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SHORT COMMUNICATIONS

Estimation by PLFA of Microbial Community Structure Associated with the Rhizosphere of *Lygeum spartum* and *Piptatherum miliaceum* Growing in Semiarid Mine Tailings

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Abstract The objective of this study was to compare the microbial community composition and biomass associated with the rhizosphere of a perennial gramineous species (*Lygeum spartum* L.) with that of an annual (*Piptatherum miliaceum* L.), both growing in semiarid mine tailings. We also established their relationship with the contents of potentially toxic metals as well as with indicators of soil quality. The total phospholipid fatty acid (PLFA) amount was significantly higher in the rhizosphere soil of the annual species than in the rhizosphere soil of the perennial species. The fungal/bacterial PLFA ratio was significantly greater in the perennial species compared to the annual species. The fatty acid 16:1ω5c, the fungal/bacterial PLFA ratio and monounsaturated/saturated PLFA ratio were correlated negatively with the soluble contents of toxic metals. The cyc/prec (cy17:0+cy19:0/16:1ω7+18:1ω7) ratio was correlated positively with the soluble contents of Pb, Zn, Al, Ni, Cd, and Cu. The results of the PLFA analysis for profiling microbial communities and their stress status of both the plant species indicate that perennial and annual gramineous species appear equally suitable for use in programmes of revegetation of semiarid mine tailings.

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Introduction

The development of appropriate revegetation techniques in semiarid mine tailings is essential to reduce erosion, protect soil against degradation, and limit the spread of metal contamination [21]. Drought-, salt- and metal-tolerant native plant species have been recommended in order to achieve a self-sustainable vegetation [6, 21]. The natural vegetation that can colonise these tailings is mainly dominated by perennial and annual gramineous species [6]. The different strategies developed by native plants to survive in environments contaminated with heavy metals can influence the biomass and diversity of microbial communities, which are known to play a key role in the mobilisation and immobilisation of metal cations [3].

Selection of the most suitable, autochthonous species based on their ability to stimulate microbial populations is a major requisite for successful reclamation of these sites.

Microbial populations and their activities are fundamental for maintaining soil quality, since they mediate the processes of organic matter turnover, nutrient cycling, and structure formation and stabilisation [28]. In particular, arbuscular mycorrhizal (AM) fungi are soil microorganisms that establish mutual symbioses with the majority of higher plants including plants growing on heavy metal-contaminated soils [29]. In these soils, AM fungi can contribute to plant growth by binding heavy metals within roots, thus restricting their translocation to shoot tissues [17]. In this way, we have shown the beneficial effects of AM fungi on plant development in Mediterranean salt marshes contaminated with mining wastes [5].

Recently, different methods have been developed for studying composition of microbial community in soil, most of which are unknown and not yet cultivated [20]. In particular, phospholipid fatty acid (PLFA) analysis is a biomarker method that can contribute to the elucidation of the structure of the in situ microbial community. Phospholipid fatty acids are potentially useful signature molecules, due to their presence in all living cells. The use of PLFA profiles of soil microbial communities provides a more sensitive measure of change at the community level than other conventional methods (cultivation techniques) as it accounts for a larger proportion of the soil microbial community. Community-level PLFA profiles have been found to be useful in detecting the responses of soil microbial communities to a variety of land uses or disturbances and certain marker PLFAs can indicate the relative amounts of certain functional or taxonomic groups of organisms in soils [18]. However, from pure-culture studies, it is well-established that membrane lipids are affected deeply by environmental factors, either changing the PLFA composition of bacterial cell membranes (phenotypic plasticity) or altering PLFA profiles resulting in shifts in the soil microbial community structure. Thus, in addition to differentiating taxonomic groups certain fatty acids and ratios of fatty acids are proposed to assess the physiological state of microbial communities [20]. Studies of microbial community responses to heavy metal pollution under natural conditions are rare [15] particularly in arid and semiarid environments where moisture availability for biotic activity is scarce. There is no previous assessment by PLFA analysis of the AM status of plants grown in semiarid soils that have been degraded by mining.

Plants can affect available soil carbon, temperature, and water content and thus have the potential to affect microbial community composition and function [25]. For this study, we have focused on two types of Mediterranean gramineous species (perennial and annual), these being the only vegetation in the mine tailings from a mine area located in Southeast Spain. We hypothesise that the type of perennial or annual vegetation can have a significant effect on patterns of microbial community structure in a soil polluted with heavy metals because perennial grasses deposit more plant litter than annual grasses. The objective of the study was to compare the microbial community composition and biomass associated with the rhizosphere of such plants and to establish their relationship with the contents of potentially toxic metals as well as with indicators of soil quality. All this information may be of great importance in the planning of remediation programmes for these heavy metal-polluted soils.

Materials and Methods

Study Area

The study was carried out in the Cartagena–La Union Mining District (110-0 m a.s.l., 37º37'20''N, 0º50'55''W-37º40'03''N, 0º48'12''W), a short coastal chain located in the Southeast of Spain, in which man has developed an intense mining activity for thousands of years. The accumulation of high amounts of mining wastes on the surface has originated the formation of mine tailings that cover 160 ha in this zone [6]. These mine tailings present high erosion problems and contain high levels of heavy metals such as lead, copper, zinc, cadmium, and arsenic. The climate is typically Mediterranean with annual rainfall around 300 mm especially concentrated in spring and autumn and with an annual temperature average of 17.5 °C. In this zone, a neutral mine tailing called "Gorguel" (U.T.M. X687480 Y4162800 Z135, length: 200–300 m, width: 95 m, height: 25 m, volume: 750,000 m^3) with an age of about 50 years was selected.

Plant Species

The plants selected were *Lygeum spartum* L. and *Piptatherum miliaceum* L., which are perennial and annual Mediterranean gramineous species, respectively. Both grasses have a high density of fine roots in the topsoil highly contributing to reduce soil erosion rates [7]. They are perfectly adapted to water stress conditions and are the most abundant plants growing in the mine tailing.

Sampling Procedures

A field sampling survey was carried out in a homogenous area measuring approximately 1,500 m^2. For this survey, 50 individual plants similar in size (25 replicates for each of the two target species) were randomly chosen. Each root system was extracted by excavating manually a

hole 40 cm wide, 40 cm long, and 20 cm deep. Soil strongly adhering to roots and collected at 0–4 mm from the root surface was defined as rhizosphere soil. To collect the rhizosphere soil the root system with rhizosphere soil adhered was introduced into a plastic bag, shaken and separated the rhizosphere soil from the root system. Field-moist rhizosphere soil samples were divided into two subsamples. One subsample was sieved at 2 mm and stored at 2 °C for PLFA analysis and the other subsample was air-dried at room temperature and sieved at 2 mm for physical–chemical and chemical analyses or at 0.25-4 mm for aggregate stability and glomalin.

Assessment of Mycorrhization

Roots were subsampled in three 2-cm cross-sections of the upper, middle, and lower root system. To assess colonisation, roots were cleared with 10% KOH and stained with 0.05% trypan blue [26]. The percentage of root length colonised by AM fungi was calculated by the gridline intersect method [11]. Positive counts for AM colonisation included the presence of vesicles or arbuscules or typical mycelium within the roots.

Soil Analyses

Soil pH and electrical conductivity were measured in a 1:5 (*w*/*v*) aqueous solution. Total organic C and total N were determined with an automatic Nitrogen and Carbon Analyser. Calcium carbonate was determined using Bernard calcimeter. In the 1:5 (*w*/*v*) soil aqueous extracts, water-soluble carbon was determined with an automatic Carbon Analyser for liquid samples (Shimadzu TOC-5050A). Water-soluble carbohydrates were determined by the method of Brink et al. [4]. Soluble metal contents were determined in a 1:5 (*w*:*v*) aqueous solution using an ICP mass atomic spectrometer (ICO-MS 4500 Agilent Technologies). Easily extractable glomalin was extracted from soil samples sieved between 0.25 and 4 mm with 20 mM sodium citrate (pH 7.0) at a rate of 250 mg of soil in 2 ml of buffer and autoclaving at 121 °C for 30 min [30]. The supernatant was removed and two additional sequential 1-h extractions were performed. All supernatants from a sample were combined, the volume was measured, an aliquot was centrifuged at 10,000×*g* for 15 min to remove soil particles and Bradford-reactive total protein was measured.

Aggregate stability percentage was quantified by placing soil macroaggregates (0.25–4 mm) on a 0.25 mm sieve, wetting the soil and applying an artificial rain with an energy of 270 Jm^{-2}. The resistant aggregates were dried and weighed after removing plant debris and sand particles based on the method described in Lax et al. [19]. Physical–chemical properties of the rhizosphere soil of both plant species are shown in Table 1.

PLFA analysis was carried out as described in Zelles and Bai [31]. Briefly, lipids were extracted (methanol, chloroform, and phosphate buffer) from a fresh soil sample equivalent to a dry matter (dm) of 50 g. The resulting lipid material was fractionated into neutral lipids, glycolipids, and phospho-(polar) lipids on a silica-bonded phase column (SPE-SI; Bond Elute, Varian, Palo Alto, USA) by elution with chloroform, acetone, and methanol, respectively. The different steps of extraction, separation, hydrolysis, and derivatization led to three fractions of ester-linked PLFA composed of saturated fatty acids (SATFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). MUFA fraction was modified with dimethyldisulfid and each fraction was analysed separately by a GC-MS system MSD 5973 (AGILENT Technologies, Wilmington, USA) equipped with a HP-5 (50 m×0.2 mm) fused silica capillary column. Individual PLFA markers were used to quantify the relative abundance of specific microbial groups. Bacteria were identified by the SATFAs: i15:0, a15:0, n15:0, i16:0, a16:0, i17:0, a17:0, i19:0, cy17:0, cy19:0 and the MUFAs: 16:1ω7 and 18:1ω7. From this selection, i15:0, a15:0, n15:0, i16:0, a16:0, i17:0, a17:0, and i19:0 are specific for Gram-positive and cy17:0, cy19:0, 16:1ω7, and 18:1ω7 for Gram-negative bacteria [32]. Other microbial groups identified with PLFA biomarkers include fungi (18:2ω6; [2]) and actinobacteria (10-methylbranched SATFAs; [32]). Physiological state of microbial communities was determined using the ratios of MUFA/SATFA and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy17:0+cy19:0/16:1ω7+18:1ω7). The fatty acid 16:1ω5c was considered as a biomarker for estimating AM biomass and distribution [24].

Fatty acids are designated according to the nomenclature used by Frostegård et al. [10].

Statistical Analysis

One-way ANOVA was used to assess the effect of plant type on total PLFA, PLFA biomarkers, and physical-chemical parameters. Correlation analysis between all the soil parameters measured was carried out using Spearman's rank correlation coefficients. Principal component analysis of the Mol% values of PLFAs was used to compare the PLFA patterns of both plant species. All statistical analyses were performed using SPSS 15.0 for Windows.

Results and Discussion

The total PLFA amount was significantly ($P<0.05$) higher in the rhizosphere soil of the annual gramineous species (*P. miliaceum*) than in that of the perennial species (*L.*

Table 1 Physical-chemical characteristics of the rhizosphere soil of *L. spartum* and *P. miliaceum*

	L. spartum	*P. miliaceum*
pH (H_2O)	6.36±1.19a[a]	6.20±0.66a
EC (1:5, dS m^{-1})	2.68±0.82a	2.73±0.53a
$CaCO_3$ (%)	<5a	<5a
Water-soluble C (μg g^{-1})	62±16a	57±6a
Water-soluble carbohydrates (μg g^{-1})	5±5a	8±5a
Total organic C (g kg^{-1})	6.0±3.3a	7.1±3.6a
Total N (g kg^{-1})	0.32±0.10a	0.40±0.10a
Aggregate stability (%)	29±13b	19±9a
Soluble Fe (mg kg^{-1})	0.18±0.41a	0.33±0.42a
Soluble Zn (mg kg^{-1})	85±123a	188±181b
Soluble Pb (mg kg^{-1})	3±5a	2±4a
Soluble Cu (mg kg^{-1})	0.10±0.03a	0.11±0.05a
Soluble Cd (mg kg^{-1})	1.27±2.56a	1.21±0.87a
Soluble Cr (mg kg^{-1})	0.01±0.01a	0.01±0.01a
Soluble Ni (mg kg^{-1})	0.15±0.19a	0.14±0.09a
Soluble Al (mg kg^{-1})	0.66±0.82b	0.30±0.16a
Soluble As (mg kg−1)	0.01±0.01a	0.09±0.11b

For each parameter, values sharing the same letter are not significantly different ($P<0.05$)

EC electrical conductivity

[a] Mean±standard deviation

spartum), as shown in Table 2. The total amount of PLFA serves as an estimate for soil microbial biomass [1]. While one might expect higher total amount of PLFA in the perennial species because this species presents a dense and fibrous root system [7] and presumably supplies higher C to the soil microbial biomass than the annual grass, this was not observed. This emphasises the fact that rhizodeposition quality is more important than its quantity for regulating microbial biomass. Thus, the root exudates deposited by the annual species were more effective for promoting microbial biomass. In contrast, the root biomass of perennial species is richer in cellulose than that of annual species [7], which is less easily degraded by soil microorganisms. A significant correlation ($r=0.416$, $P<0.01$) was registered between the total PLFA amount and the water-soluble C content, which is correlated with microbial activity. In addition, although the rhizosphere of *P. miliaceum* and that of *L. spartum* differed substantially in their PLFA contents; there were no significant differences in their PLFA fractions according to the PCA analysis (data not shown). The results imply that the microbial community structure was similar for the two rhizospheres.

The total bacterial PLFA content depended on the plant species and was higher in the rhizosphere of the annual grass (Table 2). This is due to the fact that the rhizosphere of such plants also presented higher contents of Gram-negative bacteria PLFAs and actinobacteria. It has been stated that since actinobacteria may form actinospores they better survive in relatively harsh soil environments (desiccation and heat). *P. miliaceum* is a top-rooted grass with a low root density [7]. This root system is expected to have lower ability in recovering soil water than that of perennial grasses [22], which would be consistent with higher proliferation of actinobacteria in the rhizosphere of *P. miliaceum*. We also observed a dominance of Gram-negative bacteria over Gram-positive bacteria in both rhizospheres, particularly in the rhizosphere of the annual grass, which could be due to the fact that Gram-negative bacteria are able to utilise a greater variety of C sources and adapt quicker to adverse conditions such as those of our polluted soil [15].

The fungal PLFA content (18:2ω6; [2]) did not differ significantly between the two rhizospheres, but the fungal: bacterial PLFA ratio was significantly greater in *L. spartum* compared to *P. miliaceum* (Table 2). As mentioned above, the root system of *L. spartum* presents a higher cellulose content than that of *P. miliaceum*, which favours colonisation and growth of fungi-producing extracellular cellulases [14]. The proportion of fungal PLFAs was lower than that of bacterial PLFAs in both rhizospheres. However, other investigations [16] found an increase in the fungal/bacterial ratio in soils highly contaminated with heavy metals. The fact that fungi are more affected by heavy metals might be because they dominate in larger pores and macroaggregates (>250 μm), which would make them more vulnerable to heavy metals. In contrast, bacteria are associated with smaller pores and microaggregates (<250 μm) and thus are more protected from metal contamination. Another possible explanation is that bacteria may have plasmids that encode resistance to toxic heavy metals [8]. We recorded a negative correlation between the soluble content of arsenic (As) and the fungal/bacterial ratio (Table 3).

The biomarker of AM fungi (16:1ω5c) was correlated positively with the percentage of mycorrhizal colonisation ($r=0.602$, $P<0.001$). There were no significant differences in

Table 2 Mycorrhizal colonisation and abundance of signature phospholipids fatty acids, total fatty acid contents, and class ratios of fatty acids in the rhizosphere soil of *L. spartum* and *P. miliaceum*

	L. spartum	*P. miliaceum*
Colonisation (%)	21±21a[a]	14±12a
Easily extractable glomalin (μg g^{-1} soil)	30±30a	35±27a
SATFA (nmol kg^{-1} dry wt.)	6,410±2,700a	8,517±3,975b
MUFA (nmol kg^{-1} dry wt.)	2,762±1,400a	4,296±2,600b
PUFA (nmol kg^{-1} dry wt.)	1,501±1,525a	1,054±500a
Bacterial G+PLFA (nmol kg^{-1} dry wt.)	1,243±775a	1,705±1,050a
Bacterial G- PLFA (nmol kg^{-1} dry wt.)	2,014±950a	2,738±1,425b
Actinobacteria PLFA (nmol kg^{-1} dry wt.)	890±375a	1,223±525b
Bacterial PLFA (nmol kg^{-1} dry wt.)	3,258±1,500a	4,444±2,275b
Fungal PLFA (nmol kg^{-1} dry wt.)	1,009±775a	812±375a
AM fungi PLFA (nmol kg^{-1} dry wt.)	112±75a	128±125a
Total PLFA (nmol kg^{-1} dry wt.)	10,673±4,275a	13,868±6,175b
Bacterial G+/G− PLFA	0.63±0.25a	0.67±0.25a
MUFA:SATFA	0.46±0.25a	0.56±0.25a
cyc/prec	0.92±0.75a	0.75±0.50a
Fungal:bacterial PLFA	0.33±0.25b	0.20±0.00a

For each parameter, values sharing the same letter are not significantly different ($P<0.05$)

SATFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *cyc/prec: cyclic* Gram-negative SATFA:MUFA precursor Gram-negative ratio

[a] Mean±standard deviation

this mycorrhizal marker between the rhizospheres of the two species. Several authors have demonstrated that the total percentage of mycorrhizal colonisation is correlated negatively with the concentration of soluble C fractions [23] indicating that mycorrhizal fungi act as strong sinks for photosynthates. We also found a significant negative correlation between the fatty acid 16:1ω5c and the concentration of water-soluble carbohydrates in the rhizosphere soil ($r=-0.418$, $P<0.01$). AM fungi also produce glycoprotein, a glomalin which acts as an insoluble glue to stabilise aggregates [30]. Recently, it has been postulated that glomalin plays a primary role in fungal physiology and in secondary effects in the soil environment that lead to the observed correlations of glomalin with soil aggregate stability [27]. Our results are inconsistent with this theory because there was no significant correlation between the concentration of glomalin and the percentage of stable aggregates. On the other hand, glomalin has been shown to be efficient in sequestering different heavy metals, especially Cu, Pb, and Cd [12]. In our study, only the soluble content of Cr in the soil correlated negatively with the concentration of glomalin ($r=-0.358$, $P=0.01$).

Table 3 Correlation coefficients among PLFAs and soluble metal contents of the rhizosphere soil of both plant species ($n=50$)

	Fe	Zn	Pb	Cu	Cd	Ni	Al	As	Cr
SATFA	NS	NS	−0.296*	NS	NS	NS	NS	NS	−0.292*
MUFA	NS	NS	−0.537***	NS	NS	−0.343*	NS	NS	NS
PUFA	NS	NS	NS	NS	NS	NS	NS	NS	−0.308*
Bacteria G+	NS	NS	NS	NS	NS	NS	NS	NS	−0.358**
Bacteria G−	NS	NS	−0.409**	NS	NS	NS	NS	NS	NS
Actinobacteria	NS	NS	−0.303*	0.345*	NS	NS	NS	NS	−0.390**
Total bacteria	NS	NS	−0.384**	NS	NS	NS	NS	NS	NS
Total fungi	NS	NS	NS	NS	NS	NS	0.327*	NS	−0.302*
AM fungi	NS	−0.351*	−0.599***	NS	−0.373**	−0.524***	NS	NS	NS
Total PLFA	NS	NS	−0.303*	NS	NS	NS	NS	NS	−0.316*
Bacteria G+/G−	NS	0.299*	NS	0.373**	NS	NS	NS	NS	−0.484**
MUFA:SATFA	NS	−0.324*	−0.463**	−0.402**	−0.292*	−0.379**	−0.367**	NS	0.321*
cyc/prec	NS	0.404**	0.560***	0.383**	0.403**	0.484***	0.298*	NS	−0.344*
Fungi/Bacteria	NS	NS	NS	NS	NS	NS	NS	−0.299*	NS

NS not significant, *SATFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *cyclic/prec: cyclic* Gram-negative SATFA:MUFA precursor Gram-negative ratio

* $P<0.05$; ** $P<0.01$, *** $P<0.001$

The phospholipid 16:1w5c can be particularly responsive to environmental perturbations and may be a good indicator of changes in the microbial community structure. It has been recorded as a decrease in this biomarker of AM fungi in response to the addition of heavy metals [15]. This may be due to damage to fine roots by metals that may reduce rhizosphere habitats for mycorrhizal fungi, decreasing the mycorrhizal infection of plant roots. In this sense, the AM fungi fatty acids were negatively correlated with the soluble contents of Pb, Zn, Ni, and Cd (Table 3) and the total content of Pb (r=−0.506, P<0.001).

The increase in the ratio of saturated to unsaturated fatty acids is a common adaptation mechanism developed by microorganisms in response to starvation stress. The ratio has been used as a valuable indicator of substrate availability in soil [32]. MUFA/SATFA ratios >1 have been observed in soils with high C content and organic inputs, and <1 for soils characterised by low substrate availability. Our values for the ratio of monounsaturated to saturated fatty acids were about 0.51 for both rhizospheres (Table 2), which is consistent with the low C contents in these tailings (Table 1). On the other hand, this ratio is an indicator of nutrient bio-availability in soils along gradients of metal contamination because the toxic effects of the metals can inhibit the use of nutrients [9]. This is supported by a significantly negative relationship between this ratio and the soluble contents of Pb, Zn, Al, Ni, Cd, and Cu in the soil (Table 3).

The increase in cyclopropyl fatty acids (cy17:0 and cy19:0) is another adaptation mechanism that is induced by starvation or heavy metal toxicity [10, 13] and the resultant increase in membrane fluidity. The conversion of monoenoic precursors (prec) into cyclopropyl fatty acids (cyc) helps to maintain a functional living membrane by minimising the membrane lipid losses or the changes in membrane fluidity. It has been observed that the cyc/prec ratio is >0.1 under environmental stress. In our study, the cyc/prec ratio was around 0.80 for both plant species, which suggests that the microbial communities associated with their rhizospheres were stressed by heavy metal toxicity. Accordingly, this ratio was correlated positively with the soluble contents of Pb, Zn, Al, Ni, Cd, and Cu (Table 3).

It can be concluded that the microbial communities of the rhizosphere of two gramineous species predominating in Mediterranean mine tailings in terms of biomass and community structure were strongly influenced by the metal bioavailability to microorganisms. Considering the ability of the plants for stimulating rhizosphere microbial populations, both perennial and annual gramineous species can be indistinctly used in programmes of revegetation of these heavy metals polluted soils. However, the *P. miliaceum*, representing the annuals, supported more the soil microbial biomass than the perennial species *L. spartum*. On the other hand, *L spartum* seems to be more important for development of fungal community.

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