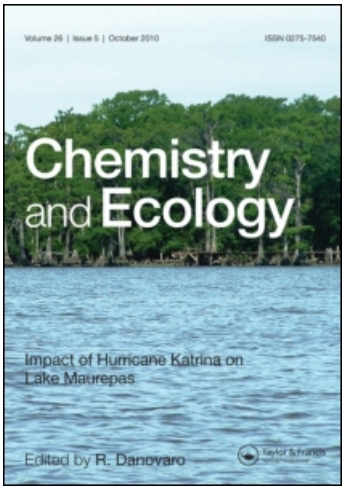

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Shifts in Microbial Communities, Microbial Biomass and Organic Matter Mineralization for Three Mediterranean Soils Contaminated By Metals

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SHIFTS IN MICROBIAL COMMUNITIES, MICROBIAL BIOMASS AND ORGANIC MATTER MINERALIZATION FOR THREE MEDITERRANEAN SOILS CONTAMINATED BY METALS

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Microbial communities (phospholipid fatty acid pattern, bacterial growing strategies, eco-physiological index (EPI) and total bacteria counts, as a number of heterotrophic culturable bacteria), substrate-induced respiration (SIR), and nitrogen mineralization were studied in three Mediterranean soils at three different depth levels (A, B and C). Soils were experimentally treated with a final concentration of 1000 ppm of trace metals (Cu^{2+}, Zn^{2+}, Al^{3+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+} and Cd^{2+}). Soils were stored in 57 l plastic containers for one year, and watered with 100 l during this period. Leachate was recovered through a bottom tap. Samples of the three depths were studied. Soil microbial communities showed different effects to other studies presented in the literature, but carried out on non-Mediterranean soils. Dramatic differences were found between treated soils and untreated ones, but not between soils or horizons. The treated soil displayed a decrease in CFUs, SIR N-mineralization and EPI together with a dominance of *r*-growing strategists. The relative moles percent of several PLFAs, especially 15:0, 16:1ω7, cy17:0, br18:0 and 18:1ω7 decreased because of the pollution of soils, whereas 10Mel6, 18:2ω6, cy19:0, i16:0 and br17:0 showed higher values than in untreated soils.

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Keywords: Heavy metal; Mediterranean soils; microbial communities; biomass; organic matter

INTRODUCTION

Soil micro-organisms are key components in the biogeochemical cycling of various elements. They play a significant role in the mineralization of organic matter (Alexander, 1973) and hence are of prime importance in maintaining the fertility of soils. As a result there is an increasing awareness of the need for a greater understanding of factors that influence either the amount of microbial biomass, the structure of microbial communities or the rate of microbial processes in soils.

The detrimental effect of heavy metal pollution on soil microbial processes has been extensively reviewed by Bååth (1989) and studies have been focused mainly on describing the toxic effect of metals and define threshold levels when biological soil processes begin to be affected.

The relative effects of heavy metal pollution on ecosystem functioning can be determined by comparing microbial biomass and activity, rates of organic matter degradation and subsequent nutrient release in contaminated and non-polluted soils. Comparative studies have reported reductions in microbial biomass or soil enzyme activity for coniferous acidic forest soil exposed to atmospheric deposition of metals from smelters (Freedman and Hutchinson, 1980; Nordgren *et al.*, 1986; Bååth *et al.*, 1992; Fritze *et al.*, 1996), for subarctic forest or loamy soils (Frostegard *et al.*, 1993; Pennanen *et al.*, 1996), for agricultural soils amended with metal-containing sewage sludge (Fliessbach *et al.*, 1994; McGrath *et al.*, 1995), and under controlled experimental conditions (Brynhildsen *et al.*, 1995; Probanza *et al.*, 1996). However, little or no information is available about effects of heavy metal addition on biological processes which take place on low organic content Mediterranean soils, as it can be concluded from the exhaustive review of Bååth (1989).

Information about this topic is extremely important in order to know the medium-term effects of multiple heavy metal pollution on such soils. This requirement has recently been highlighted by the uncontrolled leak of around 5.000 tonnes of sludge containing

metalliferous mining residues which recently happened in south-western Spain, close to a National Reserve (Parque Nacional de Doñana).

The aim of the present work is to determine the effects of a multiple metal pollution (Cu^{2+}, Zn^{2+}, Al^{3+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+} and Cd^{2+}) on three Mediterranean soils sampled at three different depths, one year after adding the metals. Changes in the structure and activity of the microbial communities (phospholipid fatty acids – PLFAs (Bååth *et al.*, 1992), bacterial growth strategies, eco-physiological index (De Leij *et al.*, 1993) and total bacteria counts, as a number of heterotrophic culturable bacteria), together with substrate-induced respiration (SIR, Nordgren *et al.*, 1988), and nitrogen mineralization (Tyler, 1975) were determined as indicators of pollution effects.

MATERIALS AND METHODS

Site Description and Soil Sampling

Soils were collected from three different zones of Madrid during December of 1996: Sevilla la Nueva, **S** (coordinates 40°22′N, 4°1′30″W), Brunete, **B** (coordinates 40°24′N, 4°W) and Monteprín-cipe, **M** (coordinates 40°25′N, 3°51′W). In each case, sampling was done at three depths: 0–25 cm (**A**), from 26 to 45 cm (**B**) and below 46 cm (**C**), separately but not as soil cores.

Experimental Treatments

Soil profiles were recreated in 57 l plastic containers (Fig. 1). Containers were located in a greenhouse in order to avoid direct rain over the experimental system and to reduce extreme temperature changes. Each soil was watered at the maximum of its water holding capacity with a mixed metals solution, containing 1000 mg l^{-1} of trace metals (Cu^{2+}, Zn^{2+}, Al^{3+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+} and Cd^{2+}), all as nitrates. A further three identical containers were prepared and watered with distilled water, and used as controls. The six containers containing treated (B*, M*, S*) and untreated (B, M,

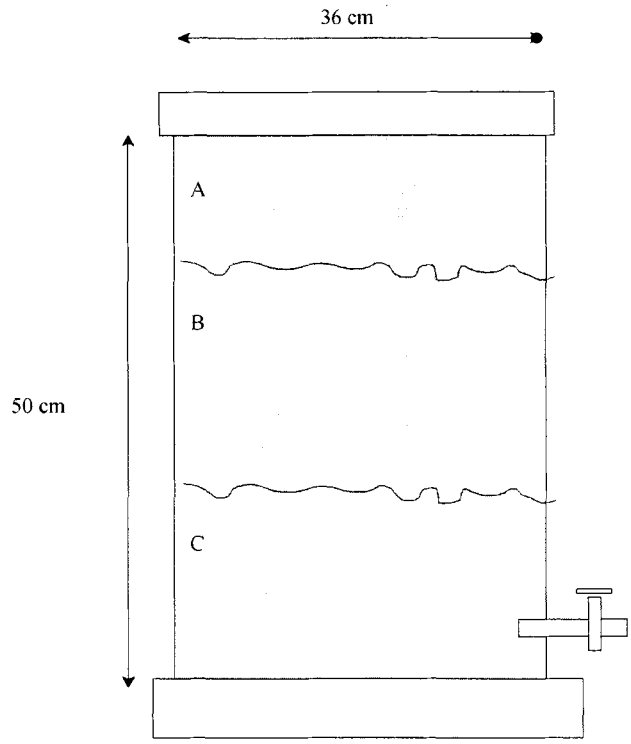

FIGURE 1 Scheme of experimental system.

and S) soils were kept for one year (January, 1997 to December, 1997) and watered weekly with sufficient distilled water to restore evaporation loses. The average of volume was 1.921 week, which varied between winter (around 1 l week^{-1}) and summer (around 4 l week^{-1}). After one year in incubation, samples of soils from each depth were taken for chemical and biological analysis and sieved through a 5 mm mesh.

Soil Chemistry

Physico-chemical Characteristics of Soils

pH was determined in distilled water : soil (2.5:1, v/w), with a Beckman 12 P/N 123134 pH meter and a Radiometer GK 2401 electrode. Texture was determined following the Boyoucos densitometry method

(Day, 1965). Total nitrogen was determined by the colorimetric method of Smith (1960), after a Kjeldahl digestion in a Prolabo Maxidigest-MX350 microwave digestor. Organic carbon was evaluated as weight loss on ignition by heating oven-dried (105°C) samples to 400°C for a minimum of 4 h. Finally, cation exchange capacity (CEC) was measured according to that described by Chung and Zarosky (1993). All parameters were assayed on each soil depth from the three soils.

Heavy Metal Content

A sequential extraction procedure of heavy metals was performed (Tessier *et al.*, 1979) except for aluminium, and followed by quantification of each metal, by using Merck colorimetric kits: copper (Spectroquant®-14767), zinc (Spectroquant®-14832), aluminium (Spectroquant®-14825), iron (Spectroquant®-14761), lead (Spectroquant®-14833), nickel (Spectroquant®-14785), manganese (Spectroquant®-14770), cadmium (Spectroquant®-14834) and chromium (Spectroquant®-14758). The detection limit of these kits is 0.01 mg l^{-1} for each metal, with a exception for chromium (0.001 mgL^{-1}). For each case, total metal fraction was determined on 5 g of soil.

Microbial Community Structure

Microbial community structure was studied using four different parameters: phospholipid fatty acid (PLFA) analysis, bacterial growth strategies, eco-physiological index and total bacteria counts (as the number of heterotrophic culturable bacteria).

PLFA Analysis

PLFA analysis was made on 0.5 g of soil. Soil samples were suspended in 1 ml of sterile distilled water. Two aliquotes were sampled: 0.1 ml of the suspension was used for the plate counts and bacterial growth strategy (see below), and 0.9 ml for the PLFA analysis.

The PLFA analysis was also carried out by washing plates with 30–40 colonies used for counts after 2–3 days of incubation at

20°C. Bacteria were collected by adding 3 ml of 0.15 M citrate buffer (pH 4.0) to the plate; then, the agar surface was gently scraped out with a glass stick, and 1.5 ml of the bacterial suspension recovered for lipid extraction.

Lipids were extracted by a procedure described previously by Frostegård *et al.* (1991), and based on the method of Bligh and Dyer (1959). Briefly, 0.9 ml of soil suspension or 1.5 ml of bacterial suspension in citrate buffer (0.15 M, pH 4.0) were extracted for 2 h with a one-phase mixture consisting of chloroform, methanol and citrate buffer, in the proportions 1 : 2 : 0.8 (v/v). After centrifugation, the pellets were washed once with the one-phase mixture, and the supernatants were combined. The supernatants were separated into two phases by adding chloroform and citrate buffer, and 2 ml (soil samples) or 1 ml (plate bacteria suspension) of the lower phase was dried at 40°C under nitrogen. The lipid material was fractionated into neutral glycolipids and polar lipids on silicic acid (100–200 mesh, Unisil) columns by elution with chloroform, acetone and methanol respectively. The phospholipid-containing polar fraction was collected and dried at 40°C under nitrogen. A 74 μM methyl nonadecanoate (Me, 19 : 0) was added to the phospholipid fraction as an internal standard. Samples were then subjected to mild alkaline methanolysis (Dowling *et al.*, 1986). The resulting fatty acid methyl esters were analyzed on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and a 50-m HPI capillary column. Hydrogen was used as a carrier gas, and injections were made in a splitless mode. The temperature programme was as follows: initial temperature of 80°C for 1 min, increasing at 20°C min^{-1} to 160°C and then increasing at 5°C min^{-1} to the final temperature of 270°C, which was kept for 5 min. All the solvents and chemicals used were of analytical grade (Scharlau, Spain). Fatty acid standards were obtained from Sigma-Aldrich (USA). Glassware was washed with distilled water and heated at 400°C overnight.

Fatty acids were designated as the total number of carbon atoms: number of double bonds, followed the position of the double bond from the methyl end (ω) of the molecule. *cis* and *trans* configurations are indicated by *c* and *t* respectively. The prefixes, *a* and *i*, indicate anteiso and isobranching; br indicates an unknown methyl branching position; 10Me indicates a methyl group on the 10th carbon atom

from the carboxyl end of the molecule; and cy refers to cyclopropane fatty acids.

Bacterial Growth Strategies

Microbial community structure was assessed by using the method of De Leij *et al.* (1993). Therefore, the second aliquote (0.1 ml) of the soil suspension (1 g ml^{-1}) was used for plate-counting and bacterial growth strategy. For this purpose, three replicates of serial ten-fold dilutions of this suspension were prepared on sterile distilled water. Then, 500 µl of each dilution was plated in a medium, containing Soil Agar (Acero *et al.*, 1990) which includes standard agar (Difco, USA), soil extract, and Winogradsky saline solution (Pochon and Tardieux, 1962). Plates were incubated at 28°C for up to 10 days, and visible colonies were marked and enumerated on a daily basis for six consecutive days and on day 10th. In this way, seven counts (or classes) were generated per plate. When plates became too crowded, the next dilution was used for enumeration. The number of bacteria in each class was expressed as a proportion (%) of the total count. The different distributions gave insight into the distribution of *r*- and *K*-strategists in each soil and horizon. Fast growing bacteria (*r*-strategists) were defined as bacteria that produce visible colonies at 28°C within 48 h, and slow growing bacteria (*K*-strategists) as those which appear later.

Eco-physiological Index

To express the distribution of 7 classes in each sample in a single number, the Shannon diversity index (H') (Shannon, 1948) was calculated,

$$H' = -\Sigma(\mathrm{pi} \cdot \log_{10}\mathrm{pi}),$$

where pi representing each of the 7 classes as a proportion of the total population in that sample; pi = population in class *i*/total population. De Leij *et al.* (1993) pointed out that H' is normally used as a measure of the number of species and abundance of each species in a community. Thus, the name diversity index is strictly speaking

not appropriate for the quantification of classes of organisms with similar development characteristics on agar, that are obviously sub-communities. Therefore, the name eco-physiological index is used.

Number of Heterotrophic Culturable Bacteria

Total counts obtained after 10 days of incubation were expressed in colony forming units (CFUs) per gram soil (dry weight).

Substrate Induced Respiration (SIR)

SIR was measured following the method described by Anderson and Domsch (1978). A 2 g of soil was amended with a powder mixture, containing 200 mg of glucose and 500 mg of talcum, which corresponded to the amount of glucose required to obtain a maximum flux of carbon dioxide. Respiration was measured in samples at 50% water-holding capacity equivalent to 5 g oven-dry soil after conditioning at 22°C for 2 days. The carbon dioxide production rate was measured after incubating samples for 24 h at 22°C, using a KONIK 3000 HRGC equipped with a KNK019-501 TC detector and a Chromosorb 101 column (Gutierrez Mañero *et al.*, 1994).

Nitrogen Mineralization

Nitrogen mineralization was measured by the method of Højberg *et al.* (1996), under the conditions proposed by Alef and Kleiner (1986). Briefly, 2 g of soil were disposed in 40 ml volume glass vials. Soil samples were mixed with 0.015 g of L-asparagine (Merck) and 24 ml of distilled water. Vials were sealed off and disposed on an orbital shaker (200 r.p.m.) at 25°C. A 1 ml of each sample was collected hourly for 5 h. An ammonium chloride content was evaluated photometrically by using the Merck kit for ammonium (Spectroquant®-14752).

Statistics

The moles percent of PLFA values from the soil samples as well as plate bacteria suspensions were normalised by $\log_{10}$ transformation before being subjected to principal component analysis (PCA;

Hartman, 1967) to elucidate major variation and covariation patterns. Multivariate calculations were preformed using the computer program SYSTAT™ v 5.0 (Systat Inc.) for Windows™. Ecophysiological index, SIR, and nitrogen mineralization values, were compared in each case using an ANOVA. CFU count comparisons between different soils and horizons were made after a log transformation, using an ANOVA. All ANOVA tests were carried out by the programme SYSTAT™ v 5.0 (Systat Inc.) for Windows™.

RESULTS

The initial physico-chemical properties of soils are shown in Table I. The soils had a similar pH ranging between 5.94 and 6.53 with a sandy texture. Organic carbon and total nitrogen contents were more variable both between soils and between horizons, and generally decreased down the profile. The cationic exchange capacity (CEC) of soil B was the highest, followed by soil S (24 to 31 meq100 g^{-1} soil) and soil M (12 to 20 meq 100 g^{-1} soil).

Table II shows the differences in the total amount of various heavy metals present in each soil sample at the end of experiment, when biological parameters (PLFA content, *K/r* strategists, EP-indexes, CFU, SIR and N-mineralization) were determined. In general, the highest concentrations of trace metals (Cu, Zn, Al, Ni, Mn, Cd and Cr) were in the upper horizon and declined with depth, the opposite trend was observed for lead, which showed a maximum at the deepest level of the treated soil M (14.28 mg g^{-1} soil); iron accumulation did not show any specific trend at the different horizons. Amounts of copper, nickel, manganese and chromium from treated soils were higher than those recovered from controls, although there was no effect of pollution on other trace metals (Zn, Al, Fe, Pb and Cd).

PCA of all PLFAs partitioned from each soil and horizon resulted in a separation of the different plots along the first component (PC 1), which explained 74.45% of the variation in the data (Fig. 2). Score plots of PCA showed a clear difference between treated and untreated samples. The most polluted soils having a high score were found to the left in the graph, which coincided with the lowest values of PC 1 and the positive ones of the second principal component (group A in

TABLE I Soils physico-chemical and sampling sites characteristics. $n = 3 \pm$ SE

Soil	*S*			*B*			*M*		
Type of soil	*Luvisol*			*Cambisol*			*Fluvisol*		
Type of vegetation shurb	*Quercus ilex forest*			*Terophitic pasture*			*Cistus ladanifer*		
Horizon pH	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
(H_2O)	6.07 ± .12	6.00 ± .08	5.94 ± .05	6.52 ± .07	6.36 ± .1	6.53 ± .09	6.52 ± .08	6.36 ± .07	6.53 ± .09
Clay (%)	18.41	15.78	10.52	28.93	31.56	28.93	10.52	7.89	13.15
Lime (%)	17.95	11.49	7.66	10.46	16.92	16.52	16.75	13.32	14.12
Sand (%)	63.64	72.73	81.82	60.61	51.52	54.44	72.73	78.79	72.73
Organic C (%)	1.878 ± .23	1.232 ± .41	0.590 ± .16	2.012 ± .38	1.552 ± .33	1.642 ± .46	2.760 ± .54	0.682 ± .17	0.644 ± .18
Total nitrogen (%)	0.210 ± .04	0.050 ± .009	0.040 ± .01	0.212 ± .05	0.066 ± .01	0.090 ± .008	0.230 ± .06	0.050 ± .0006	0.036 ± .002
CEC (meq/100 g)	37.22 ± 1.98	31.67 ± 2.12	24.67 ± 1.8	58.89 ± 3.89	57.78 ± 4.21	59.44 ± 4.45	20.00 ± 2.0	12.78 ± 1.34	16.67 ± 1.23

TABLE II Soils (by horizons) metal content after 1 year, Units: mg metal g^{-1} soil. Chemical symbol plus asterisk means treated soil, and without asterisk control soil. $n=3 \pm$ SE

Soil	*S*			*B*			*M*		
Horizon	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Cu	0.051 ± .003	0.043 ± .008	0.074 ± .002	0.066 ± .002	0.073 ± .002	0.115 ± .017	0.053 ± .004	0.058 ± .002	0.074 ± .008
Cu*	0.270 ± .041	0.087 ± .013	0.070 ± .001	0.236 ± .051	0.256 ± .032	0.082 ± .009	0.138 ± .009	0.159 ± .024	0.110 ± .011
Zn	0.462 ± .082	0.418 ± .091	0.385 ± .007	0.642 ± .062	0.529 ± .044	0.515 ± .054	0.426 ± .018	0.418 ± .087	0.409 ± .021
Zn*	0.537 ± .075	0.443 ± .063	0.414 ± .009	0.622 ± .059	0.640 ± .051	0.470 ± .032	0.444 ± .015	0.444 ± .074	0.416 ± .012
Al	67.46 ± 3.45	65.89 ± 4.36	41.28 ± 1.66	122.24 ± 8.32	206.38 ± 10.22	200.67 ± 9.65	27.95 ± 3.97	23.81 ± 2.97	38.05 ± 4.87
Al*	57.61 ± 2.23	63.14 ± 3.98	64.44 ± 2.98	193.75 ± 8.99	319.75 ± 11.02	111.66 ± 2.39	29.38 ± 4.32	36.99 ± 3.26	33.34 ± 4.21
Fe	81.99 ± 5.38	62.09 ± 4.11	58.35 ± 2.67	141.29 ± 6.54	148.88 ± 3.99	147.32 ± 3.21	5.13 ± 1.25	5.47 ± 1.11	7.30 ± 2.01
Fe*	41.79 ± 1.35	95.20 ± 6.54	88.72 ± 3.99	142.80 ± 6.87	160.11 ± 4.28	107.67 ± 3.11	7.37 ± 1.54	8.42 ± 1.36	6.84 ± 1.89
Pb	5.08 ± .95	4.93 ± .89	4.41 ± .29	4.10 ± 1.01	4.19 ± 1.01	4.57 ± 1.08	10.05 ± 2.12	5.24 ± 0.96	5.47 ± 1.85
Pb*	5.75 ± 1.03	4.49 ± .77	4.95 ± .32	4.64 ± 1.14	5.04 ± 1.11	4.89 ± 1.17	5.27 ± 1.06	5.44 ± 1.21	14.28 ± 3.11
Ni	0.070 ± .002	0.100 ± .011	0.060 ± .006	0.120 ± .033	0.110 ± .012	0.110 ± .019	0.090 ± .005	0.060 ± .005	0.070 ± .002
Ni*	0.290 ± .032	0.171 ± .037	0.144 ± .046	0.370 ± .023	0.300 ± .045	0.180 ± .020	0.180 ± .068	0.140 ± .012	0.140 ± .008
Mn	0.881 ± .121	0.910 ± .082	0.870 ± .068	0.300 ± .054	0.240 ± .033	0.230 ± .098	1.330 ± .24	0.830 ± .023	0.981 ± .021
Mn*	0.510 ± .072	0.341 ± .041	0.300 ± .022	0.560 ± .112	0.271 ± .022	0.372 ± .101	1.301 ± .21	1.350 ± .37	0.581 ± .013
Cd	0.10 ± .01	0.02 ± .008	0.04 ± .003	0.04 ± .001	0.03 ± .006	0.01 ± .005	0.11 ± .013	0.10 ± .02	0.07 ± .01
Cd*	0.04 ± .001	0.03 ± .003	0.03 ± .005	0.07 ± .0011	0.06 ± .007	0.04 ± .003	0.12 ± .014	0.05 ± .006	0.04 ± .009
Cr	0.015 ± .0011	0.012 ± .001	0.020 ± .002	0.023 ± .009	0.013 ± .008	0.013 ± .001	0.040 ± .002	0.040 ± .004	0.020 ± .002
Cr*	0.120 ± .012	0.022 ± .002	0.010 ± .001	0.125 ± .021	0.015 ± .007	0.108 ± .011	0.055 ± .001	0.065 ± .007	0.049 ± .006

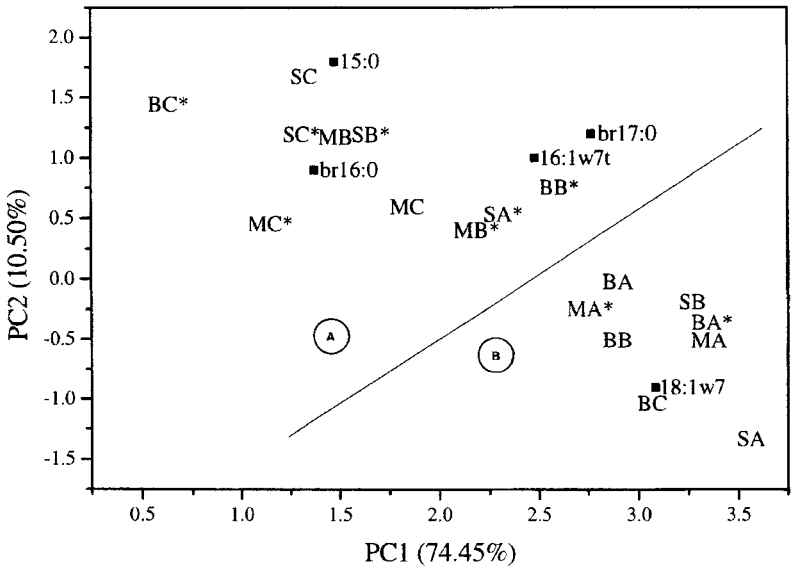

FIGURE 2 PCA showing variation in scores of soil PLFAs and loading values for some individual PLFAs (square symbols) in the different soils and horizons, both in treated and control soils. M, B and S designate the soils and A, B and C the horizon where samples were taken; asterisk: treated soils. For PLFAs, nomenclature see material and methods.

Fig. 2). Therefore, the untreated samples have been gathered in group B (Fig. 2) at the highest values of PC 1 and the lowest ones of the second component (PC 2). Two treated samples (MA* and BA*) appeared associated to the later group. These two groups appeared to be separated along PC 2 (explaining 10.5% of the total variation). This indicated that the scores for the different plots along components 1 and 2 revealed the effect of pollution. However, it was not possible to detect any trend in the ordination of samples regarding to either soil or horizon, neither in treated soils nor in controls. To investigate if the metal pollution at the three areas induced the same changes in the PLFA patterns, the most disperse loadings for the individual PLFAs for the first PC component (indicating the pollution) were plotted against each other (Fig. 2). PLFAs that increased in proportional abundance at higher pollution levels are found in the upper half of the plot, while those that increased, it untreated soils are plotted at the lower right corner of the graph.

Table III illustrates the results (% mol) of the most representative PLFAs which were detected in control and untreated soils. Differences in the relative abundance of some fatty acids recovered from contaminated soils were observed. In that way, the relative mole percent of several PLFAs, especially 15 : 0, 16 : 1ω7, brl7 : 0, cyl7 : 0, brl8 : 0 and 18 : 1ω7 decreased mostly due to increased metal content. On the other hand, the PLFAs, l0Mel6, 18 : 2ω6, cyl9 : 0 and i16 : 0, showed higher values in treated soils. However, there were some variations considering type of soil or horizon.

The study of PLFAs obtained from plates, used previously for examining visible colony forming units, represents only a fraction of the whole soil community. Actually, these strains are not only the culturable fraction but also the most metabolically active in the soil at the moment of sampling. PCA of those PLFAs is shown in Figure 3. This analysis shows a separation of the different plots along the first two principal components, which explained 75.33% and 9.41% of the variation in the data (Fig. 3). Once more, scores of PCA showed a clear difference between treated (group A) and control soils (group B). A second grouping is distributed along the second component and the lowest values of the first one, and corresponded to untreated soils (group B in Fig. 3). In spite of the notable effect of pollution on plate PLFAs ordination, it was not possible to detect any trend in the distribution of samples either by soil or horizon criteria. In a similar way that it was represented for soil PLFAs, in this case (plate PLFAs), the most disperse loadings for the individual fatty acids were graphed against each other (Fig. 3). PLFAs which increased at higher pollution levels are found at the right corner of the plot, whereas those that increased at untreated soils are plotted in one half of the graph along both principal components. The bacterial community structure in microcosms of the three soils are represented in Figures 4, 5 and 6. Treated soils were dominated by fast growing bacteria (*r*-strategists). In contrast, it was detected an enhancement in *K*-strategist bacteria, which correspond to slow growing microorganisms, as derived from the absence of heavy metals in soils. The only exception to this behaviour was the soil SB (Fig. 6b), where microbial communities had changed toward slower growth as a result of pollution, while in controls the microbial population structure had turned into faster growth. Bacteria that produced visible colonies

TABLE III Proportion of some PLFAs (mean for all three horizons) in treated and control soils. Units (% mol). $n = 3 \pm$ SE

Soil	*SN*		*B*		*M*	
PLFA	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
15:0	8.35 ± 0.77	7.46 ± 0.85	5.35 ± 1.87	7.04 ± 0.17	7.28 ± 0.41	3.80 ± 1.02
i16:0	1.28 ± 0.57	2.38 ± 0.45	1.07 ± 0.09	2.30 ± 0.48	1.68 ± 0.25	2.09 ± 0.47
16:1ω7t	6.55 ± 0.92	5.61 ± 0.75	8.12 ± 0.29	6.04 ± 0.90	5.23 ± 0.91	2.98 ± 0.50
br17:0	4.05 ± 2.09	1.58 ± 0.36	4.84 ± 1.41	2.79 ± 1.53	3.22 ± 2.14	1.04 ± 0.18
10Me16:0	0.53 ± 0.24	0.99 ± 0.18	0.44 ± 0.03	0.96 ± 0.20	1.01 ± 0.35	0.87 ± 0.20
cy17:0	2.37 ± 0.63	2.56 ± 0.52	3.46 ± 0.58	2.05 ± 0.50	1.59 ± 0.35	1.25 ± 0.31
br18:0	1.02 ± 0.47	0.63 ± 0.11	1.60 ± 0.24	0.61 ± 0.12	0.97 ± 0.39	0.65 ± 0.07
18:2ω6	2.29 ± 0.36	8.97 ± 1.32	4.03 ± 0.32	6.10 ± 1.07	15.09 ± 5.18	15.75 ± 3.92
18:1ω7	11.69 ± 2.12	7.87 ± 1.57	12.89 ± 0.73	9.81 ± 2.55	10.09 ± 1.70	9.86 ± 1.10
10Me18:0	4.11 ± 2.12	3.84 ± 0.70	4.91 ± 0.40	4.22 ± 0.59	3.53 ± 0.27	3.64 ± 0.46
cy19:0	1.42 ± 0.64	2.66 ± 0.50	1.94 ± 0.21	2.56 ± 0.53	1.59 ± 0.43	2.32 ± 0.53

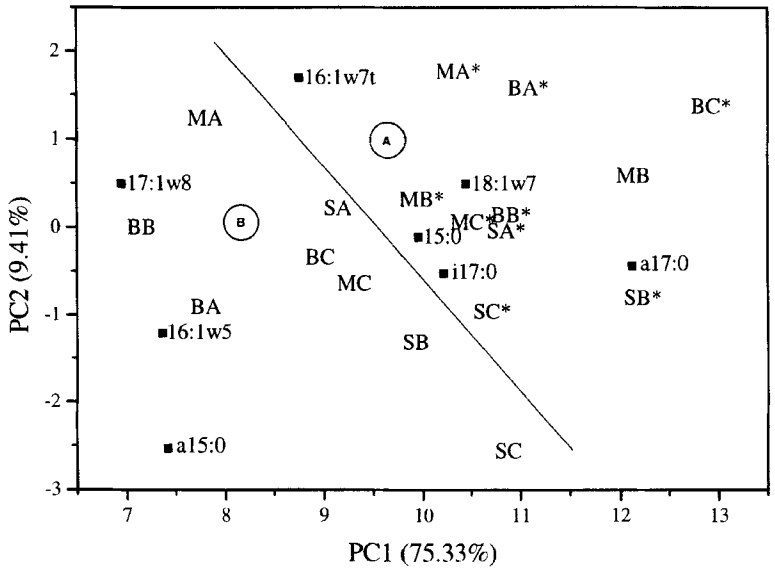

FIGURE 3 PCA showing variation in scores of petri dishes PLFA and loading values for some individual PLFAs (square symbols) in the different soils and horizons, both in treated and control soils. M, B and S designate the soils and A, B and C, the horizon where samples were taken; asterisk: treated soils. For PLFAs, nomenclature see material and methods.

after 2 days of incubation were the most dominant ones (Figs. 4, 5 and 6).

Total bacteria counts per gram soil were lower and significantly different ($p < 0.001$) for treated compared to untreated soils (Tab. IVa). Any case, the results were not statistically significant when comparing samples by soils or horizons.

EP-indexes on soils were always greater ($p < 0.001$) for control than for treated samples (Tab. IVb). Although this were not significant differences in EP-index on soils or horizons; this value increased at the top layers of uncontaminated soils.

The mean substrate-induced respiration rates of controls varied between 1.7 and 17.8 nmol carbon dioxide $h^{-1} g^{-1}$ soil (Tab. IVc). This range of variation was smaller for treated soils. In addition, statistical differences were highly significant for control and treated soils as well as for horizons and interaction ($p < 0.001$ in all cases).

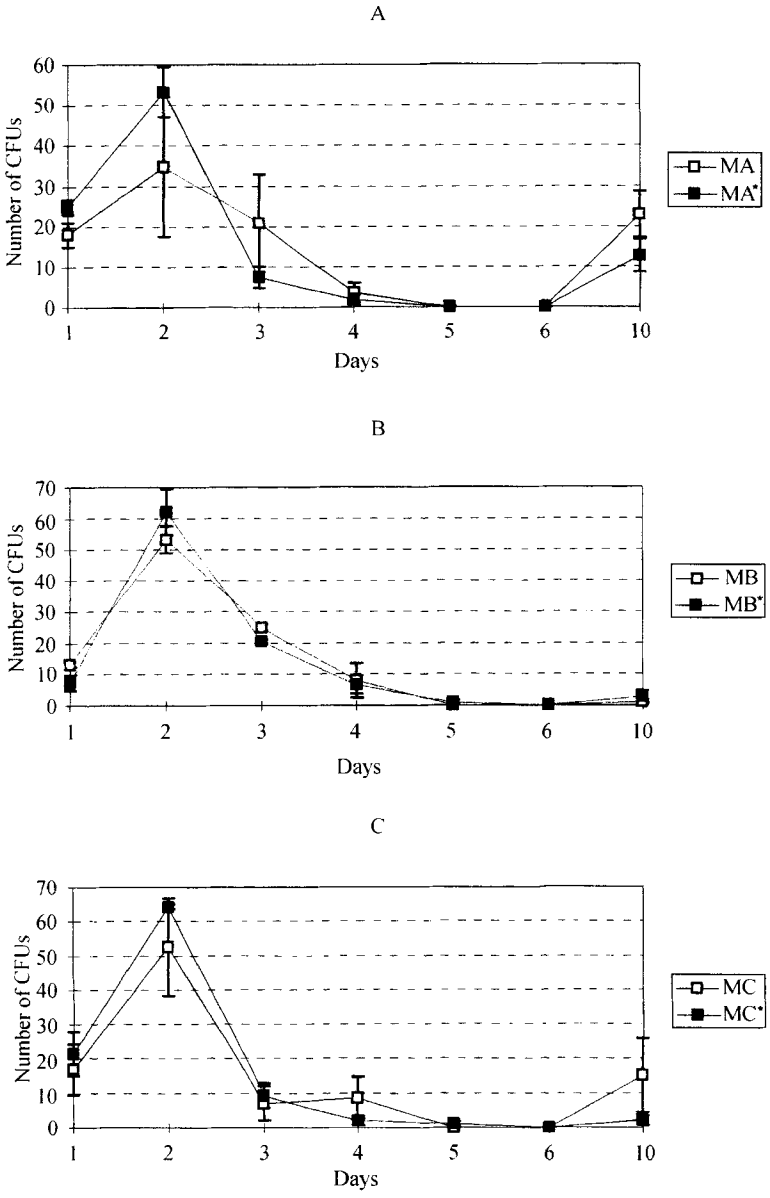

FIGURE 4 Microbial community structure in Monteprincipe soil (M), both in treated (solid symbols) and control soils (open symbols), at different horizons (A, B and C). Data derived from bacterial colonies appearing on soil agar over a period of 10 days. Bars represent SE. $n = 2$.

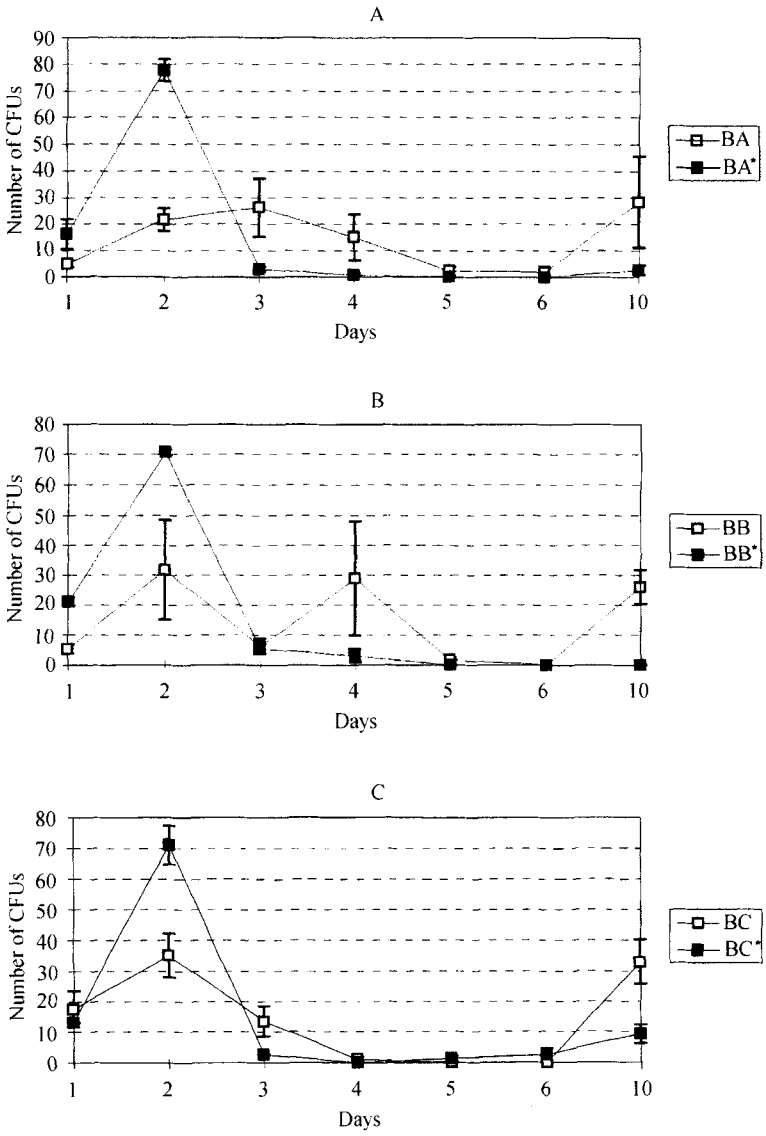

FIGURE 5 Microbial community structure in Brunete (B), both in treated (solid symbols) and control soils (open symbols), at different horizons (A, B, and C). Data derived from bacterial colonies appearing on soil agar over a period of 10 days. Bars represent SE. $n = 2$.

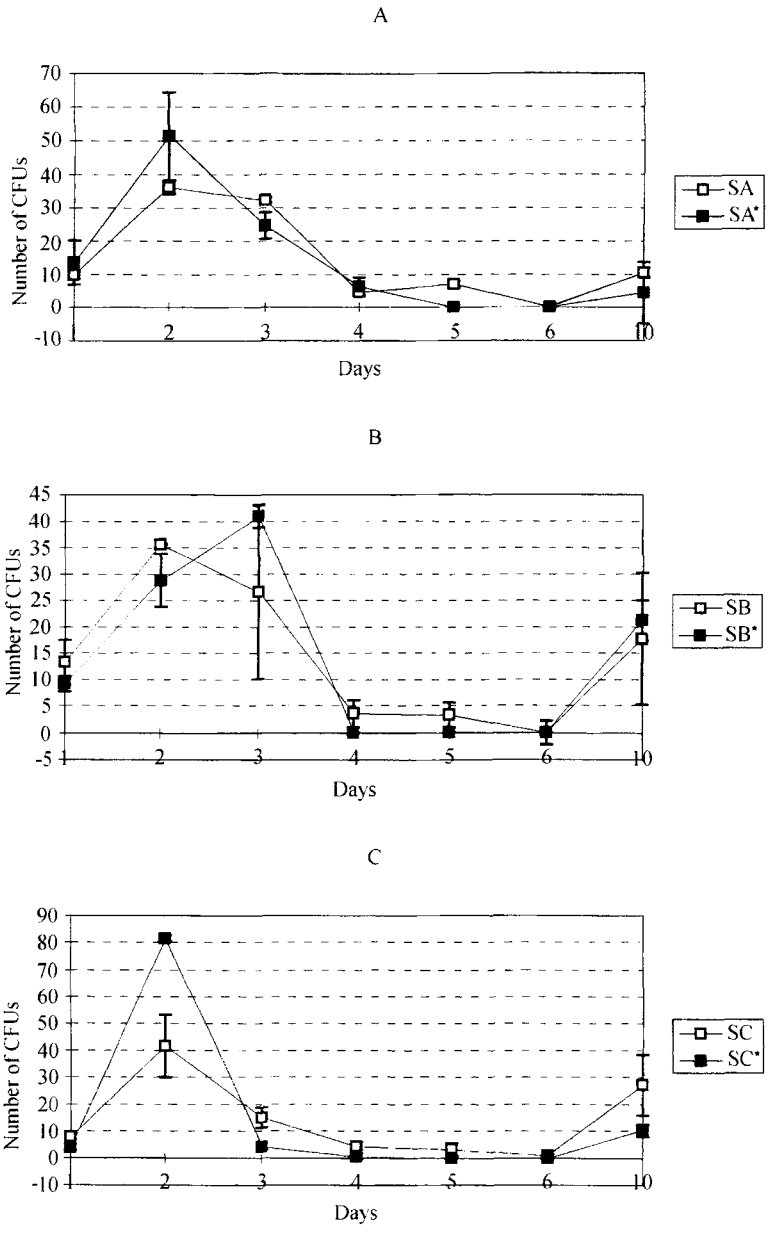

FIGURE 6 Microbial community structure in Sevilla la Nueva (S), both in treated (solid symbols) and control soils (open symbols), at different horizons (A, B, and C). Data derived from bacterial colonies appearing on soil agar over a period of 10 days. Bars represent SE.

TABLE IV Total bacterial counts (log_{10} CFUs) (a) Eco-physiological index for bacteria (b) SIR (c) and nitrogen mineralization (d) in soils and horizons of treated and untreated soils, compared by a factorial model, *i.e.*, 3 soils × 3 horizons. $n = 3 \pm$ SE. NS, not significant

Soil	*SN*			*B*			*M*		
Horizon	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
(a)									
Treated	1.38 ± .02	1.09 ± .04	2.02 ± .01	1.63 ± 14	1.46 ± .03	1.58 ± .01	1.45 ± .01	1.78 ± .01	1.32 ± .01
Control	1.62 ± .04	1.50 ± .01	1.72 ± .05	2.01 ± 08	1.91 ± .08	1.57 ± .05	1.44 ± .01	1.72 ± .01	1.85 ± .01
Treated *vs.* Control =					$p < 0.001$				
between soils (S) =					NS				
between horizons (H) =					NS				
between interactions $S \times H$ =					$p < 0.001$				
(b)									
Treated	0.49 ± .007	0.43 ± .054	0.29 ± .000	0.29 ± .011	0.33 ± .019	0.40 ± .078	0.43 ± .015	0.37 ± .024	0.30 ± .02
Control	0.65 ± .027	0.58 ± .021	0.59 ± .006	0.58 ± .047	0.51 ± .036	0.55 ± .018	0.61 ± .007	0.58 ± .008	0.57 ± .04
Treated *vs.* Control =					$p < 0.001$				
between soils (S) =					NS				
between horizons (H) =					NS				
between interaction $S \times H$ =					NS				
(c)									
Treated	6.7 ± .31	3.1 ± .05	2.0 ± .31	3.8 ± .40	3.9 ± .24	3.3 ± .29	3.8 ± .13	3.1 ± .14	2.3 ± .11
Control	11.2 ± .35	8.1 ± .40	1.7 ± .03	8.1 ± 2.14	5.1 ± .10	10.3 ± .24	17.8 ± .93	5.5 ± .80	6.8 ± .56
Treated *vs.* Control =					$p < 0.001$				
between soils (S) =					$p < 0.05$				
between horizons (H) =					$p < 0.001$				
between interaction $S \times H$ =					$p < 0.001$				
(d)									
Treated	.012 ± .00	.003 ± .00	.017 ± .00	.012 ± .00	.010 ± .00	.036 ± .00	.024 ± .00	.012 ± .00	.013 ± .00
Control	.045 ± .01	.025 ± .01	.026 ± .00	.030 ± .00	.017 ± .01	.037 ± .01	.039 ± .00	.038 ± .00	.022 ± .00
Treated *vs.* Control =					$p < 0.001$				
between soils (S) =					NS				
between horizons (H) =					NS				
between interaction $S \times H$ =					NS				

The observed N-mineralization rates of the three soils are shown in Table IVd. There was a decrease in the net N-mineralization for treated soils, which was significant ($p < 0.001$).

DISCUSSION

Giller *et al.* (1998) classify this kind of studies into three groups: (i) laboratory ecotoxicology assays, (ii) "field" ecotoxicology assays, and (iii) environmental monitoring. The present study is a hybrid between the two first examples, since it is formally a laboratory exercise, where the toxicity of a combination of metals to particular microbial populations or processes are compared, but also a field ecotoxicology study, where samples of contaminated and control soils are taken to apply an ecotoxicological test once time is after adding the pollutants.

An initial approach to detect possible changes in soil microbial community, in a nonselective way is analysis of PLFA composition of the soil. The PLFA profile does not give an actual species composition, but instead gives an overall landscape of the community structure. However, changes in the concentration of certain PLFAs may be related to changes in more specific groups of organisms. On the basis of PLFA analysis, the effects of alkaline and heavy metal pollution as far as different management practices have been detected on soil microbial communities (Pennanen *et al.*, 1996; McGrath *et al.*, 1995).

Both treated and control soils used in the present study have somewhat different PLFA profile (Fig. 2). Total variation explained by the first principal component is almost 85%, suggesting that much of the variation in the data was due to heavy metal pollution at these sites coupled with some other environmental factors Treated samples MA* and BA* (both from the topsoil) appears associated with untreated soils. This can be due the fact that this horizon was subject for a longer time to leaching. Pollution resulted in a decrease of the fatty acids, 16:1ω7t, br17:0, cy17:0, br18:0, 18:1ω7 and 10Me18:0, while the largest proportional increase in the treated samples was found for those i16:0, 10Me16:0, 18:2ω6 and cy19:0 (Tab. III). In both cases, there were fatty acids usually found either on gram-positive or gram-negative ones, as well as those originated

from fungi and actinomycetes (Kropendstedt, 1985; O'Leary and Wilkinson, 1988; Wilkinson, 1988). This indicates that the separation between gram-negative and gram-positive bacteria in assayed soils by PLFA patterns is not straightforward. This was also the case in a study of the effect of liming on the PLFA pattern of soil micro-organisms (Frostegård *et al.*, 1993). Anyhow, there is a predominanze of gram-negative fatty acids on heavy metal treated soils about others untreated, where they surpass the gram-positive bacteria. Nevertheless, in the case of fungi, there are not homogeneous results, since some characteristic fatty acids can be detected in all experimented soils (Bååth *et al.*, 1988).

According to our results, the fatty acids, i16 : 0, 18 : 2ω6 and cyl9 : 0, increase as a consequence of heavy metal pollution. Those findings have been also reported by the authors cited, who found that 16 : 1ω7t, 18 : 1ω7 and l0Mel8 : 0 appear at lower concentrations on polluted soils. However, our results apparently contradict that obtained by these authors, since cyl9 : 0 decreases after pollution and the branched fatty acids, brl7 : 0 and brl8 : 0, and cyl7 : 0, increase on polluted soils. These differences could be related to some physico-chemical properties, such as pH and organic matter of soils, where the observed values were 4.0 and 80%, respectively. In the present study, pH was nearly neutral and the organic carbon was 1.5% (Tab. I). There are two fatty acids which engage a special attention: l0Mel8 : 0 and 18 : 2ω6. The tuberculoesteraric acid (l0Mel8 : 0) is found almost exclusively in actinomycetes (Lechevalier, 1977). If l0Mel8 : 0 is considered as an indicator of actinomycetes, our results reveal that this group of organisms decreased in response to metals. This diminution agrees with that described by Babich and Stotzky (1977) but contradicts that of Jordan and Lechevalier (1975). On the other hand, in agreement with Frostegård *et al.* (1993), our experiment shows increased proportions of the fungal PLFA 18 : 2ω6 in metal-polluted soils. We can hypothesize two reasons to explain why fungi predominate in polluted samples. (i) After pollution, fungi are more competitive than bacteria and consequently displace them. This competition could be verified through various mechanisms, such as capability or availability of nutrients to be taken up, biological diversity before pollution, and tolerance and/or adaptability of these micro-organisms (ii) Heavy metals can kill bacteria rather than fungi, and these can occupy both

space and function that previously belong to bacteria. It is generally agreed that fungi are less sensitive to pollution than bacteria (Doleman, 1985; Fritze, 1991; Hiroki, 1992). These reports support these two hypotheses, although we cannot rule out any of them because metal toxicity depends notably on the physico-chemical properties of the soil (Doleman and Haanstra, 1986; Collins and Stotzky, 1989), which are related to microbial communities.

The effect of heavy metal pollution on plate PLFA pattern was also studied, and is clearly seen as the PCA of these fatty acids (Fig. 3), where 75.33% of the variation in the PLFA data was explained by the first principal component. PLFAs recovered from plates represent a small fraction of total soil bacteria. Indeed, some authors have estimated that plate culturable bacteria recovered from soil suspension mean a 0.3–20% of the total (Alexander, 1973; Torsvik *et al.*, 1990). Even so, PCA resulted from the analysis of plate PLFAs shows a quite similar distribution pattern of samples to that obtained with soil PLFAs, proceeding from the whole soil microbial community (Figs. 2 and 3). Therefore, we can conclude that heavy metal pollution, under the assayed conditions, affects not only soil culturable microorganisms (plate PLFAs). This fact supposes that these viable bacteria are quite enough to evaluate switches which strike upon heavy metal pollution in the soil coenotic composition.

The concept of *r*/*K*-strategy is derived from evolutionary ecology and producers that this has a genetic differences between organisms on their ability to exploit and survive in different kinds of environments (Pianka, 1970; Luckinbill, 1978). This method proved to be simple, reproducible and highly sensitive for characterization of microbial communities (De Leij *et al.*, 1993; Ikeda *et al.*, 1997). In this paper, it is observed a clear dominance of *r*-strategists in polluted soils, whereas there is an enhancement in *K*-strategist bacteria in controls (Figs. 4, 5 and 6). In general, *r*-strategists are characteristic for unstable environments, while *K*-strategists match better in stable environments. Since the perturbation of the experimental system has been pictured along one year, after adding a mixture of metals, it could be affirmed that the soil system is typically unstable, which could explain the dominance of *r*-populations in polluted soils. According to these results, it can be elucidated that the effect of metal pollution on the assayed Mediterranean soils implies a small resilience

and a high persistence of the system, considering both concepts as defined by Pimme (1991). This explanation could be put out in more simple terms: selective pressure of metal pollution enhances *r*-strategists (metal resistant micro-organisms), whereas *K*-strategists disappear. Taking in our account of the time of sampling (one year after pollution), it seems that heavy metal pollution turns in an irreversible situation for the system, at least concerning in growth strategies.

Bacterial abundance has been estimated as colony forming units (CFUs) using plate count techniques, and they have been extensive to study the effects of pollution on soil microbial communities. Total bacteria counts were lower in treated soils than in controls (Tab. IVa). Our results are opposite to those showed by other authors (Bååth *et al.*, 1980, 1992). Nevertheless, it should be considered that several studies have shown that there exist some factors which affect the toxicity of metals (Babich and Stotzky, 1985) and it is generally argued that metal toxicity is lower at higher pH values as far as in sandy loam soils with low organic matter. However, the decreased number of CFUs in treated soils might also be due to a change in species composition (as it was indicated by PLFA pattern); bacterial species in untreated samples could be better able to form visible colonies. A striking result was achieved on growth strategies of contaminated soils as it has been previously discussed, which consists in a dominance of *r*-strategists (Tab. IVb) despite a low number of CFUs. This result could be traced to a low pool of bacteria (most of these results to be killed by heavy metals), although they exhibit a notable fast growing pattern.

It appears that the use of diversity indices, as a means of studying the impact of heavy metal pollution does not give as much information as using the species composition *per se* (Bååth, 1989). Nevertheless, the Shannon diversity index is a widely used algorithm in order to calculate biological diversity and results are specially meaningful in using simultaneously with growing *r*/*K*-strategists. In the experiments presented in this paper, EP-index supported the findings derived from microbial community structure data, and untreated soils have higher values than those found on contaminated samples. In addition, top layers of uncontaminated soils show more diversity than the deepest horizons (Tab. IVb). This behaviour was also revealed for treated

samples. As derived from our results, it can be deduced that pollution of soils exerts a selective pressure on the environment, which affects some populations of micro-organisms, and consequently implies a reduction in EP-index (biological diversity). This response agrees with a low resilience of the system as well as with the observed growth strategies of bacteria in treated soils. Reber (1992) observed that in most of the assayed soils with a long term metal exposure exhibited an increase in bacterial diversity. This controversy with our results is in relation to the evolutive adaptation (natural selection) of soil bacteria. In our case, it can be supposed that it has not been time to take place these evolutionary phenomena. Moreover, only when the modification of the system consists in a single perturbation (such as an unitary addition of the pollutants through the experiment), biological diversity behaves quite similarly to that obtained in this paper, as proposed by Ikeda *et al.* (1997), studying an abnormal situation such as soil compaction.

Substrate-induced respiration (SIR), as far as N-mineralization, are easy to measure and appear to be sensitive techniques to detect the effect of heavy metal pollution on metabolism, either with stress or disturbance, and especially under standardized conditions (Chang and Broadbent, 1982; Brookes, 1993; Wardle and Ghani, 1995). In the present study a clear reduction of SIR values on treated soils is shown (Table IVc). These results are in a stark opposition with those obtained by Fliessbach *et al.* (1994), who found an increase on soil base respiration in the most contaminated arable soils they tested. This apparent contradiction could be explained by two reasons: (i) the response of *C*-mineralization to metals is dependent on the chemical nature of the available substrate (Palmborg *et al.*, 1988), and the required amount for respiration can be decreased through the formation of complexes with the added metals; and (ii) in most of studies, this sort of response suggests a complex effect to disturbance (an immediate acute toxicity situation), where soil respiration fluctuates above and below respiration rate with an amplitude decreasing with time (Schinner and Brunner, 1984). In disturbance, heavy metal toxicity kills a part of the soil microbial biomass, resulting in a flush of decomposition of micro-organisms (Leita *et al.*, 1995), which increase the carbon dioxide production. In our case, the lower carbon dioxide accumulation detected in treated soils could be the result

of a persistent toxicity effect (stress). On stress, the microbial biomass is constituted by those survival populations which occur along a selection process (metal tolerant bacteria). In that sense, total biomass is lower on treated soils (see CFU results in Tab. IVa) and for this reason, SIR values are higher in untreated samples.

All considerations made above for substrate-induced respiration response can be exactly transferred for the results derived from N-mineralization (Tab. IVd). Our results show that net N-mineralization was inversely related to the presence of heavy metals in soils. This agrees with Witter and Marstrop (unpublished, cited in Giller *et al.* 1998), who reported a similar effect by adding *L*-phenylalanine as a nitrogen source into field polluted soils. However, most investigations have been based on laboratory experiments, where amendments of different heavy metals provoke the same effect (Bååth, 1989; Chang and Broadbent, 1982). Despite previous arguments, we can rule out that the mechanisms of metal tolerance, that should be taking place in a stress situation, carry invariably out an additional energy cost to micro-organisms. Such a spending can result in a decrease in the amount of substrate that is used for respiration. However, a part of the carbon substrate were used by bacteria to develop their own tolerance mechanisms, such as the binding of metals to extra-cellular polymers to the cell wall (Ross, 1993).

CONCLUSIONS

1. This study shows that heavy metal soil pollution is primarily responsible of all changes detected in the structural and functional characteristics of the Mediterranean assayed soils. Indeed, the present data are occasionally different to those observed by other authors who have been working outside the Mediterranean area, but these are characterized by having high organic matter and lower pH values.
2. The response observed as a consequence of the presence of heavy metals is not correlated with the type of soil nor horizon.
3. Pollution of heavy metals provoked a decrease of CFUs, substrate induced respiration, N-mineralization and ecophysiological indexes, and revealed a clear dominance of *r*-growing strategists.

4. In addition, soils supplemented with heavy metals showed different relative mole percent of several PLFAs, so that 15:0, 16:1ω7, cyl7:0, brl8:0 and 18:1ω7 decreased on polluted soils, whereas l0Mel6, 18:2ω6, cyl9:0, i16:0 and brl7:0 were higher after pollution, which means that fungi predominated in treated samples.

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