the method of Trevors (1984), using INT (2 (*p*-iodophenyl)-3-(*p*-nitrophenol) 5-phenyl tetrazolium chloride) as the electron acceptor (García et al. 1993). Arylsulphatase activity (Aryl) was determined as proposed by Tabatabai and Bremmer (1970) after soil incubation with *p*-nitrophenyl sulphate and measurement of *p*-nitrophenol absorbance at 400 nm. β-glucosidase activity (β-gluc) was measured as indicated by Tabatabai (1982) after soil incubation with p-nitrophenyl glucoside and measurement of *p*-nitrophenol absorbance at 400 nm. Acid phosphatase activity (Phosph) was measured after soil incubation with *p*-nitrophenyl phosphate disodium in a 0.5 M maleate buffer (pH 6.5) and measurement of PNP absorbance at 398 nm (Nannipieri et al. 1980). Protease activity (Prot) was calculated after incubation of soil with casein and measurement of the absorbance of the extracted tyrosine at 700 nm (Ladd and Butler 1972).

DH was expressed in mg INTF kg^{-1} dw h^{-1}. Aryl, β-gluc and Phosph activities were expressed in mg PNP kg^{-1} dw h^{-1}. Prot was expressed in mg Tyrosine kg^{-1} dw 2 h^{-1}.

2.5 Statistical Analysis

All statistical analyses were carried out with the program SPPS 15.0 for Windows. A normality test was carried out for all variables prior to analysis of the variance. If necessary, non-normal distributed data was transformed accordingly. Differences between treatments within each sampling event for each variable were tested using ANOVA. Post-hoc analysis was performed using Tukey's test for equal variances and Games-Howell's test for unequal variances. To test for time-related differences within the same treatment microbiological data was also analysed by ANOVA for repeated measures using time as factor. Validity of the repeated factor for ANOVA was tested using Mauchly's sphericity test. The Greenhouse-Geisser correction was used for violations of sphericity. Significant differences for all

variables between the different sampling events within the same treatment were established by Students t-test pairwise comparisons using the Bonferroni correction. A t-test for unequal variances was computed when these were heterogenous. A correlation matrix between all chemical and biochemical parameters was calculated. The significance level reported ($\alpha = 0.01$ and $\alpha = 0.05$) is based on Pearson's coefficients.

3 Results and Discussion

In this study we focused on time-related patterns of various intra-and extracellular enzymatic activities in a soil moderately contaminated with trace elements in which different amendments were applied. Since solubility of As and Pb was very low, metal-enzyme interactions were only reported for Cd, Cu and Zn. Detailed information on biotic and abiotic properties within each sampling can be found elsewhere (Pérez de Mora et al. 2005; Pérez-de-Mora et al. 2006a, b).

Results from microbial biomass C (MBC) estimations showed various interesting trends. Firstly, the incorporation of amendments into the soil resulted in higher MBC concentrations in all samplings compared to both controls (Fig. 1a). In the second sampling and subsequently higher microbial biomass yields were recorded in the control with plants than in the control without plants (Fig. 1a). Finally, in most treatments, except LEO and BC, microbial biomass was highest in the second sampling and subsequently decreases (Fig. 1a). The incorporation of amendments and the presence of a rhizosphere in the soil had therefore a stimulatory effect on the soil microbial community. This could be related to the alkalinising effect of the amendments and the incorporation of nutrients and readily degradable organic substrates as suggested by the positive correlations between MBC and soil pH and nutrient-related properties (N-Kjeldahl, available-P and water soluble C) (Table 2) (Sakamoto and Oba 1991). The growth of a vegetation cover between the first and second sampling could partly explain why for most treatments MBC was highest in the second sampling. Plants excrete 10–20% of their photosynthetates as root exudates, which can be used up by numerous microorganisms as substrates for growth (Salt et al. 1998). In subsequent samplings, however, microbial biomass generally decreased although higher plant yields were achieved (Pérez-de-Mora et al. 2006c). More complex mechanisms may also be involved which may account for this trend such as competition between plants and soil microorganisms for nutrients and/or different degradability of organic matter incorporated with the amendments.

Since microbial biomass is generally higher in soils with higher organic matter content and amendments may also incorporate microorganisms into the soil, it may be useful to normalize shifts in soil microbial biomass C by using the ratio microbial biomass C/total organic C (MBC/TOC) (Insam and Merschack 1997). A high ratio can be attributed to a highly active microbial population or to easily degradable organic sources, at least for that population (Spargling 1992). Common

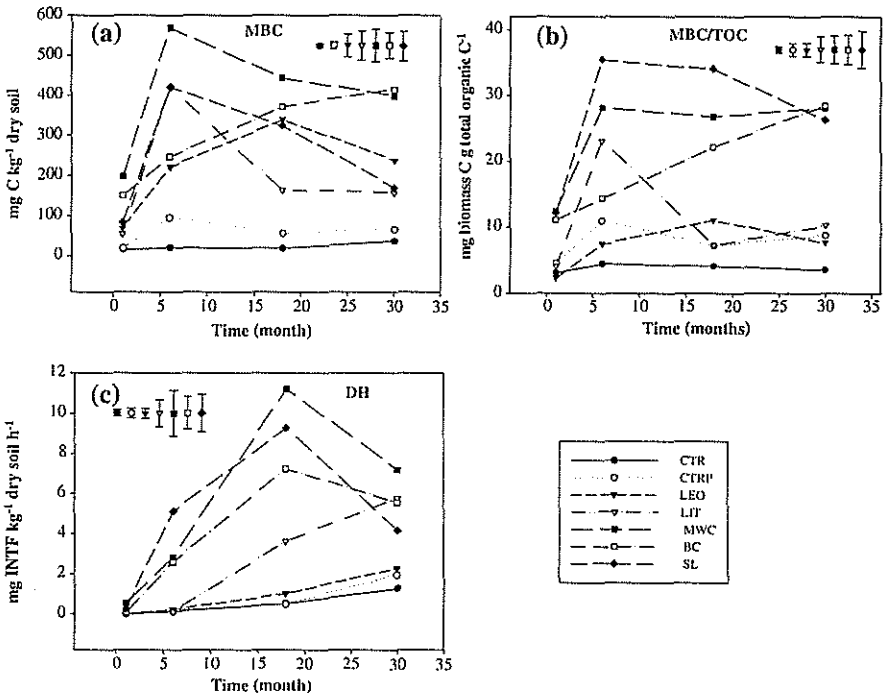

Fig. 1 Temporal dynamics of a microbial biomass C, **b** microbial biomass C/total organic C and **c** dehydrogenase activity. Symbols represent mean values. Error bars represent the maximum standard deviations within one treatment during the whole experiment

values ranged from 10 to 40 mg MBC g^{-1} TOC (Gigliotti and Farini 2002). The ratio showed a very similar pattern for all treatments to that of MBC (Fig. 1b). In the case of the compost and sugarbeet lime amended soils the ratio was significantly higher in all samplings compared to both controls. Nonetheless, in soils amended with litter and leonardite differences with the controls were not significant. On occasions similar values were observed in the control with plant and the amended soils. These results suggest that microorganisms may be incorporated with the amendments (hence higher MBC yields), but also that litter and leonardite are not as good as composts or lime to stimulate microbial growth in degraded soils. The fact that plant litter needs first to be cut down into smaller fractions for more effective microbial attack and the recalcitrant nature of organic C from the leonardite may explain these results.

Enzymatic patterns were enzyme-and treatment-dependent showing the extreme complexity of ecological reactions occurring in the soil. Dehydrogenase activity has been used to assess heavy metal toxicity in soils (Rossel et al. 1997) and microbial activity in semiarid Mediterranean areas (García et al. 1997). During the experiment higher DH was recorded in all treated soils compared to controls, except LEO (Fig. 1c). This enzyme was positively correlated with MBC, but there

Table 2 Correlation coefficients between chemical and biochemical properties

	MBC/TOC	MBC	DH	Aryl	β−gluc	Phosph	Prot
pH	0.565[a]	0.450[a]	0.616[a]	0.379[a]	−0.007	−0.322[a]	0.318[a]
Sol-Cd	−0.415[a]	−0.411[a]	−0.561[a]	−0.343[a]	−0.159	−0.023	−0.482[a]
So-Cu	−0.368[a]	−0.428[a]	−0.403[a]	−0.254[a]	−0.308[a]	−0.213[b]	−0.449[a]
Sol-Zn	−0.502[a]	−0.471[a]	−0.616[a]	−0.360[a]	−0.246[a]	−0.080	−0.530[a]
TOC	−0.221[b]	−0.202[b]	−0.223[b]	−0.117	−0.105	0.034	−0.011
N-Kjeld	0.312[a]	0.574[a]	0.465[a]	0.632[a]	0.384[a]	0.208[b]	0.266[a]
Avail-P	0.304[a]	0.250[a]	0.223[b]	0.059	−0.181	−0.287[a]	−0.054
WC	0.527[a]	0.665[a]	0.356[a]	0.687[a]	0.544[a]	0.388[a]	0.330[a]
MBC/TOC	1	0.852[a]	0.598[a]	0.510[a]	0.192[b]	−0.088	0.266[a]
MBC		1	0.498[a]	0.568[a]	0.384[a]	0.156	0.310[a]
DH			1	0.585[a]	0.227[b]	−0.093	0.485[a]
Aryl				1	0.383[a]	0.143	0.224[b]
β-glu					1	0.724[a]	0.393[a]
Phosph						1	0.331[a]
Prot							1

[a] Correlation is significant at the 0.01 level
[b] Correlation is significant at the 0.05 level

seemed to be a delay in DH response to remediation practices compared to MBC; in most cases DH values were highest in the third sampling (Fig. 1c). Due to its intracellular nature DH is usually better correlated to microbial biomass dynamics than extracellular enzymes (Pérez-de-Mora et al. 2008). Values of DH were significantly higher in the third and fourth samplings for most of the treatments except for CRT. These results may be therefore influenced by the higher sensitivity of DH to soil acidity compared to extracellular enzymes as the positive correlation between this activity and soil pH suggests (Table 2). In fact, soil pH in all treatments increased as time progressed including control soils (Pérez-de-Mora et al. 2006d).

Temporal dynamics of extracellular activities were very variable. Similarities with the activity patterns of other biochemical parameters estimated such as MBC and DH were only found for Aryl (Fig. 2). Here soils amended with composts and SL showed the largest activity (Fig. 2a). As observed for DH, the highest activity values were recorded in the third sampling. In the other treatments (LIT, LEO, CTR, CTRP) little variation was found during the experiment (Fig. 2a). Temporal and treatment induced differences for Aryl may be related to changes in soil pH and trace element availability as the correlations between these parameters indicate (Table 2). In fact, this activity has been outlined as a very sensitive enzyme in tracing heavy metal effects (Hinojosa et al. 2008). Given that SO_4^{-2} availability is enhanced as soil pH increases and sulphides from the spill should act as the main source of SO_4^{-2} anions in all treatments, data suggests that Aryl was less affected by sulphate concentrations in soil than by the pH or metal concentrations.

Results from β-gluc, Phosph and Prot differed from the rest of biological parameters studied (Fig. 2b–d). This may be explained by the fact that

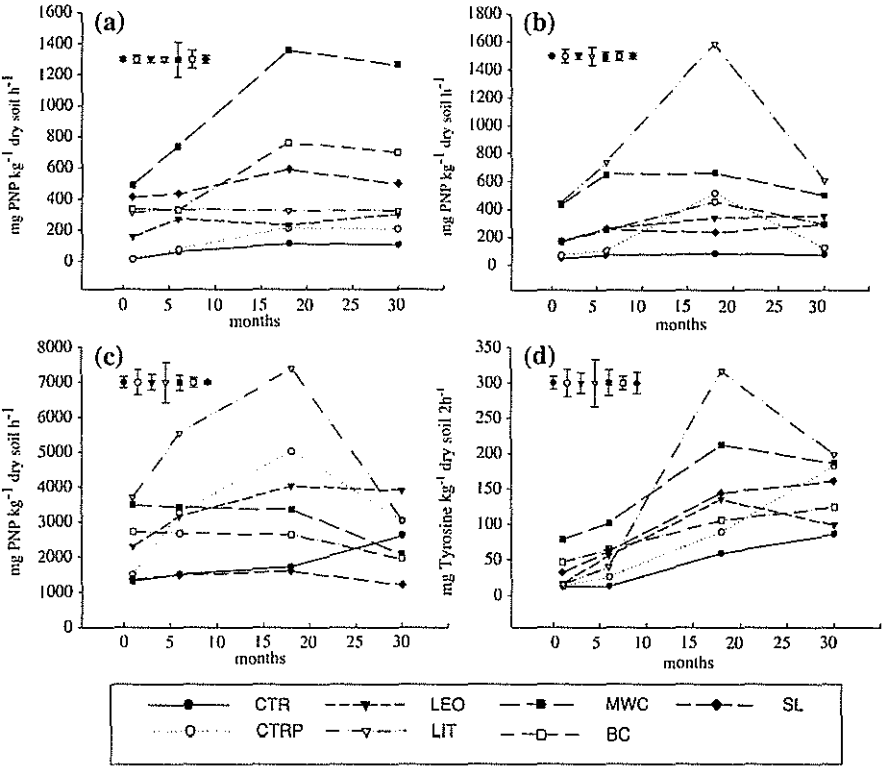

Fig. 2 Temporal dynamics of **a** aryl-sulphatase C, **b** β-glucosidase **c** acid-phosphatase and **d** protease. Symbols represent mean values. Error bars represent the maximum standard deviations within one treatment during the whole experiment

concentrations of the substrates and the final products resulting from these activities could be highly influenced by the incorporation of amendments and the development of a plant cover. In the first case, enzymatic activity would be promoted as more substrate is present. On the other hand, if the final products of enzymatic reactions accumulate and are readily available, enzymatic release and hence potential activity might decrease (feedback inhibition). In the case of β-gluc little temporal variations were recorded during the experiment, except in the LIT and CTRP treatments in which mean values for the third sampling event were significantly higher than those found in the other treatments (Fig. 2b). Both LIT and CTRP showed a significant increase in β-gluc between the second and the third samplings followed by a sharp decrease in activity towards the end of the experiment (Fig. 2b). This trend could be related to a significant improvement in vegetation cover in these two treatments between the second and the third sampling (Pérez-de-Mora et al. 2006c). In the other amended soils incorporation of C substrates through the addition of amendments and the presence of a healthy plant

cover from the beginning of the experiment could account for a more homogeneous C pool in the soil and thus a more constant pattern of β-gluc over time.

Acid-phosphatase showed a similar trend as β-gluc in the LIT and CTRP treatments (Fig. 2c). For the other soils different results were observed: in the case of LEO, Phosph increased as time progressed, whereas in CTR potential activity remained low until the third sampling and it increased sharply towards the end of the experiment. It is possible that improved soil conditions in these treatments at the end of the experiment (f.ex. less acid pH in CTR) could account for higher potential activity values (Fig. 2c). In contrast, soils amended with composts and SL showed a decrease in Phosph at the end of the experiment (Fig. 2c). This might be due to feedback inhibition of the enzyme by inorganic phosphate as suggested by the negative correlation between acid-phosphatase and available-P (Table 2). These results are also in agreement with data reported by other authors for other soils into which amendments with a high P content were incorporated (Madejón et al. 2003, Plaza et al. 2004).

Protease activity generally increased in all treatments as time progressed, except in LIT, where a similar pattern as for β-gluc and Phosph was observed (Fig. 2d). Prot was negatively correlated with soluble heavy metal concentrations and positively correlated with soil pH (Table 2). Therefore amelioration of soil properties and metal availability could partly account for this general trend. In addition, temporal variations in N availability related to amendment incorporation, plant maturity and mineralization processes could also influence Prot in the soil as the positive correlation between protease and N-Kjeldahl indicates (Table 2). Other authors have also reported positive but rather weak correlations between these two variables arguing that the complex nature of organic N (Schulten and Schnitzer 1998, Wick et al. 2002) and the substrate specificity of the various extracellular proteases (Kalisz 1988) may account for this observation.

4 Conclusions

- The incorporation of amendments and/or development of a plant cover show great potential for stabilizing trace elements in soil in the mid-term, enhancing nutrient cycling and microbial activity at the same time.
- Due to their alkaline nature, composts and sugarbeet lime performed better than other amendments and thus have more potential for field application. However, once a vegetation cover has established, amendment additions may be unnecessary or kept at a minimum.
- Remediation practices showed a positive effect on soil functionality which is enzyme and treatment dependent. Enzymatic activities such as dehydrogenase, aryl-suplhatase and protease seem to be more sensitive to changes in soil pH and trace element availability than β-glucosidase and acid-phosphatase, which seem to be more strongly influenced by substrate or enzymatic product availability.

- The utilization of microbial enzymatic assays for assessing soil remediation practices can be very helpful, but various activities are required for diagnostic monitoring of soil restoration.

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