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A MICROBIAL APPROACH IN SOILS FROM CONTAMINATED MINE AREAS: THE JALES MINE (PORTUGAL) CASE STUDY

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SUMMARY

Microorganisms play a crucial role in decomposition processes and nutrient cycling and, therefore, in soil quality. Soil enzymes have shown sensitivity to contaminants, such as heavy metals, due to their interaction with the specific reaction sites, thereby reducing the formation of the reaction products. In this study, several enzymatic bioassays were applied (dehydrogenase, acid phosphatase, arylsulfatase, urease and ß-glucosidase) to soils with different heavy metal contents, from the abandoned Jales mine (Portugal), before, during and after a rehabilitation process (years 2002, 2003 and 2004, respectively). Additionally, the mineralization of N and the microbial biomass of C and N were measured.

The results obtained in this study indicated that dehydrogenase, arylsulfatase and N-mineralization activities demonstrated that there had been a recovery in soil microbial numbers, but provided no information on the influence of contaminants in soils. Microbial biomass C and N also presented an increase from 2002 to 2004, and soil organic matter and pH influenced the enzymatic activities, mainly dehydrogenase, acid phosphatase and arylsulfatase.

An increase of microbial activities was observed in 2003, with several soil enzymes showing recoveries in their activities. Therefore, nutrient cycles have probably benefited from this, improving soil quality.

KEYWORDS:
heavy metals, soil enzyme, microbial biomass, soil contamination.

INTRODUCTION

Soil quality is intimately related to both physicochemical soil properties and soil biological functions, and their evaluation will help to characterize the fertility and productivity of soils. Recently, some attention has been also directed to soil habitat function, and the assessment of factors that influence toxicity towards microorganisms and all processes that they mediate need to be taken into consideration [1].

Some authors [2-4] have suggested that biological or biochemical properties that have been demonstrated to be useful for the detection of soil quality changes are related to nutrient cycles. Soil enzymatic activity can be used as a sensitive index addressing heavy metals contamination. Nitrification or respiration are usually recommended as toxicity endpoints in specific guidelines, but other endpoints, e.g. soil enzymatic activities, have also the advantages of having rapid and cost-effective assays [5]. Metals are able to reduce enzyme activity by: 1) interacting with the enzyme-substrate complex, 2) denaturing the protein by the interaction with its active sites or 3) by simply affecting the microbial cells that produce the enzymes [6-8]. Not all metal species can induce similar effects, so metal speciation in the solid or liquid phases, and consequently their bioavailability, will be of great importance on producing effects in soil enzymatic processes [9].

The use of single enzyme bioassays has been criticized by several authors [2, 3, 10, 11], because each enzyme catalyses a specific reaction, using a specific substrate. Therefore, measuring different enzyme activities is advisable in soil quality studies.

This study is integrated in a larger work carried out in Jales mine, where ecotoxicological approaches evaluated soil toxicity and contamination in two soils of this area [12-14]. Over 3 years, four enzymatic activities, important in different nutrient cycles in soil and decomposition processes (arylsulfatase, urease, acid phosphatase and ß-glucosidase) were determined. Nitrogen mineralization was also determined and the soil dehydrogenase activity was measured because it is considered to be an endocellular enzyme, playing an integral part in microbial metabolism and, therefore, an indicator of physiologically active organisms. Such an activity is also involved in the oxidation of organic matter [15-17].

Two hypotheses were raised in this study: 1) are single enzymatic activities able to mirror soil contamination? 2) are soil enzymes able to detect slight contamination changes/ status during short-term periods?

MATERIAL AND METHODS

Study area

The study was carried out in the abandoned Jales mine vicinities (near Vila Pouca de Aguiar) in the northeast of Portugal. During 1993, the mine was abandoned and from September 2002 to late 2003 the mine spoil was rehabilitated by an environmental concessionary hired by the Portuguese government, and acting independently from this study. In this process, the mine spoil was impermeabilized using geosynthetic layers to prevent the spreading of spoil particles. This procedure prevented silt-sand-clay materials contaminated with As, Cd, Pb, Zn and Cu to flow over the surrounding area of the mine spoil, and with a consequent input of contamination.

TABLE 1
Physico-chemical properties, biological characteristics and metal content in JNC and JC soils from Mina de Jales (Portugal) in 2002.

Parameters	units	JNC soil	JC soil
pH	-	4.14	4.47
Dry matter	%	70.25	66.19
Soil Organic Matter	%	5.07	2.88
Cation Exchange Capacity	cmol/Kg	4115	5492
Max. Water Holding Capacity	%	36.5	35.1
Redox Potencial	mV	59	47
Sand	%	20.96	23.08
Clay	%	16.8	13.6
Silt	%	27.51	25.02
Total Carbon	%	8.55	4.61
Total Nitrogen	%	0.4	0.4
Sulfur	%	0.02	0.03
Calcium	mg/Kg	441	1840
Potassium	mg/Kg	1290	2670
Microbian Biomass N	mg/Kg	40.0	84.4
Microbian Biomass C	mg/Kg	96.9	792.2

Heavy metal (mg/Kg)	JNC soil	JC soil
As	71	251
Ag	<0.2	1.5
Al	14 000	10 000
Be	2.0	3.1
Cd	1.9	8.2
Co	n.d.	n.d
Cr	6.0	15.0
Cu	8.0	24.0
Fe	7 370	17 800
Hg	<0.05	<0.05
Mn	99	255
Ni	5.0	9.0
Pb	33.0	209.0
Sb	0.37	2.29
Se	0.47	0.38
Zn	33	97

Two distinct areas were chosen due to their different heavy metal content. The fields nearby the mine spoil are used for agricultural purposes, but the sampling area (±400m²) chosen has not been used for the past years for agricultural nor pastoral purposes. Cambisoil collected from this area (N 41° 27' 53.9''; W 07° 34' 50.6''), hereafter identified as JC soil, has high heavy metal concentrations (Table 1). This area was harvested every summer. The other selected sampling area (±800m²) was located 3 km from the mine spoil and was mainly used for cow and horse pasture (N 41° 28' 36.1''; W 07° 34' 14.0''). The cambisoil collected from this area, hereafter identified as JNC soil, has lower heavy metal contents when compared to the JC soil (Table 1), with the exception of aluminium content. The water-soluble metal content from soil elutriates is given in Loureiro *et al.* [13].

The vicinities of the mine spoil enclose agriculture and pasture fields with vegetation composed by *Alnus glutinosa* (L.) Gaertn., *Carpinus betulus* L., *Castanea sativa* Miller, *Frangula alnus* Miller, *Fraxinus angustifolia* Vahl, *Malus domestica* Bork, *Pinus pinaster* Aiton, *Pinus sylvestris* L., *Quercus pyrenaica* Willd., *Quercus robur* L., and *Salix atrocinerea* Brot..

Soil sampling and soil analysis

In this study, the sampling was made by a composite sampling procedure due to possible high heterogeneity of soil properties within a sampling area.

In each selected area, 10 soil samples of the top 0-10 cm (Ø 20 cm) were collected randomly, stored in open plastic bags and then transported to the laboratory. In the lab, the litter layer was removed and the soils sieved on a <2-mm mesh, and physico-chemical analyses were then conducted. Soil samples were pooled and stored in closed plastic bags at 4 °C until enzymatic analysis was completed. As recommended, soil enzymatic activities were processed without freezing, drying out or waterlogged during storage [18]. Soil pHs were determined in a KCl (1M) solution [19], and soil moisture was measured by the difference in weight be-fore and after drying the soil, at 105 °C, in an oven, overnight.

The organic matter content was determined by the loss-ignition method, after the determination of soil moisture content, from loss in weight after 5 h at 540 °C (adapted from [20]). Microbial biomass of C and N were measured, as based in a standardized protocol ISO 14240-2 [21], and the total N was determined by a Kjeldahl's digestion.

Heavy metal content was only analysed in the soil samples collected in 2002 because, as the spoil was being impermeabilized, no more metal input was expected. Also, the heavy metal content was similar to the values found by other authors some years before [22]. Nitrogen content and microbial biomass of C and N were evaluated in 2002 and 2004. Heavy metal analysis was performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES, Perkin Elmer 4300 DV). Soil digestion was made in HCl and HNO_3 (3:1).

Enzymatic Activities

In this study, we measured the enzymatic activities in the two soils at different stages of rehabilitation: in February 2002 (no rehabilitation), February 2003 (during rehabilitation) and February 2004 (after rehabilitation), and discuss the effects of contamination and rehabilitation on soil quality.

Dehydrogenase (DHA) (EC 1.1.1.49) activity was determined by the suspension of soil samples in a triphenyltetrazolium chloride (TTC) solution and incubated for 24 h at 40 °C. The triphenyl formazan (µg TPF / g.dm / h) produced was extracted with acetone and measured photomet-

rically at 546 nm. This methodology is similar to the ISO draft that is being developed by ISO [23], and was based on the methods of Schinner *et al.* [24]. To determine acid phosphatase (EC 3.1.3.2) activity, soil samples were suspended in a buffered p-nitrophenyl phosphate solution (pH= 5) and incubated for 2 h at 35 °C. The p-nitrophenol produced (µg pNP / g.dm / h) was coloured and measured photometrically at 405 nm in a microplate reader [24-26]. Arylsulfatase (EC 3.1.6.1) activity was determined by incubating soil samples in a buffered potassium-p-nitrophenylsulfate solution (pH=5.8) at 37 °C, for 1 h. The accumulation of p-nitrophenol (µg pNP / g.dm / h) was measured as in the phosphatase protocol, based on Schinner *et al.* [24]. The method for ß-glucosidase (EC 3.2.1.21) activity determination was adapted from methodologies of Tabatabai [26], where soil samples were incubated in a buffered p-nitrophenyl-ß-D-glucoside solution (pH=6). The measurement of p-nitrophenol (µg pNP / g.dm / h) was done as previously explained. Urease (EC 3.5.1.5) activity was determined following the protocol previously described by Schinner *et al.* [24], and Kandeler and Gerber [27], where soil samples were suspended in a borate buffer (pH=10) and urea solutions incubated for 2 h at 37 °C. The release of NH_4^+ (µg N / g.dm / 2 h) was measured in a microplate reader at 690 nm. For the N-mineralization determination, soil samples were incubated in water at 40 °C, and 1 week later NH_4^+ was extracted with potassium chloride. NH_4^+ was measured as in the urease methodology [24].

All measurements were performed in 5 sub-replicates plus 3 controls.

Statistical analysis

Soil enzyme activities were compared by one-way analysis of variance ANOVA [28], using the SigmaStat statistical package [29]. To compare the differences in their enzymatic activity between soils and sampling time, a two-way ANOVA was performed. When significant differences were found, following the analysis of variance, a post-hoc multiple comparison Tukey test was used to assess differences between soils and sampling times. The Dunn's test was applied when there was a positive result ($p \leq 0.05$) after a Kruskal-Wallis one-way analysis of variance on ranks (when data had not a normal distribution).

To evaluate the weight of abiotic environmental properties on the ecosystems biotic fraction, multivariate analysis has been used in ecology, but this type of analysis is not widely used in ecotoxicology [30]. In this study, Redundancy Analysis (RDA) was performed with CANOCO [31], using enzymatic activities as species and organic matter content, pH and soil moisture content as environmental properties. Enzymatic data were transformed with the internal function ln (x + 1) to standardize and normalize all the data. The Monte Carlo Permutation method was used to assess marginal effects associated with environmental factors.

RESULTS

Changes in pH values and organic matter content in JNC and JC soils throughout the sampling periods, and also summer and autumn months in 2003, are presented in Fig. 1. There was an increase in soil pH from 2002 to 2004 and, on the other hand, a decrease in soil organic matter in the same period. JC soil pH also suffered some fluctuations within the year of 2003, with its higher value in November 2003.

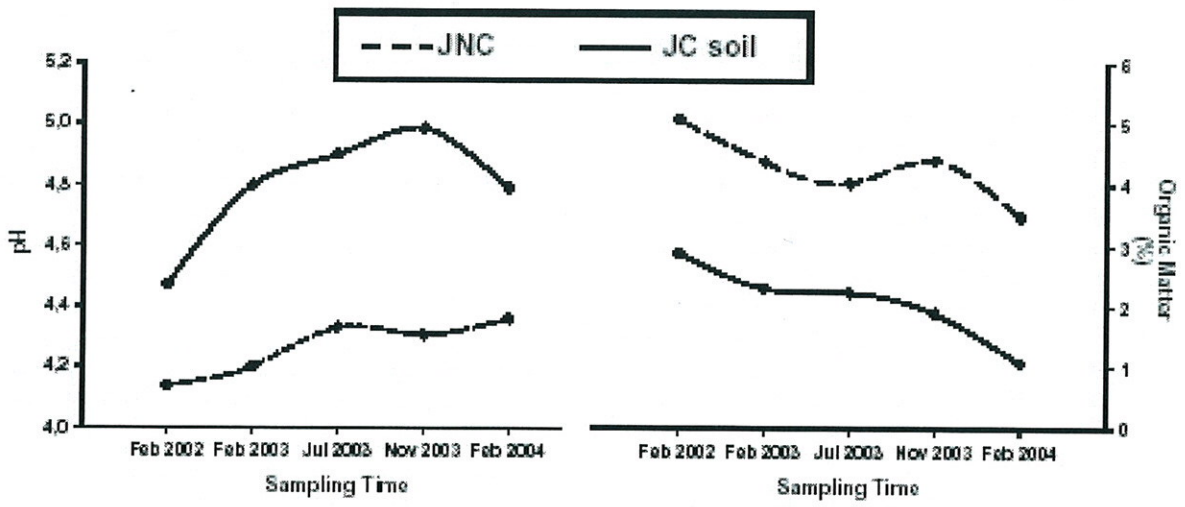

FIGURE 1- pH values and organic matter content (%) of JNC and JC soils collected from Jales Mine (Portugal) recorded during all sampling period (from February 2002 till 2004).

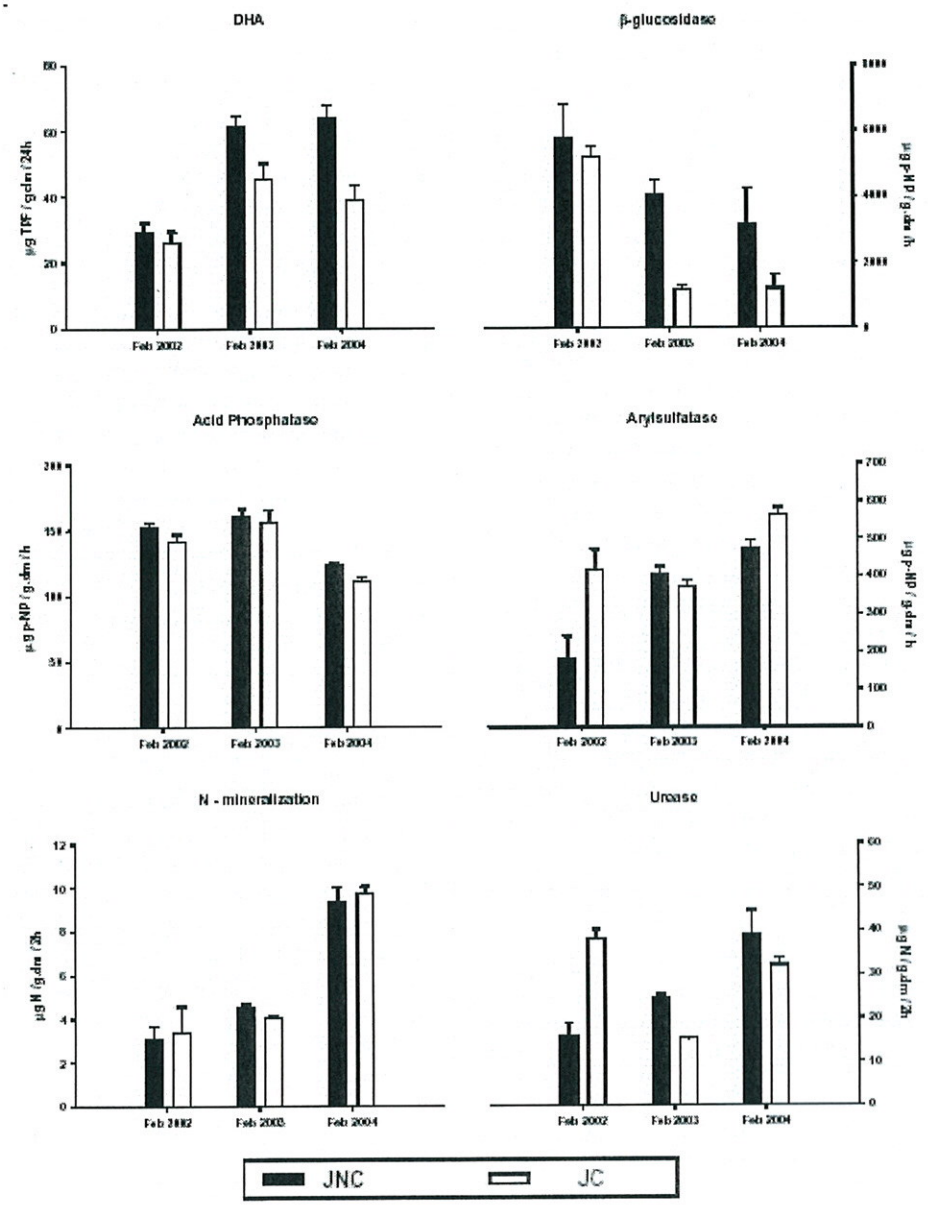

FIGURE 2- Enzymatic activities (average ± standard error) of JNC and JC soils collected from Jales Mine (Portugal) before, during and after the rehabilitation process (February 2002, February 2003 and February 2004).

Soil enzymes showed different patterns of activity before, during and after the mine's rehabilitation (Fig. 2). Additionally, microbial biomass of N increased from 2002 to 2004 from 40 to 84 mg/Kg in JNC soil, and was maintained in JC soil (84.4 to 91.40 mg/Kg) in the same period. Microbial biomass of C showed a significant increase in JNC soil from 96.90 to 361.50 mg/Kg, and also maintained its values in JC soil (792.20 mg/Kg in 2002 and 637.03 mg/Kg in 2004). Microbial biomass C and N increased significantly four and two times, respectively, in 2004 in JNC soil.

During the same period, the DHA activity increased significantly two-fold in the JNC soil and 1.5 times in the JC soil in 2004, when compared to 2002 (two-way ANOVA, $F_{1,24}=21.162$, $p\leq0.05$). Before rehabilitation (2002), the two soils had similar activities, but during the first year of rehabilitation (2003) JNC soil showed a higher increase in activity, which turned out to be significantly different from the activity in JC soil (two-way ANOVA, $F_{2,24}= 27.513$, $p\leq0.05$).

During this 3-years' period both soils showed a decrease in their ß-glucosidase activity (two-way ANOVA, $F_{2,17}=18.913$, $p\leq0.05$). JNC soil decreased almost two-fold and JC soil 4 times from 2002 till 2004. This led to a significant difference between both soils only within 2003 and 2004 samplings (two-way ANOVA, $F_{1,17}=14.507$, $p\leq 0.05$).

During the three sampling years, there were no significant differences in the acid phosphatase activity between the JNC and the JC soils (two-way ANOVA, $F_{1,24}$= 4.168, p>0.05). Both soils showed a significant decrease in their phosphatase activity in February 2004, when compared to the two previous years (two-way ANOVA $F_{2,24}$= 31.106, p<0.05; Tukey test, p≤0.05).

In the JNC soil, the arylsulfatase activity increased significantly in 2004, reaching mean values 2.5 times higher than those from 2002 (one-way ANOVA, $F_{2,12}$= 16.419, p≤0.05; Tukey test, p≤0.05). The JC soil also showed a significant increase, but by 1.3 times, when compared to 2002 (one-way ANOVA, $F_{2,12}$=9.105, p<0.05; Tukey test, p≤ 0.05).

Although with similar N-mineralization rates, both soils showed markedly greater rates in 2004 when compared to 2002 and 2003 (two-way ANOVA, $F_{2,16}$=49.392, p≤0.05). The increased rate was three times higher than that before rehabilitation (February 2002).

The urease activity did not show significant differences between soils in 2004 (two-way ANOVA $F_{2,18}$= 0.503, p>0.05). This activity increased significantly in 2004 in the JNC soil, reaching twice the value obtained before the beginning of mine rehabilitation (Kruskal-Wallis one-way analysis of variance on ranks, H=6,838, DF= 2, p≤0.05). Urease activity of JC soil showed a different behaviour, decreasing in 2003, when compared to 2002, but recovering again to similar values in February 2004 (Kruskal-Wallis one-way analysis of variance on ranks, H=8,938, DF= 2, p≤0.05).

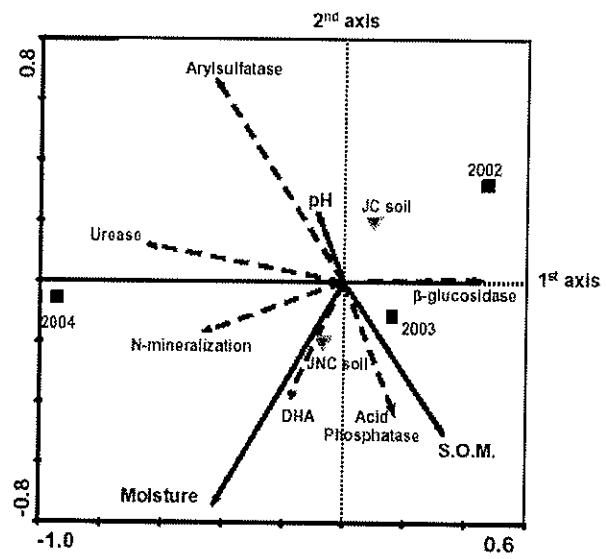

FIGURE 3 - Redundancy Analysis (RDA): triplot of enzymatic activities and environmental variables (centroid for soil type; centroid for sampling year; - - - enzymatic activity; _____ environmental parameters) of JNC and JC soils in February 2002, 2003 and 2004.

The RDA analysis showed that 91.6% of the variability of our data is explained by the 1st axis (19.4% is associated with the enzymes, and the remaining 72.2% is explained by the interaction between enzymes and environmental factors). The 2nd axis explains 6.6% of the variability (1.4% is associated with the enzymes, and the remaining 5.2% is explained by the interaction between enzymes and environmental factors). The eigenvalues for the 1st and 2nd axis were 0.194 and 0.014, respectively (Fig. 3).

From the triplot of Fig. 3, it can be observed that the 1st axis explains the distribution of the sampling years, placing the year 2002 in an opposite part of the triplot when compared to the year 2004. This distribution occurs along a gradient defined by urease and N-mineralization (associated with 2004), and ß-glucosidase (associated with 2002).

It can also be observed that both axes separate clearly the two soils. The 2nd axis separates also the enzymes that showed recovery (increase in theiractivities) along the 3 years (arylsulfatase, urease, DHA and N-minaralization) from the ones that showed even a decrease in their activity in 2004, when compared to 2002 (ß-glucosidase and acid-phosphatase).

DISCUSSION

In both soils, the rehabilitation process induced an increase in soil enzymatic activities, with the exception of ß-glucosidase and acid phosphatase.

JNC soil denoted higher enzymatic activities and also an increase in the microbial biomass C and N in 2004, showing a recovery of microbial biomass, during and at the end of the rehabilitation process. Changes in the acid phosphatase activity were related to changes of pH (decreasing with the increase of pH values), and directly related with SOM contents, which was also reported by several authors [32, 33]. The major reason for this decrease of acid phosphatase with the increase of pH might be the fact that this enzyme is more predominant in acid soils, whereas alkaline phosphatase is predominant in more alkaline soils. So, as pH increases from 2002 till 2003 and maintained in 2004 (comparing only between the February months), the acid phosphatase activity decreases as the alkaline phosphatase increases. These authors also reported that arylsulfatase presented the opposite trend with pH values, increasing with the increase of pH.

DHA and arylsulfatase activities and N-mineralization rate showed a better and positive response to the mine spoil rehabilitation process, while urease behaved differently; acid phosphatase and ß-glucosidase produced a decrease in their activity afterwards. DHA is related to active microorganisms and has been used as an indicator of soil quality [15, 16]; it generally increases with the increase of soil pH. The increase of the mineralization process will improve the transformation of organic forms to mineral forms, which is of extreme importance to plants. Consequently, their increase is a good indication for soil quality improvement. Also SO_4^{2-} is essential for plants and also immobilized by

edaphic organisms; its production is promoted by arylsulfatase activity which also presented an increase in both soils from the mine area [34].

From 2002 until 2004, there was an increase in the microbial biomass C in JNC soil that might be responsible for the decrease/ degradation of the organic matter content in both soils collected from the mine. But it also induced the decrease in ß-glucosidase activity that was unexpected and contradictory to what was found in several studies where this enzyme was always positively and highly related to the microbial biomass C content [4, 32, 35].

The increase in urease activity and N-mineralization rate was also related to the increase of the microbial biomass N, which doubled its value in February 2004, when compared to 2003, in JNC soil. When decomposition processes are taking place in soil, the C/N ratio will decrease with time because carbon biomass is lost as CO_2, while N is reused by microorganisms [34]. The decrease in the N biomass will reach a point when the microbial activity will also decrease, due to organic carbon loss. In good quality soils, this loss will take short periods to recover, depending only on the time that the organic material needs to be degraded [34, 36, 37].

The high concentration of Fe found in JC soil elutriates [13] can act as an inhibitor of enzymes, diminishing their activities, but also can make complexes with other ions like SO_4^{2-} and other heavy metal complexes, reducing the heavy metal toxicity [9]. Aluminium concentration in JNC soil elutriates can also play an important role in this soil toxicity to microorganisms and soil quality [13].

In this study, pH value and microbial biomass C and N were the parameters that seemed to influence the most the recovery of soil enzymes along time. Heavy metal speciation varies with slight pH changes and, therefore, changes in pH in both soils might be one of the responsible factors for differences in enzymatic activities. The increases of microbial biomass C and N are probably related to the significant increase of soil enzymatic activity in JNC soil.

CONCLUSIONS

In this study, DHA activity, an indication for living cells` presence in soils, showed a good recovery after the rehabilitation of the mine spill. This is usually considered to be a marker of soil quality. Additionally, arylsulfatase activity and N mineralization rate showed a good upturn in 2004, improving N and S cycles that are crucial for plants. With a different trend, enzymatic activities related to the C and P cycles showed regression in 2004. With these results we can state that a range of enzymes must be analysed to have a complete profile of microbial behaviour in soils. Otherwise, wrong assumptions can be taken as premises. In this study, it was observed that some soil enzymes responded to changes due to the mine rehabilitation.

The study of enzymatic activities in soil is a useful tool to check how nutrients are involved in the recycling of soils and how contamination or other stressors are affecting soil quality. Nevertheless, this assessment should be done as part of an integrative test battery, to fulfil the microbial gaps that usually exist in the ERA, but can not probably be used by their own as "a single enzymatic battery".

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REFERENCES

[1] Stuczynski, T.I., McCarty, G.W., and Siebielec, G. (2003) Response of soil microbiological activities to cadmium, lead and zinc salt amendments. J. Environ. Qual. 32, 1346-1355.

[2] Nannipieri, P., Kandeler, E., and Ruggiero, P. (2002) Enzyme activities and microbiological and biochemical processes in soil.In R.G. Burns and R.P. Dick (Ed-s.) Enzymes in the Environment: Activity, Ecology and Applications, Marcel Dekker, Inc., New York, Marcel Dekker, Inc., 1-33.

[3] Cepeda, C.T., Leirós, M.C., Seoane, S., and Sotres, F.G. (2000) Limitations of soil enzymes as indicators of soil pollution. Soil Biol. Biochem. 32, 1867-1875.

[4] Ajwa, H.A., Dell, C.J., and Rice, C.W. (1999) Changes in enzyme activities and microbial biomass of tallgrass prairie soil as related to burning and nitrogen fertilization. Soil Biol. Biochem. 31, 769-777.

[5] Carbonell, G., Pablos, M.V., García, P., Ramos, C., Sánchez, P., Fernández, C., and Tarazona, J.V. (2000) Rapid and cost-effective multiparameter toxicity tests for soil microorganisms. The Science of the total Environment. 247, 143-150.

[6] Vig, K., Megharaj, M., Sethunathan, N., and Naidu, R. (2003) Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Advances in Environmental Research. 8, 121-135.

[7] Guettes, R., Dott, W., and Eisentraeger, A. (2002) Determination of Urease activity in soils by carbon dioxide release for Ecotoxicological Evaluation of contaminated soils. Ecotoxicology. 11, 357-364.

[8] Madejón, E., Burgos, P., López, R., and Cabrera, F. (2001) Soilenzymatic response to addition of heavy metals with organic residues. Biol. Fertil. Soils. 34, 144-150.

[9] Allen, H.E. (2002) Bioavailability of Metals in Terrestrial Ecosystems: Importance of Partitioning for Bioavailability to Invertebrates, Microbes, and Plants. In: Lee, C. (Ed.) Metal and the Environment Series, SETAC, New York, SETAC, 158.

[10] Cepeda, C.T., Leirós, M.C., Sotres, F.G., and Seoane, S. (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol. Fertil. Soils. 26, 100-106.

[11] Leirós, M.C., Cepeda, C.T., Fernández, F.G., and Sotres, F.G. (1999) Defining the validity of a biochemical index of soil quality. Biol. Fertil.Soils. 30, 140-146.

[12] Loureiro, S., Soares, A.M.V.M., and Nogueira, A.J.A. (2005) Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut. 138, 121-131.

[13] Loureiro, S., Ferreira, A.L.G., Soares, A.M.V.M., and Nogueira, A.J.A. (2005) Evaluation of the Toxicity of Two Soils from Jales mine (Portugal) Using Aquatic Bioassays. Chemosphere. 61(2), 168-177.

[14] Loureiro, S., Santos, C., Pinto, G., Costa, A., Monteiro, M., Nogueira, A.J.A., and Soares, A.M.V.M. (2006) Toxicity Assessment of Two Soils from Jales Mine (Portugal) Using Plants: Growth and Biochemical Parameters. Arch. Environ. Contam. Toxicol. 50, 182–190.

[15] Rossel, D. and Tarradellas, J. (1991) Dehydrogenase activity of soil microflora: significance in ecotoxicological tests. Environmental Toxicology and Water Quality: An International Journal. 6, 17-33.

[16] Rossel, D., Tarradellas, J., Bitton, G., and Morel, J.-L. (1996) Use of enzymes in soil ecotoxicology: a case of dehydrogenase and hydrolytic enzymes. Soil Ecotoxicology, Lewis Publishers, Lewis Publishers, 179-206.

[17] Römbke, J., Bauer, C., and Marschner, A. (1996) Hazard assessment of chemicals in soil. Proposed ecotoxicological test strategy. Environ. Sci. Pollut. Res. 3(2), 78-82.

[18] ISO, Soil quality- Sampling- Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory. 1993, ISO- The International Organization for Standardization: Genève. p. 4.

[19] ISO, Soil quality- Determination of pH. 1994, ISO- The International Organization for Standardization: Genève. p. 5.

[20] Storer, D.A. (1984) A simple high sample volume ashing procedure for determination of soil organic matter. Commun. in Soil Sci. Plant Anal. 15(7), 759-772.

[21] ISO, Soil quality -- Determination of soil microbial biomass -- Part 2: Fumigation-extraction method. 1997, ISO- The International Organization for Standardization: Genève. p. 12.

[22] Santos Oliveira, J.M. and Ávila, P.F. (1995) Avaliação do impacto químico ambiental provocado por uma exploração mineira. Um caso de estudo na Mina de Jales. Estudos, Notas e Trabalhos, I. G. M. 37, 25-50.

[23] ISO, Soil Quality- determination of dehydrogenase activity in soils-Part 1: Method with TTC. Draft (Enquiry stage), ISO- The International Organization for Standardization: Genève. p. 5.

[24] Schinner, F., Ohlinger, R., Kandeler, E., and Margesin, R. (1996) Methods in Soil Biology. Springer- Verlag, Berlin, Springer- Verlag, 426.

[25] Dick, R.P., Breakwell, D.P., and Turco, R.F. (1996) Soil Enzymes activity and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W. and Jones, A.J. (Eds.) Methods for assessing soil quality, Soil Science Society of America, Inc., Madison, Wisconsin, Soil Science Society of America, Inc., 247-272.

[26] Tabatabai, M.A. (1994) Soil Enzymes. In: Weaver, R.W., Angle, J.S. and Bottomley, P.S. (Eds.) Methods of soil Analysis, Part 2. Microbiological and biochemical Properties, Soil Science Society of America, Madison, Soil Science Society of America, 775-833.

[27] Kandeler, E. and Gerber, H. (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fertil. Soils. 6, 68-72.

[28] Zar, J.H. (1996) Biostatistical Analysis (Ed-s.), Prentice-Hall International, Inc., NJ, USA, Prentice-Hall International, Inc.,

[29] SPSS, SigmaStat for Windows (version 2.03), S. Science, Editor. 1995: IL.

[30] Van den Brink, P.J., Van den Brink, N.W., and Ter Braak, C.J.F. (2003) Multivariate analysis of ecotoxicological data using ordination: demonstrations of utility on the bases of various examples. Australian Journal of Ecotoxicology. 9, 141-156.

[31] Ter Braak, C.J.F. and Smilauer, P. (2002) CANOCO Reference manual and CanoDraw for Windows User's guide: Software for Canonical Community Ordination (version 4.5). In: Power, M. (Ed.), Ithaca, USA.

[32] Mullen, M.D., Melhorm, C.G., Tyler, D.D., and Duck, B.N. (1998) Biological and biochemical soil properties in no-till corn with different cover crops. Journal of Soil and Water Conservation. 53(3), 219-224.

[33] Ekenler, M. and Tabatabai, M.A. (2003) Responses of phosphatases and arylsulfatase in soils to liming and tillage systems. J. Plant Nutr. Soil Sci. 166, 281-290.

[34] Varennes, A. (2003) Productividade dos Solos e Ambiente. In: Varennes, A. (Ed.) Escolar Editora. Lisboa, Escolar Editora, 490.

[35] Turner, B.L., Hopkins, D.W., Haygarth, P.M., and Ostle, N. (2002) b-Glucosidase activity in pasture soils. Applied Soil Ecology. 20, 157-162.

[36] Stevenson, F.J. and Cole, M.A. (Eds.) (1999) Cycles of Soil. John Wiley & Sons, Inc., New York, John Wiley & Sons, Inc., 427.

[37] Knoepp, J.D., Coleman, D.C., Crossley Jr., D.A., and Clark, J.S. (2000) Biological indices of soil quality: an ecosystem case study of their use. Forest Ecology and Management. 138, 357-368.

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