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# The combination of quarry restoration strategies in semiarid climate induces different responses in biochemical and microbiological soil properties

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ABSTRACT

Mining activities generate loss of environmental and landscape quality, especially in arid and semiarid Mediterranean regions. A precondition for ecosystem reclamation in such highly disturbed areas is the development of functional soils with sufficient amount of organic matter. In a restoration experiment in limestone quarries in the Sierra de Gádor (Almería), SE Spain, several combinations of organic amendments (sewage sludge and compost from domestic organic waste) and mulches (gravel and woodchip) were tested and native plants (*Anthyllis cytisoides, A. terniflora* and *Macrochloa tenacissima*) were planted. After five years, the effect of each treatment on the soil chemical properties, basal respiration, four enzyme activities (dehydrogenase, urease, β-glucosidase and alkaline phosphatase) and the microbial community composition was analysed. Analysis of phospholipid fatty acids (PLFAs) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting were used to analyse the microbial communities (bacteria and fungi). Undisturbed natural soils adjacent to the mining area were used as soil quality references. Organic amendments, particularly compost, improved soil chemical and biochemical properties as well as microbial biomass. However, the effects of mulch application did not show a clear trend with respect to soil functionality and did not increase the microbial biomass. Soils treated with sewage sludge and compost showed bacterial PLFA concentrations similar to those of reference soils, but compost treatments presented fungal PLFA concentrations that were much higher. Each combination of organic amendment and mulch was selective for a proper microbial community. Nevertheless, increases in soil functionality and microbial biomass were not related to changes in microbial diversity. After five years, the microbial properties of restored soils had not yet converged to values recorded in the reference soils. However, the combination of mulches and organic amendments, particularly compost treatment, is suggested to be beneficial for restoring degraded soils from quarrying areas because they stimulate microbial growth and activity, with positive implications for the increase in both soil fertility and quality.



## 1. Introduction

The quality of landscape and environment is negatively affected by mining. Open-pit limestone mining is particularly harmful because of the type of extraction, which does not generate reject material. Thus, reclamation of these degraded lands must begin with an inert substrate with easily friable materials on steep slopes

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that favour soil erosion (Gunn and Bailey, 1993). Moreover, natural restoration in arid and semiarid areas is very slow because of the limiting climate conditions in these regions, such as low rainfall and intense solar radiation, submitting plants to severe water stress. All these factors contribute to increasing soil erosion and degradation (Miralles et al., 2009; Solé-Benet et al., 2009). The most effective way to face these conditions is through the rehabilitation of both the constructed substrate and plant cover as soon as possible to accelerate ecological restoration (Jorba and Andrés, 2000). However, these new constructed soils do not have the necessary physical, chemical and biological characteristics to support the development of natural vegetation (Sort and Alcañiz, 1996). Many authors agree that the first step should be the formation of a fertile soil layer, which enhances both biological activity and biogeochemical cycles, and activates and accelerates the regeneration of plant communities (Caravaca et al., 2002; Ros et al., 2003; Heneghan et al., 2008; Domene et al., 2009; Soliveres et al., 2012). Indeed, the generation of an active and sustainable microbial community is fundamental to soil bioreclamation.

In recent years, restoration strategies have been developed to optimize the cost/results ratio. Some of these strategies aim to improve soil fertility, e.g. using organic amendments from organic waste (Alcañiz et al., 1996; Navas et al., 1999; Moreno-Peñaranda et al., 2004; Domene et al., 2009; Maisto et al., 2010; Ortiz et al., 2012). Others, such as mulch incorporation, act at the landscape level, reducing soil erosion and protecting soil from extreme climatic conditions by reducing evaporation and increasing infiltration (Tejedor et al., 2003; Cook et al., 2011; Benigno et al., 2013; Hueso-González et al., 2015). Finally, Clemente et al. (2004) described the need to use plant species adapted to nutrient and water stress because they have evolved morphological and physiological adaptations that enable them to survive and grow under harsh conditions, such as those in arid and semiarid areas.

Criteria for evaluating the effects of different quarry restoration strategies are frequently based on visually distinguishable indicators, such as soil erosion and plant communities (Mummey et al., 2002). However, the interactions between the main components of the soil system, feedback between aboveground and belowground processes, and the resilience of the ecosystem to disturbance must all be known to improve restoration efforts (Heneghan et al., 2008). Several authors have suggested that secondary succession is driven by the interaction between plants and soil microbial communities (Kardol et al., 2013; Lozano et al., 2014).

The addition of an external source of organic matter stimulates soil microbial community growth and activity in soils degraded by mining or agriculture, resulting in mineralization of nutrients available to plants (Tejada et al., 2006; De Varennes et al., 2010; Asensio et al., 2013; Alvarenga et al., 2014) and increasing soil fertility and quality (Miralles et al., 2009; Diacono and Montemurro, 2010). Soil microbiota plays essential functions in soil structure formation, plant establishment and soil organic matter transformation (Díaz et al., 1994 Zink and Allen, 1998). Soil enzyme activities have been successfully used as soil quality indicators because enzymes respond to changes in soil much faster than other physical or chemical properties (García et al., 1994; Trasar-Cepeda et al., 1998). Kandeler et al. (1996) suggested that modifications in the soil microbial community due to environmental factors should be reflected in the level of soil enzymatic activity. More recently, the disconnect between bacterial community structure and potential enzyme activities has been reported in mining soils (Frossard et al., 2012), whereas Li et al. (2015) suggested that changes in soil function may indicate the evolution of the microbial community after manure application. In this sense, joint research on soil enzymatic activity and microbial communities could play a crucial role in restoration practices because these communities are very sensitive and respond more rapidly to environmental changes than plants (Claassens et al., 2006; Harris, 2009). However, many biotic and abiotic soil factors, such as the characteristics of soil organic matter, soil moisture and temperature, can affect the biomass (Brockett et al., 2012; Bastida et al., 2013a; Zhou et al., 2014) and structure of soil microbial communities (Mastrogianni et al., 2014; You et al., 2014; Hortal et al., 2015). Currently, a variety of high-throughput tools are available for the assessment of the composition, structure and functional activity of microbial community in the natural environment, such as next generation sequencing (NGS, Shokralla et al., 2012) or GeoChip (He et al., 2010). Although these technologies have expanded our understanding of microbial assembly, gaining comprehensive knowledge of soil microbial community is still difficult because microorganisms are governed by a multitude of abiotic factors (Schloter et al., 2003) and subjected to stochastic effects (Dini-Andreote et al., 2015). For this reason, integrative studies that involve a variety of methods for exploring the response of soil communities in terms of biomass, activity and diversity are required to properly assess the success of restoration programs.

Hueso et al. (2011) showed that in semiarid Mediterranean areas, indigenous microbial communities are well adapted to severe climate conditions such as high temperatures and scarce rainfall, whereas new soil microbial communities from organic waste are more sensitive to water stress than native soil microbiota. Organic amendments can also produce changes in the autochthonous soil microbial populations due to the diverse available substrate that they provide. A thorough characterization of soil activity and microbial communities after restoration with organic amendments in quarry restoration areas has not yet been done and could be crucial to understanding soils' response to climatic constraints such as water scarcity. A few field studies have addressed how the soil microbiota responds to restoration strategies. Nevertheless, an improved understanding of the response of microbial communities to different restoration treatments is needed to evaluate management success in limestone quarry restoration in a semiarid climate (Garris et al., 2016).

According to the above mentioned framework, the purpose of this study was to determine the effects of various mined-soil restoration strategies on soil activity, biomass and genetic structure of soil microbial communities (bacteria and fungi). Nine restoration treatments consisting of different combinations of organic amendments and mulches were evaluated in a semiarid Mediterranean ecosystem five years from the beginning of a soil restoration project relative to undisturbed natural soils as a reference. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) linked to sequencing-based approaches was used to describe the composition of microbial communities, whereas phospholipid fatty acids (PLFAs) were utilized to assess microbial biomass. Microbial general activity was determined by measuring basal respiration and dehydrogenase activity. Specific enzyme activities related to N, P and C cycles in the soil were determined to evaluate the effect of quarry restoration on soil nutrient dynamics. We hypothesized that the addition of organic amendments would positively affect soil functionality and the abundance of microorganisms but that this effect could be altered by mulches. Understanding how soil biochemical and microbial properties respond to different strategies may help strengthen management practices in quarry restoration.

## 2. Materials and methods

### 2.1. Description of the study area and experimental design

The study was conducted in a limestone quarry located at the southeastern edge of the Gádor massif (36°55′20″N, 2°30′29″W), Almería (SE Spain). The climate is semiarid thermo-Mediterranean with a mean annual temperature of 17.6 °C. The mean annual

rainfall is 245 mm mostly in autumn and winter, and the mean potential evapotranspiration is 1225 mm year$^{-1}$. The experiment was set up in 2008 on a hillside with a 19% mean slope at 370 m a.s.l.

In this restoration experiment, a factorial design was used to test the effect of two factors, organic amendment and mulch, and each factor consisted of three levels. The organic amendment factor levels were a) thermally dried sewage sludge from urban waste water, b) compost from domestic organic waste, and c) no amendment. The thermally dried sewage sludge was collected from a municipal wastewater treatment plant in Almería and was added at a rate of 28 kg m$^{-1}$ (Total organic C = 351.5 g kg$^{-1}$ and moisture = 59%). Compost was collected from a treatment plant close to the quarries and was added at a rate of 34 kg m$^{-1}$ (Total organic C = 196.5 g kg$^{-1}$ and moisture = 40%). The amount of applied organic amendments was calculated according to their carbon content to increase the initial organic matter content in each plot by 2%. The mulch factor levels were a) gravel mulch, b) woodchip mulch, and c) no mulch. Gravel mulch was siliceous fine gravel with a fine earth content from a nearby quarry, and the organic mulch was *Pinus halepensis* woodchip (1 cm thick × 2 to 15 cm). Nine treatments resulting from the combination of the two factors were established randomly. Plots measured 5.0 m × 5.0 m and were laid out in three replicates nearby to facilitate the treatment application by the heavy machinery used in the quarry. However, the differences or similarities among soil samples should not be attributed to their spatial distribution but rather to the applied treatments because the experimental area was homogeneous from a physiographic point of view (soil, slope gradient, orientation and microclimate). First, the different organic amendments treatments were spread on the soil surface and mixed into the soil with a mechanical backhoe, and then mulch was spread to a thickness of 3–5 cm. Finally, 25 plants of the 3 most abundant native species were planted in the same proportion as they are present around the quarry, 12 *Macrochloa tenacissima* (L.) Kunth, 8 *Anthyllis terniflora* (Lag) Pau and 5 *Anthyllis cytisoides* L.), at a planting distance of 1 m with the species alternated. Plants were irrigated during the first summer.

Five years after the beginning of the experiment, eight soil samples were collected from each plot from the top 15-cm layer. Subsequently, the eight samples were combined in a sterile plastic bag to form a single composite soil sample for each plot that was transported to the laboratory, where each sample was homogenized and passed through a 2-mm sieve. The border effect was avoided during sampling on each plot. Each soil sample was divided into different parts during storage: at 4 °C for biochemical and PLFA analysis; at −20 °C for molecular analysis; and at room temperature for chemical analysis. The same procedure was performed for soil samples used as reference soil; three different points in a nearby natural, undisturbed area were sampled to use as proxy of a microbial community in equilibrium with its physical, chemical, and biological environment (Worm and Duffy, 2003).

### 2.2. Chemical and biochemical soil characteristics

Basic soil chemical property analyses were performed following standard procedures. Electrical conductivity (EC) of aqueous extract 1/5 (w/v) was measured using a digital conductivity meter (Basic 30, Crison, Carpi, Italy), and pH was determined in an aqueous solution 1/2 (w/v) in a micropH 2002Crison pHmeter (Crison, Barcelona, Spain). Total organic C (TOC) was determined by the Nelson and Sommers (1996) method. Total N content (TN) was determined using a LECO Truspec C/N analyser (St. Joseph, MI, USA), and total calcium carbonate content was measured with a Bernard calcimeter. Total P and K contents were determined after acid digestion by inductively coupled plasma (ICP) emission spectrometry using an ICAP 6500 DUO Thermo (Thermo Scientific, Wilmington, DE, USA).

Soil respiration was analysed by placing 50 g of soil moistened to 40–50% of its water-holding capacity in sealed flasks and incubated for 47 days at 28 °C. The $CO_2$ released was measured weekly using an infrared gas analyser (CheckmateII; PBI Dansensor, Ringsted, Denmark). The cumulative amount of $CO_2$ released during the incubation period was calculated as the sum of all the weekly measurements (Hernández and García, 2003). Basal soil respiration was expressed as mg $CO_2$–C kg$^{-1}$ soil day$^{-1}$. The activity of four soil enzymes was measured. Dehydrogenase activity was determined by using 1 g of soil and the reduction of 2-*p*-iodo-3-nitrophenyl-5-phenyl tetrazolium chloride to iodonitrophenyl formazan (García et al., 1997). Urease activity was determined as the amount of $NH_4^+$ liberated during hydrolysis using urea as a substrate (Kandeler and Gerber, 1988). β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as a substrate (Eivazi and Zakaria, 1993), and alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate as a substrate (Tabatabai et al., 1969).

### 2.3. Phospholipid fatty acid (PLFA) analysis

PLFAs were extracted from soil samples (3 g) by chloroform-methanol extraction following protocols described by Frostegård et al. (1993). Phospholipid fractions were saponified and methylated to fatty acid methyl esters (FAMEs) by alkaline methanolysis and quantified by gas chromatography (Trace GC-Ultra Thermo Scientific, Austin TX, USA). Separations were carried out in a 30−m capillary column (Thermo TR-FAME 30 m × 0.25 mm i.d. × 0.25 μm film) operated under a constant helium flow. During analysis, the oven temperature was kept at 150 °C for 0.5 min, ramped up at 2 °C/min to 180 °C and then to 240 °C at 4 °C/min. Fatty acids were assigned to bacterial (Gram-positive bacteria, Gram-negative bacteria and actinobacteria) and fungi groups as described by Bastida et al. (2015a). The ratio of bacterial to fungal PLFAs reflects the ratio between bacterial and fungal biomass (Bardgett et al., 1996).

### 2.4. Soil DNA extraction, PCR and DGGE analysis

Total DNA was extracted using the Fast DNA Spin Kit for soil (MP Biomedicals, Solon OH) following the manufacturer's instructions. The V6-V8 region of the bacterial 16S rDNA and the V7-V8 region of the fungal 18S rDNA were amplified by PCR employing the specific 986F-1401R (Felske et al., 1998) and EF390-FR1 (Vainio and Hantula, 2000) primer sets, respectively. For DGGE analysis, a GC-clamp was added to the 986F and FR1 primers. Three independent PCR amplifications were performed for each set, and each soil sample and the triplicate amplification products were pooled to minimize the effect of PCR biases. DGGEs were performed with the INGENY phorU-2 System (Ingeny International BV) on a 6% polyacrylamide gel [30% acrylamide/bisacrylamide (37.5:1) (Merck Millipore)], with denaturing gradients (ranging from 46% to 60% for bacteria and from 30% to 55% for fungi) obtained with a 100% denaturing solution containing 40% formamide (v/v) and 7 M urea. After DGGE electrophoresis, the gels were stained with SYBR®GOLD (Molecular Probes, Eugene, OR, USA), and gel images were digitalized using the Chemidoc Apparatus (Bio-Rad, CA, USA).

### 2.5. Cloning and sequencing

Representative 16S and 18S rDNA-DGGEs bands were excised and eluted from gels, according to the method of Pastorelli et al. (2011). The pGEM®-T Vector System I (Promega, Madison WI, USA) was used for cloning PCR products, and transformation of ligated

products was performed using *Escherichia coli* JM109 high-efficiency competent cells (Promega). Transformants were selected on LB/ampicillin plates. Five positive clones were chosen from each excised DGGE band, screened by DGGE and subjected to directly sequencing by the Macrogen Service (Macrogen LTD., The Netherlands, http://www.macrogen.com). The nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers KT803729–KT803772.

### 2.6. Plant cover

The percentage of total planted and spontaneous plant was estimated using the point-intercept frame method proposed by Floyd and Anderson (1982) with the following modifications: a 400-point grid with 1-cm cell spacing (20 × 20 cm) was used. To estimate plant cover, we established three randomly 20 × 20-cm quadrats in each plot. One grid point count represented 1 $cm^2$ of cover. We counted the grids occupied by plant cover, and percent cover was determined in relation to the total grids (400-point grid per quadrat).

### 2.7. Data analyses

A two-way ANOVA was performed to determine the effects of organic amendments, mulches and their interaction on soil chemical characteristics, enzyme activities, PLFAs and DGGE profile diversity indices. One-way ANOVA was also carried out to compare the characteristics of both restored and reference soils. Data were tested for homogeneity of variance, and differences between individual means were evaluated by Tukey's post hoc test at $P < 0.05$. Pearson correlations were also calculated to determine the relationships of soil chemical properties and microbial biomass with soil enzyme activities. For all abovementioned statistical analyses, SPSS v.19.0 (SPSS, Inc.) was used. Band migration distance and the intensity within each DGGE profile were evaluated using Gel Compare II software v 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). Bacterial and fungal community diversity were characterized by calculating three indicators from DGGE profiles: community richness (number of bands) and Shannon-Weiner and Simpson indices assessed as described by Pastorelli et al. (2011). A cluster analysis based on position and the presence/absence of bands in the different profiles was carried out to determine the similarity between DGGE profiles using the Dice product moment correlation coefficient and the unweighted pair group method using the arithmetic average (UPGMA) algorithm by GelCompare II. The DGGE banding patterns, extracted as quantitative band matching tables, were standardized by calculating the relative intensity of each band (ratio of intensity of each band versus the total band intensity) and imported into the PAST software (Hammer et al., 2009; http://folk.uio.no/ohammer/past). An analysis of similarity (ANOSIM) was used to examine the statistical significance of DGGE profiles and determine the differences in microbial community structure of different soils. ANOSIM generates a statistical test, R, the magnitude of which indicates the degree of differences among the groups: a score of 1 indicates complete separation and 0 no separation. ANOSIM was performed using PAST with the Bray-Curtis distance measure and 9999 permutation tests.

The 16S and 18S rDNA sequence chromatograms were edited using Chromas Lite software (v2.1.1; Technelysium Pty Ltd; Tewantin, Old, AU; http://www.technelysium.com.au/chromas_lite.htm) to verify the absence of ambiguous peaks and convert them to the FASTA format. The DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras hidden in the 16S rDNA sequences. Nucleotide sequences were compared against all sequences stored in the GenBank database using the Web-based BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) to find closely related nucleotide sequences. Nucleotide sequences were aligned with other sequences of equivalent length from environmental clones and axenic cultures retrieved from the GenBank database for phylogenetic analysis, using the ClustalX 2.0.11 multiple sequence alignment software (Larkin et al., 2007). Distance analysis was performed according to the method of Jukes and Cantor (1969) followed by phylogenetic tree construction performed by the neighbour-joining algorithm (Saitou and Nei, 1987) using TREECON 1.3b (Van de Peer and DeWachter, 1994). The robustness of associations among samples (node) was evaluated by bootstrap analysis with 1000 replicates.

**Table 1**
Average soil chemical values and plant cover (standard error in parenthesis) in different soils. Different letters indicate significant differences among soil treatments at $P < 0.05$.

| Treatments | CaCO3 (%) | EC[a] (dS m-1) | pH | TOC (g kg-1) | Total N (g kg-1) | Total P (g kg-1) | Total K (g kg-1) | C/N | Plant Cover (%) |
|---|---|---|---|---|---|---|---|---|---|
| No amendment – No mulch | 49.06 (3.88)ab | 1.18 (0.10)cd | 8.86 (0.02)a | 1.13 (0.06)g | 0.21 (0.00)g | 0.39 (0.02)f | 5.46 (0.17)cd | 5.33 (0.48)b | 8.3 (1.6)c |
| No amendment – Gravel | 28.62 (3.25)cd | 1.68 (0.14)bc | 8.95 (0.08)a | 2.79 (0.19)f | 0.23 (0.00) fg | 0.28 (0.01)g | 4.54 (0.08)e | 12.08 (0.86)b | 66.0 (12.4) abc |
| No amendment – Woodchip | 50.49 (2.97)ab | 1.46 (0.05)bc | 8.70 (0.02)ab | 3.78 (0.12)e | 0.29 (0.01)fg | 0.50 (0.01)e | 5.41 (0.07)d | 12.71 (1.18)c | 22.7 (2.8)bc |
| Sludge – No mulch | 55.31 (1.26)a | 1.52 (0.21)bc | 8.53 (0.05) abc | 8.65 (0.18)c | 0.87 (0.04)e | 0.69 (0.02) d | 6.58 (0.20)a | 9.89 (0.71) cde | 52.8 (10.1)abc |
| Sludge – Gravel | 24.83 (8.20)d | 1.36 (0.15)bc | 8.70 (0.03)ab | 3.49 (0.14)ef | 0.44 (0.04) fg | 0,21 (0.01)g | 3.26 (0.02)g | 7.99 (0.57)a | 72.1 (23.6)ab |
| Sludge – Woodchip | 50.64 (0.12)ab | 1.47 (0.1)bc | 8.23 (0.27)c | 7.54 (0.26)d | 0.48 (0.02)f | 0.58 (0.00) e | 6.01 (0.07)b | 15.49 (0.29) cd | 55.4 (2.7)abc |
| Compost – No mulch | 46.74 (1.16)ab | 2.46 (0.44)ab | 8.24 (0.10)c | 29.11 (0.45)b | 2.94 (0.04)c | 1.06 (0.03)c | 5.76 (0.01) bcd | 9.86 (0.28)d | 65.9 (11.1)abc |
| Compost – Gravel | 40.18 (2.20) abc | 2.06 (0.28) abc | 8.40 (0.03)bc | 41.34 (0.25)a | 5.29 (0.03)a | 1.41 (0.05)a | 3.91 (0.08)f | 7.79 (0.01)ef | 92.9 (12.1)a |
| Compost – Woodchip | 42.99 (1.20) abc | 2.92 (0.18)a | 8.22 (0.05)c | 29.91 (0.15)b | 4.47 (0.07)b | 1.24 (0.01 b | 4.77 (0.03)e | 6.67 (0.07)b | 64.5 (18.8) abc |
| Reference soil | 36.67 (2.61)bc | 0.20 (0.02)d | 8.27 (0.06)c | 29.31 (0.35) b | 2.40 (0.27)d | 0.29 (0.00) g | 5.80 (0.25)bc | 12.46 (1.31)f | 94.3 (7.9)a |

[a] EC (Electrical conductivity).

## 3. Results

### 3.1. Chemical soil properties and plant cover

The main soil chemical characteristics of the different treatments are presented in Table 1. pH values were not affected by the treatments (amendments and mulches), whereas EC increased significantly in plots amended with compost ($P < 0.05$) (Tables 1 and S1). Gravel mulch caused a reduction in carbonate content (Table 1). Factorial ANOVA showed a significant effect of organic amendments, mulches and their interaction on TOC, TN, P, K and C/N ratio. In general, all treated soils showed higher TOC and TN contents than the non-amended soil with statistically significant differences ($P < 0.001$) (Table S1, 1). Plots treated with compost showed the highest values for these two parameters, particularly in combination with gravel mulch. Sludge also increased the soil TOC and TN contents but to a much lesser extent than compost addition, which led to TOC and TN contents similar to those of the reference soil (Table 1). Different C/N ratios were found in each treatment, the highest ($P < 0.05$) being obtained with the combination of sludge and woodchip (Table 1). Organic amendments significantly increased total soil P content, particularly in compost-amended soils, in which it far surpassed that of the reference soil. In contrast, the K content was lower in the compost treatments.

Five years after the start of the experiment, the percentage of plant cover was higher in amended soils than in unamended soils ($P < 0.01$) (Table S1 and 1). Likewise, under gravel mulch, the

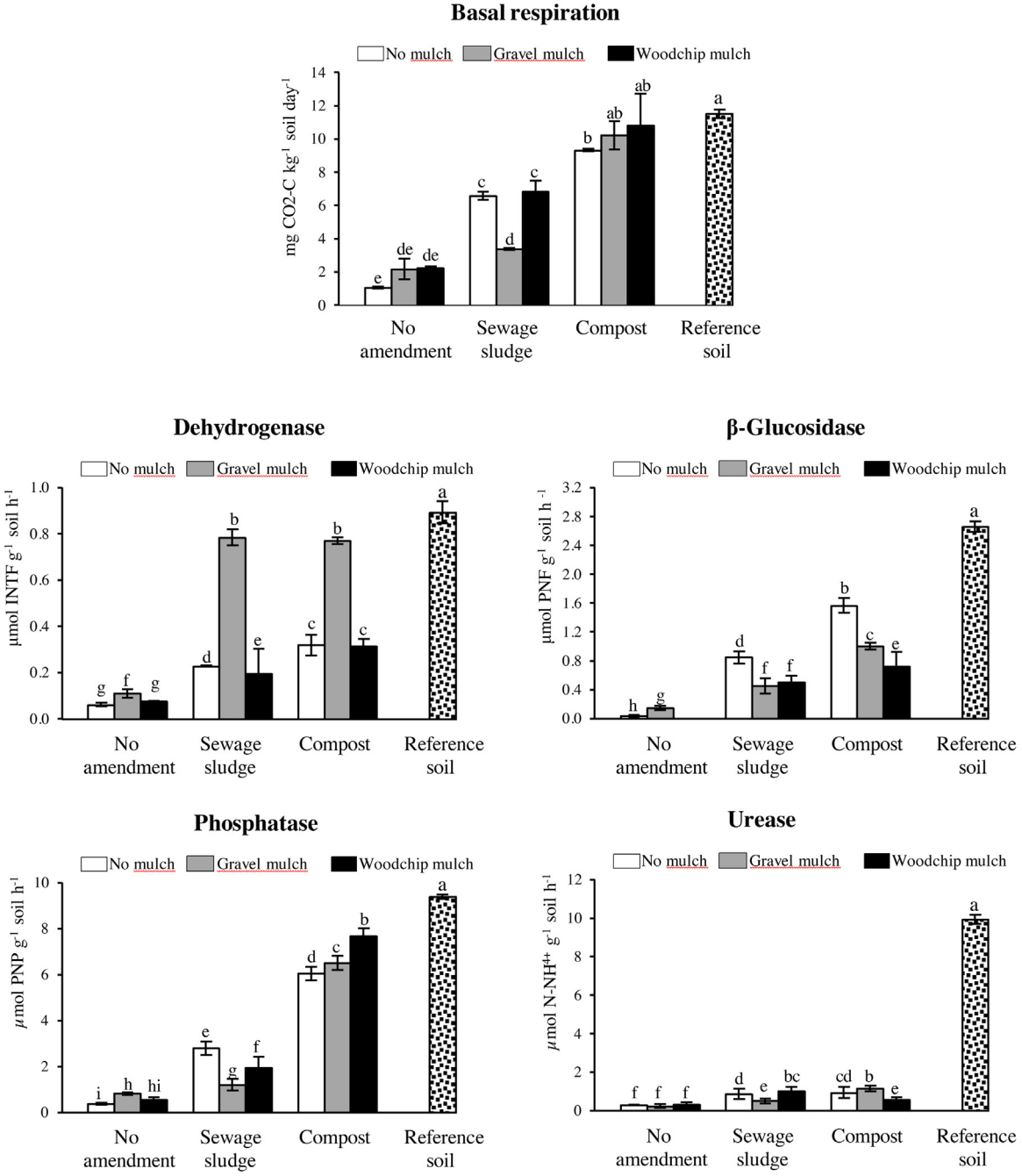


**Fig. 1.** Basal respiration and enzymatic activity in soil samples. Values are means (n = 3) ± S.E.M. Different letters indicate significant differences among soil treatments at $P < 0.05$.

vegetation cover was higher than in woodchip and no mulch (Table 1).

### 3.2. Microbiological properties and enzymatic activity analysis

Organic amendments significantly improved soil microbiological properties (Table S1 and Fig. 1). Soil respiration was positively affected by organic amendments and was more pronounced in soils treated with compost than in those treated with sludge (Table S1 and Fig. 1). Mulches affected enzyme activities, showing different trends depending on the treatments (Table S1 and Fig. 1). Dehydrogenase activity was affected by all treatments (Table S1). A significant positive effect was observed for the combination of gravel mulch with organic amendments, reaching values close to those of the reference soil (Fig. 1). β-glucosidase activity and alkaline phosphatase showed significant increases in treatments with organic amendments, particularly in the compost treatment (Table S1 and Fig. 1). Gravel and woodchip mulch had negative effects on the β-glucosidase activity in amended soils, whereas they had negative or positive effects on alkaline phosphatase activity depending on which organic amendment was used: negative effects when mulch was used in combination with sludge; positive effects when used in combination with compost. Urease activity also showed a positive increase in the two types of organic amendment (Table S1 and Fig. 1). Nevertheless, the combination of mulch with organic amendments had different effects. Under sludge treatments, woodchip mulch had a positive effect on urease activity and gravel mulch had a negative effect. The opposite effects were observed with compost treatments (Fig. 1).

The Pearson's correlation analysis showed that all soil enzyme activities were significantly correlated with the main soil chemical parameters (Table 2). Of all the variables analysed, EC and total K had the weakest effect on soil functionality.

### 3.3. PLFA analysis

The results of the two-way ANOVA showed that PLFAs were significantly ($P < 0.01$) affected by the organic amendments. Only actinobacterial community and the $Gram^+/Gram^-$ ratio were significantly affected by the combination of factors (Table S2). As expected, organic amendments significantly increased the concentrations of both fungal and bacterial PLFAs (Fig. 2). Experimental plots with compost showed significantly higher fungal PLFAs than those with sludge and no amendment, even higher than the reference soil (Fig. 2). No plot with organic amendments had as high a bacterial PLFA content as the reference soils, which was particularly true for $Gram^+$ fatty acids. Sludge treatments increased the total amount of actinobacterial PLFAs compared with compost and no amendment (Fig. 2). Nevertheless, undisturbed natural soils showed more than twice the concentration of actinobacterial PLFAs as sludge treatments. The ratio of Gram-positive to Gram-negative PLFA content was higher ($P < 0.05$) in amended soils than in unamended soils.

A strong positive correlation was observed between enzyme activities and the content of microbial PLFAs (Table 2). However, the correlation between the dehydrogenase activity and fungal PLFAs was not significant.

### 3.4. PCR-DGGE analysis

The DGGEs generated fingerprints with a high number of bands well spread throughout the gels (see Fig. S1, S2). Both organic amendments and mulches had a significant effect on bacterial and fungal community diversity ($P < 0.01$), as did their interaction ($P < 0.05$) (Table S2). The richness and Shannon-Wiener index of the bacterial community were highest for the samples taken from unamended soils and gravel mulch (Table 3). A comparison of these indices in the fungal DGGE profiles showed that they were highest in samples taken from treatments with compost but without mulch.

Cluster analysis of 16S-DGGE profiles revealed that the bacterial community structure was mainly affected by the type of amendments (Fig. 3a). Samples from soil with sludge amendment grouped together into a high-similarity (67%) cluster. The bacterial communities of natural soils appeared very different from the other treatments. Cluster analysis performed on 18S-DGGE profiles divided fungal communities into four groups, mainly depending on the organic amendment (Fig. 3b). Plots with compost had fungal community similarities exceeding 38.5%, and sludge plots generated one group with a similarity of 47.5%. Unamended plots with woodchip and without mulch were also grouped together with natural soils, in which the similarity exceeded 35%. Within each amendment group, gravel mulch generated fungal community structures with fewer similarities than the other mulch treatments.

Testing all groups independently (10 groups: three organic amendments combined with three mulches and reference soil) by one-way analysis of similarity (ANOSIM) (Table 4) revealed significant differences in the patterns between groups, with an R value of 0.8581 for bacteria and an R value of 0.988 for fungi.

**Table 2**
Correlation matrix and significance of soil chemical, basal respiration, enzymatic activities and microbial biomass (PLFAs).[a,b]

| | EC | TOC | Total N | Total P | Total K | RESP | DHA | BA | UA | PA | Fungal PLFAs | Bacterial PLFAs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TOC | 0.715** | | | | | | | | | | | |
| Total N | 0.730** | 0.981** | | | | | | | | | | |
| Total P | 0.709** | 0.965** | 0.955** | | | | | | | | | |
| Total K | −0.055 | −0.187 | −0.266 | 0.013 | | | | | | | | |
| RESP | 0.664** | 0.870** | 0.831** | 0.864** | 0.014 | | | | | | | |
| DHA | 0.181 | 0.503** | 0.509** | 0.325 | −0.744** | 0.455* | | | | | | |
| BA | 0.523** | 0.741** | 0.646** | 0.693** | 0.093 | 0.844** | 0.475* | | | | | |
| UA | 0.187 | 0.500** | 0.429* | 0.580** | 0.441* | 0.665** | 0.124 | 0.668** | | | | |
| PA | 0.814** | 0.949** | 0.950** | 0.934** | −0.107 | 0.880** | 0.406* | 0.749** | 0.473* | | | |
| Fungal PLFAs | 0.678** | 0.823** | 0.840** | 0.849** | −0.023 | 0.876** | 0.351 | 0.666** | 0.516** | 0.892** | | |
| Bacterial PLFAs | 0.230 | 0.464* | 0.417* | 0.479* | 0.159 | 0.675** | 0.384* | 0.671** | 0.628** | 0.508** | 0.584** | |
| Total PLFAs | 0.517** | 0.724** | 0.678** | 0.728** | 0.108 | 0.909** | 0.418* | 0.815** | 0.680** | 0.768** | 0.874** | 0.856** |

[a] Correlations were considered significant (*) at $P < 0.05$ and highly significant (**) at $P < 0.01$.
[b] EC: electrical conductivity, TOC: total organic C, RESP: soil basal respiration, DHA: Dehydrogenase activity, BA: β-glucosidase activity, UA: Urease activity, PA: Phosphatase activity.

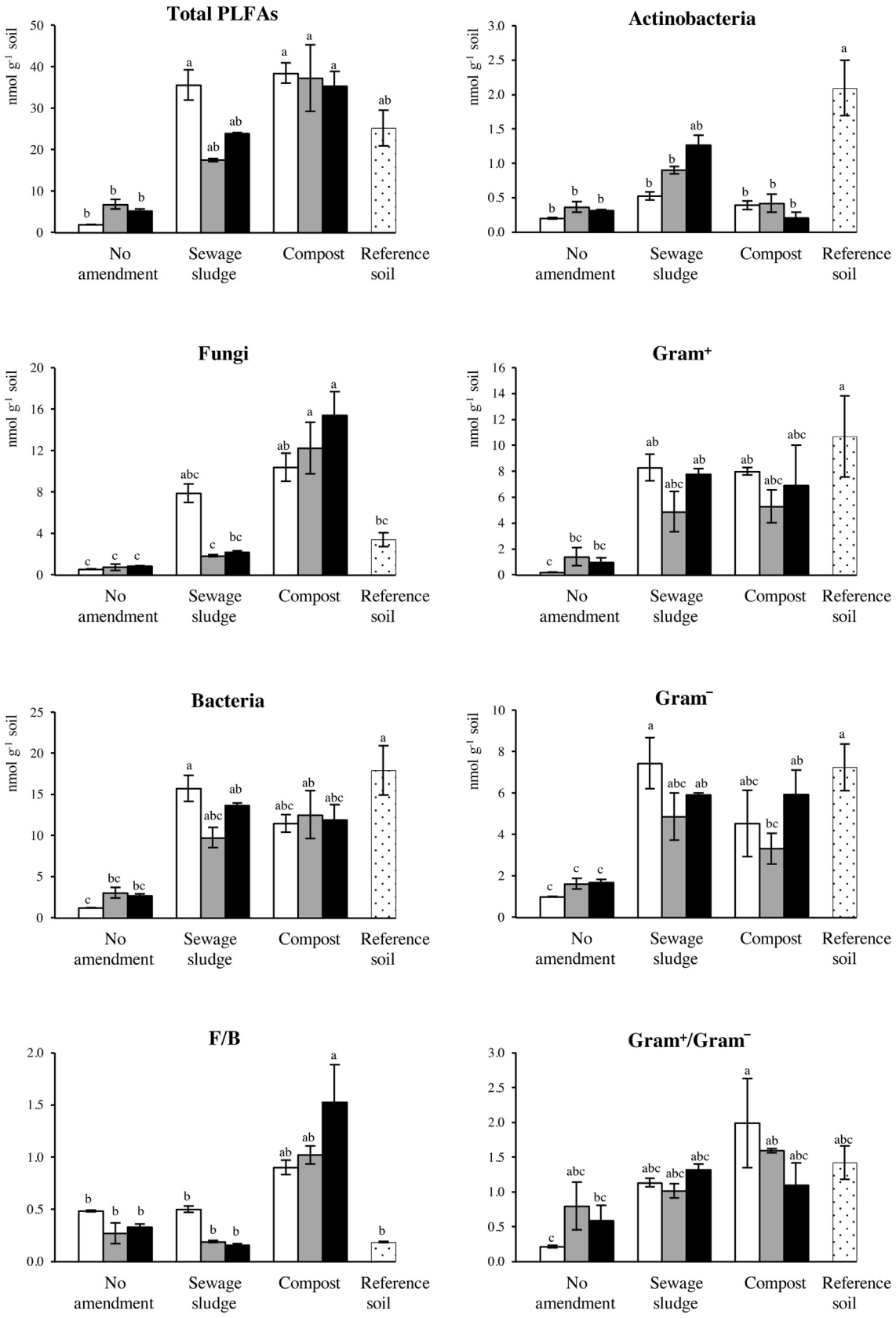


**Fig. 2.** Total PLFA, bacterial and fungal PLFA concentrations, ratio of fungi to bacterial PLFAs (F/B) and ratio of Gram-positive to Gram-negative bacterial PLFAs ($Gram^+/Gram^-$). Different letters indicate significant differences among soil treatments at $P < 0.05$.

**Table 3**
Average species richness (R), Shannon-Wiener index of diversity (H') and Simpson index of dominance (D) for bacterial and fungal communities (standard error in parenthesis). Different letters indicate significant differences among soil treatments at $P < 0.05$.

| Treatments | Bacteria | | | Fungi | | |
|---|---|---|---|---|---|---|
| | Bacteria | Shannon-Wiener | Simpson | Richness | Shannon-Wiener | Simpson |
| No amendment – No mulch | 40.33 (0.88)ab | 3.64 (0.00)ab | 0.027 (0.001)abc | 21.66 (0.33)c | 3.04 (0.01)bcd | 0.048 (0.000)cd |
| No amendment – Gravel | 41.66 (0.66)a | 3.71 (0.01)a | 0.024 (0.000)d | 11.33 (1.66)f | 2.35 (0.14)g | 0.101 (0.013)a |
| No amendment – Woodchip | 40 (1.15)ab | 3.66 (0.02)ab | 0.026 (0.000)cd | 13.66 (0.33)ef | 2.59 (0.02)fg | 0.076 (0.001)b |
| Sludge – No mulch | 35.33 (1.20)bc | 3.54 (0.04)bc | 0.029 (0.001)abc | 16.66 (1.66)def | 2.77 (0.09)def | 0.064 (0.006)bc |
| Sludge – Gravel | 36.33 (0.66)bc | 3.56 (0.01)abc | 0.028 (0.000)abc | 18.66 (0.33)cde | 2.91 (0.01)cde | 0.054 (0.001)bcd |
| Sludge – Woodchip | 34.66 (0.88)c | 3.51 (0.03)bc | 0.030 (0.001)ab | 15.33 (0.33)ef | 2.69 (0.01)ef | 0.069 (0.001)bc |
| Compost – No mulch | 32 (0)cd | 3.45 (0.00)c | 0.032 (0.000)b | 30.33 (0.33)a | 3.39 (0.00)a | 0.034 (0.000)d |
| Compost – Gravel | 36.33 (0.88)bc | 3.56 (0.02)abc | 0.028 (0.000)abc | 23 (1.52)abc | 3.11 (0.07)abc | 0.045 (0.003)cd |
| Compost – Woodchip | 27.33 (2.18)cd | 3,27 (0.07)d | 0.039 (0.002)a | 27,33 (0.33)ab | 3.29 (0.01)ab | 0.037 (0.000)d |
| Reference soil | 37 (0.57)abc | 3.24 (0.32)abc | 0.029 (0.045)abc | 19 (0.57)cde | 2.90 (0.01)cde | 0.056 (0.000)bcd |

These results were confirmed in a two-way crossed ANOSIM, which revealed that the influence of the amendment and mulch factors on the DGGE patterns of the soil bacterial communities was significant (R = 0.992 and 0.945, respectively). Similarly, amendment (R = 1) and mulch (R = 0.997) showed significant differences in fungal patterns (Table 4).

### 3.5. Clone sequence analysis

A total of 44 bands from PCR-DGGE were sequenced, 22 from 16S-DGGE and 22 from 18S-DGGE. BLAST analysis showed that the 16S-DGGE fragments sequenced are similar to the 16S rDNA sequences of known bacterial species with maximum coverage ranging from 81% to 99% and maximum identity ranging from 87% to 99%. The phylogenetic analysis indicated that sequenced clones are related to sequences retrieved from culturable bacteria in the *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia, Chlamydiae* and *Actinobacteria* phyla and with unculturable environmental species detected in soil (Fig. 4). Gram-negative bacteria were the most abundant bacteria identified, among which *Acidobacteria* were predominant (bands: B5, B6, B7, B10, B11, B12, B25, B26). Two bands were present in almost all 16S-DGGE profiles (B28 and B30) and were closely correlated to the *Actinobacteria* group and, in particular, to *Pseudonocardia rhizophila* (98% sequence homology) and *Microbacterium schleiferi* (99% sequence homology), respectively.

BLAST analysis of 18S-DGGE fragments sequenced showed similarity with 18S rDNA sequences of known fungal species, with maximum coverage ranging from 93% to 100% and maximum identity ranging from 95% to 99%. The phylogenetic analysis showed the predominance of sequenced clones related to sequences retrieved from reference fungal strains in the *Ascomycota* phylum and, within it, to the *Dothideomycetes* (bands: F1, F2, F5, F18, F23), *Eurotiomycetes* (bands: F14 and F17.4), *Sordariomycetes* (bands: F3, F4, F12, F13) and *Pezizomycetes* (band F23.3) classes (Fig. 5). In particular, two dominant bands (F3 and F4) closely related to the *Chaetomium globosum* (97% sequence homology) and *Fusarium* species (100% sequence homology), respectively, were present in all 18S-DGGE profiles. The other relatives belonged to *Basidiomycota*, *Blastocladiomycota* and *Chytridiomycota* phyla. Two bands (F8 and F9) were related to the *Mucorales* order (fungi incertae sedis). Each phylogenetic tree was supported by high bootstrap values.

In general, no particular relationship between any specific microbial taxon and soil treatment could be found, with the exception of the DGGE bands related to the *Acidobacteria* group, which were prevalent in the organic amendment soil samples profiles.

## 4. Discussion

### 4.1. Soil chemical properties

Soil chemical parameters as well as biochemical and microbial properties were affected by organic amendments. Although the same organic matter content was initially added to the soils with the organic amendments (compost and sludge), the content observed five years later varied depending on the type of amendment. Plots amended with compost showed similar TOC, even higher than the undisturbed natural soil, whereas in soils with sludge amendments, TOC was lower than that in undisturbed natural soils. According to Tejada et al. (2006), the effect of organic amendments on soil TOC depends on the chemical composition of the amendments, which determines the rate of their mineralization by soil microorganisms (Hahn and Quideau, 2013). The organic C fraction from sewage sludge is more biodegradable than that in compost and can be quickly hydrolyzed by enzymes (Cook and Allan, 1992). In our study, the highest TOC, N and P were found in the treatment with compost and gravel mulch, in which there was also more plant cover, even higher than in treatments with woodchip (Table 1). The increase in the TOC content of the compost amendment was caused by the stable nature of the amendment and likely to inputs from plant residues such as litter and fine root biomass, which have a higher decomposition rate than the woodchip (García et al., 1992; Ros et al., 2003; Dearden et al., 2006). In samples in which compost was applied, we observed very high EC, which decreased over time (data not shown). This increase in EC could have resulted from the production of low-molecular-weight organic ions or from the release of salts during the decomposition of organic substances (González-Ubierna et al., 2012; Mingorance et al., 2014), whereas the decrease in EC might have been due to the lixiviation of ions by rainfall, contributing to lower soil salinity (González-Ubierna et al., 2012).

### 4.2. Microbial activity

Microbial activity was generally enhanced by applying organic amendments, particularly compost, as demonstrated by previous studies on degraded soils from semiarid areas (Bastida et al., 2007; Alvarenga et al., 2008; Tejada et al., 2009; Hernández et al., 2014). Increases in TOC and total N have been associated with increases in basal respiration and dehydrogenase activity, both parameters accepted as indicators of total soil microbial activity (García et al., 1997; Bastida et al., 2006). Basal soil respiration was only sensitive to the application of amendments, particularly compost, which was similar to that of the reference natural soil. On the other hand, dehydrogenase activity was much higher in the combination of

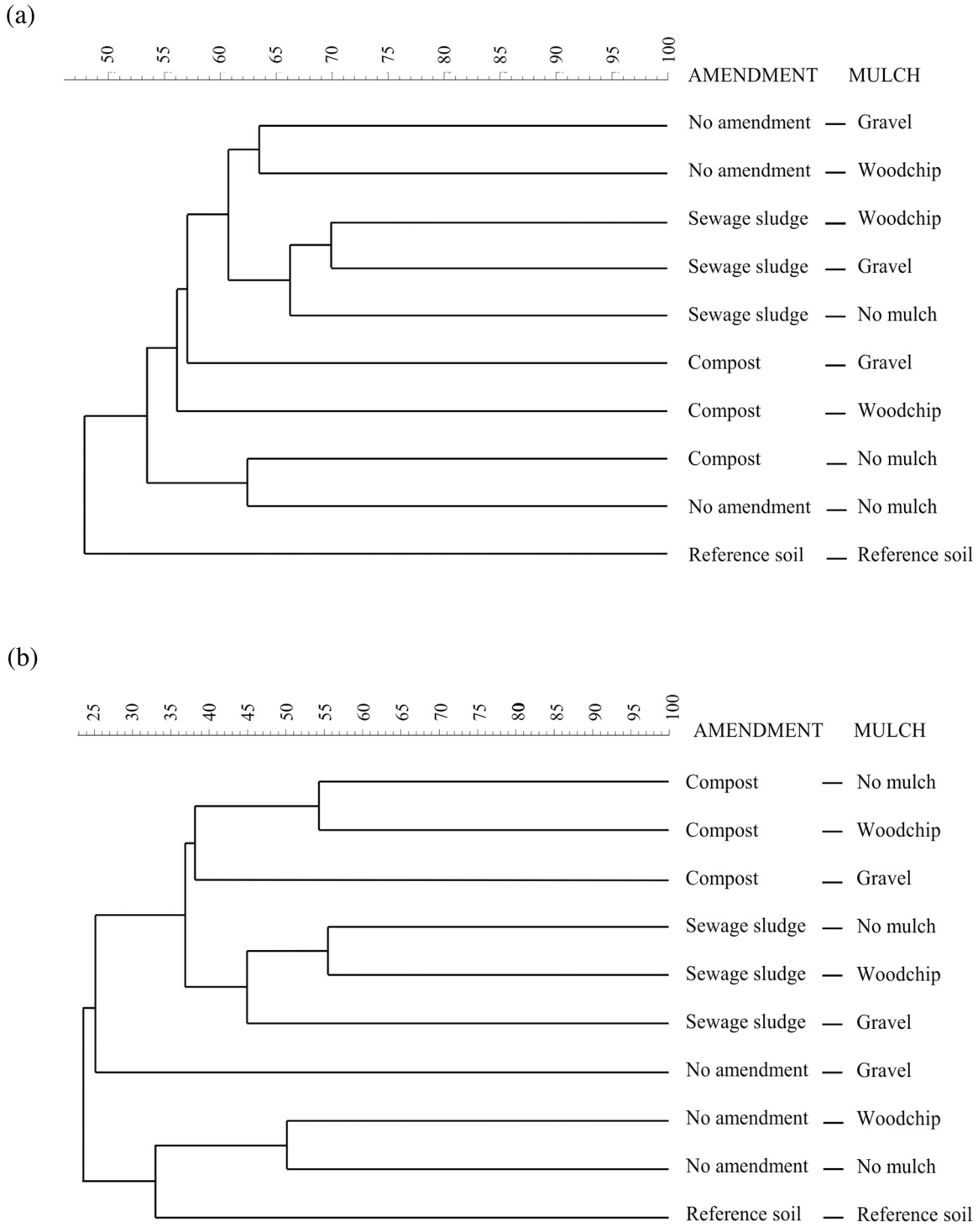


**Fig. 3.** Dendrogams constructed using Dice correlations index and UPGMA clustering of each of bacterial (a) and fungal (b) community DGGE banding patterns in soil samples.

organic amendments and gravel mulch. This fact may be correlated to an increase of plant cover under gravel mulch, as observed by other authors (Masciandaro et al., 2004; Mukhopadhyay et al., 2016). Organic amendments increased the enzyme activities (β-glucosidase, alkaline phosphatase and urease) related to the biogeochemical cycles of the elements in soil (Pascual et al., 1997; Ros et al., 2003; Bastida et al., 2007; Tejada et al., 2009; Santos et al., 2014). Mulches had a negative effect on the glucosidase activity of the amended soils five years after application, suggesting soil quality degradation over time because mulches can prevent inputs of organic matter caused by the barrier created by the mulch itself (Qiu et al., 2014). Depending of the combination of mulches with organic amendments, different effects on the alkaline phosphatase and urease activities were observed. Certain combinations had a positive effect, but in some cases the effect was negative. Therefore, mulch application may not provide the expected beneficial effects on soil microbial activity. Although our findings are based on one specific enzyme for each element cycle and it is known that the evaluation of soil quality is complex (Bastida et al., 2008; Schloter et al., 2003; Nannipieri et al., 2003),

**Table 4**
Summary of ANOSIM analyses based on the Bray–Curtis similarity matrix. In the one-way ANOSIM, the ten groups were analysed independently (nine experimental treatments and reference soil), whereas only the two factors (organic amendments and mulches) were analysed by a two-way crossed ANOSIM.

| | Bacteria | | Fungi | |
|---|---|---|---|---|
| | Sample statistic R | *P* value | Sample statistic R | *P* value |
| ANOSIM (one-way) | | | | |
| Global test | 0.8581 | 0.0001 | 0.9878 | 0.0001 |
| ANOSIM (two-way crossed) | | | | |
| Amendment | 0.99177 | 0.0001 | 1 | 0.0001 |
| Mulch | 0.94513 | 0.0001 | 0.99726 | 0.0001 |

all enzyme activities were positively correlated with TOC and TN content, suggesting that the compost amendment, in which these two parameters were the highest, produced a soil with enhanced productivity and fertility. It is noteworthy that mulch generally exerted a significant negative effect on enzyme activities related to C and P (β-glucosidase and alkaline phosphatase, respectively), whereas microbial biomass was not significantly affected by mulch type. Hence, given that these enzymes are frequently immobilized in clay and humic fractions (Nannipieri, 2006; Bastida et al., 2012), it can be suggested that mulching has a stronger impact on extracellular environment than on microbial growth.

### 4.3. Microbial biomass (PLFA)

Organic amendments had a strong effect on PLFA content, stimulating bacterial and fungal proliferation, as demonstrated by other authors (Marschner et al., 2003; Bastida et al., 2008; García-Orenes et al., 2013; Lazcano et al., 2013). PLFA profiles were positively correlated with the TOC, total N and total P contents. In this study, the microbial PLFA content in samples treated with compost was significantly higher than that treated with sludge and no-amended plots, which could be related to the higher organic matter content in plots amended with compost, as well as the stimulation of plant development that provides organic matter inputs to soil (Bastida et al., 2008). However, mulch treatments did not affect the PLFA content. This trend was also observed by Marschner et al. (2003), who reported that changes in microbial community composition were not accompanied by changes in soil enzymatic activity. Nevertheless, not only variations in microbial biomass but also changes in community structure and composition (as shown by genetic fingerprinting or fatty acid ratios) might influence enzyme activity (Kandeler et al., 1996; Bausenwein et al., 2008; Chang et al., 2014).

Compost increased microbial biomass and altered community composition towards a more fungi-dominated community. Fungi are capable of degrading more recalcitrant material (Boer et al., 2005); thus, an increase in fungal biomass in compost-amended soils could be attributed to the presence of stabilized substrates giving a competitive advantage to fungi. Moreover, the greater development of plant cover in compost-treated soils could contribute cellulose and lignin inputs to soil. It is known that fungi are capable of degrading these carbon polymers through their enzymatic systems (Boer et al., 2005; Baldrian et al., 2010). Bacterial PLFAs and the $Gram^+/Gram^-$ ratio were increased in amended plots, reaching values similar to those observed in reference undisturbed soils. $Gram^+$ bacteria communities are more resistant to drying/rewetting than $Gram^-$ bacteria because of their physiological characteristics, i.e., the presence of a strong, thick and interlinked peptidoglycan cell wall (Schimel et al., 2007). Indeed, the $Gram^+/Gram^-$ ratio has been suggested as an indicator of resistance to perturbations in microbial communities (De Vries and Shade, 2013). A change towards a more dominant Gram+ microbial community (high values of $Gram^+/Gram^-$) can be viewed as a mechanism for adapting to a semiarid climate. Sludge-amended soils also showed higher concentrations of actinobacteria PLFAs, which can likely be attributed to the high actinobacterial content commonly found in wastewater treatment plants (Bitton, 2005; Wang et al., 2014). However, the content and proportion of microbial biomass, both in restored soils and reference soils, may have changed from the start of the experiment because soil microorganisms respond to seasonal changes and vegetation (Bastida et al., 2007; Baldrian et al., 2010).

### 4.4. Diversity and composition of soil microbial communities

Our study showed clear differences in the structures of the bacterial and fungal communities according to the different combinations of organic amendments and mulches. Richness and two diversity indices (Shannon-Weiner and Simpson) indicated that the bacterial community was consistently more diverse in unamended soils than in natural reference soils or amended soils. The addition of an organic substrate to the amended soils could contribute to the selection of copiotrophic microbial populations (Bastida et al., 2015a) and hence decrease the diversity in this treatment. Other studies have shown that poor pore connectivity in soil and low water potential contribute to high bacterial diversity (Carson et al., 2010). Natural undisturbed soil showed the lowest Shannon-Weiner index value, indicating a stable bacterial community. It was hypothesized by Garbeva et al. (2004) that in a stable system each microhabitat is occupied by organisms that are best adapted to colonize that niche, playing an active role in biochemical processes of soil. Soil harbours an extremely rich microbial community (Torsvik and Øvreås, 2002) with a relatively small number of predominant species and a plethora of microbial species present in low number or in a reversible state of dormancy, ready to be triggered into activity by changes in substrate availability (Elshahed et al., 2008). The relationship between the diversity and functionality of the soil microbial communities remains controversial. The redundancy of microbial processes can explain why variations of microbial diversity are not always associated with changes in functionality (Wertz et al., 2007). Indeed, it has been observed that restored soils or soils with a higher quality than degraded soils show higher microbial activity, which is not necessarily linked to higher microbial diversity (Bastida et al., 2013b, 2015b). We found differences among the microbial communities in soils amended with compost, sludge and unamended soils, in contrast with other authors who found no differences (Crecchio et al., 2001; Ros et al., 2006). It should be stressed, in fact, that the bacterial species in undisturbed natural soils were far different from those observed in unamended and amended mine-soils.

The cluster analysis of fungal DGGE patterns showed fundamental variations with different organic amendments, revealing that fungi are sensitive to organic fertilizers. These results suggest that the increase in fungal biomass (observed by PLFA analysis) occurred simultaneously with a change in fungal community structure. New fungal species, which were very different from those observed in both unamended and undisturbed natural soils, thrived with organic treatments. This effect could be attributed to the presence of an external carbon source (Swer et al., 2011). Nevertheless, gravel mulch had a stronger effect than the other mulch treatment on the fungal community in every amendment treatment. Changes in physical and chemical soil properties caused by gravel mulch could have generated changes in fungal diversity (Spedding et al., 2004; Hartmann et al., 2014). Overall, our results suggest that plant cover and the new physical-chemical soil conditions created by the restoration treatments may have strongly influenced the richness and diversity, as well as the

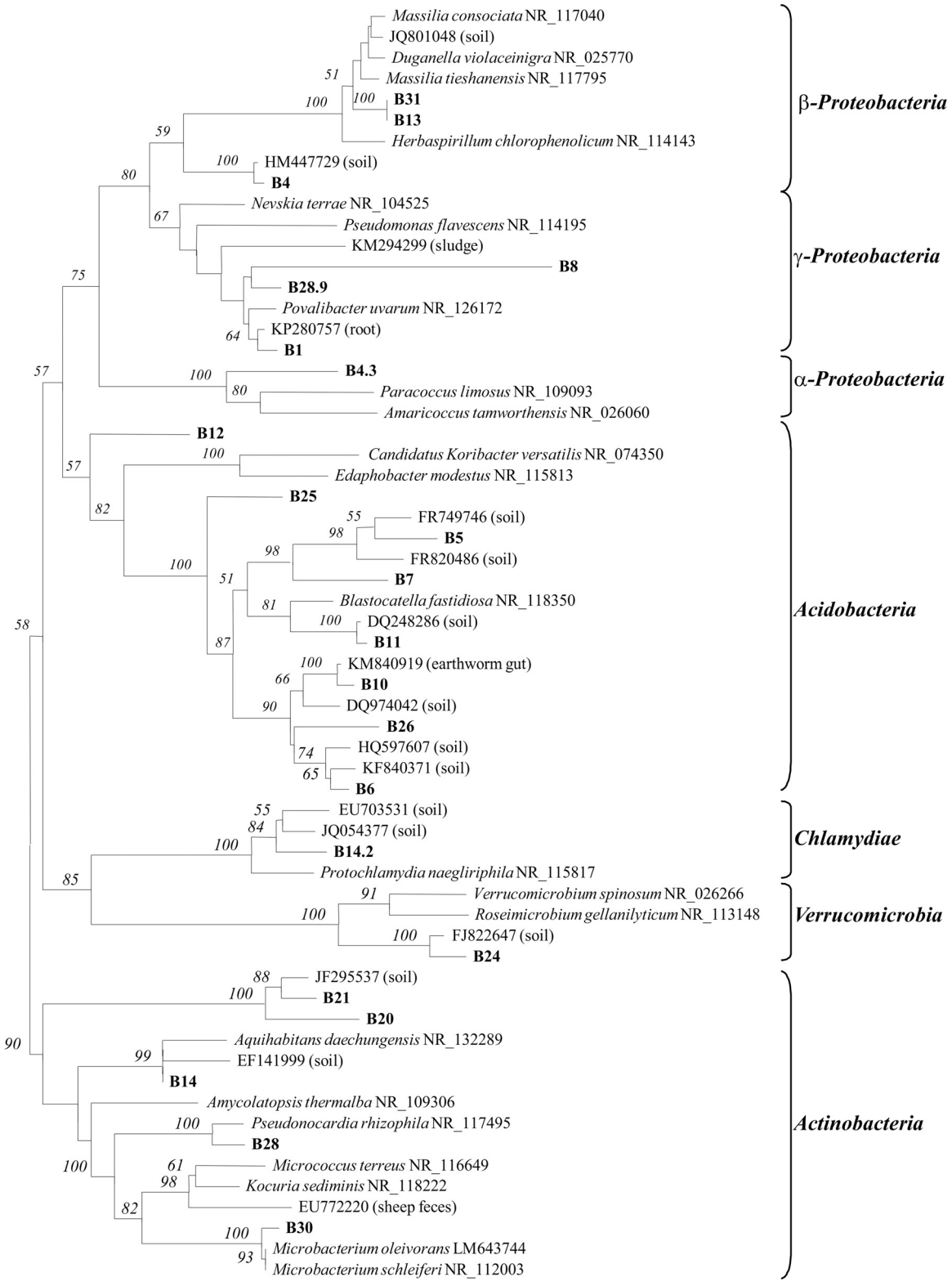


**Fig. 4.** Neighbour-joining tree for 16S rDNA PCR products sequenced. Sequences found in this study are shown in boldface. Sequences of 10 uncultured soil bacteria, 2 other uncultured environmental bacteria and 23 reference strains of α – *Proteobacteria* (2), β – *Proteobacteria* (5), γ – *Proteobacteria* (3), *Firmicutes* (2), *Verrucomicrobia* (2), *Acidobacteria* (2) and *Actinobacteria* (7) available in the GeneBank database are also included, and their accession numbers are given. Numbers at nodes represent bootstrap values after 1000 replicates. Values below 50% are omitted.

composition, of resident microbial communities by changing the abundance of particular members (Yang et al., 2003; Marschner et al., 2003). In particular, the external inputs of organic matter through organic amendments or even hypothetically through residues released from plant cover play a crucial role in shaping these soil microbial communities.

Sequenced clones related to various bacterial and fungal classes, mainly *Proteobacteria*, *Acidobacteria*, *Actinobacteria*,

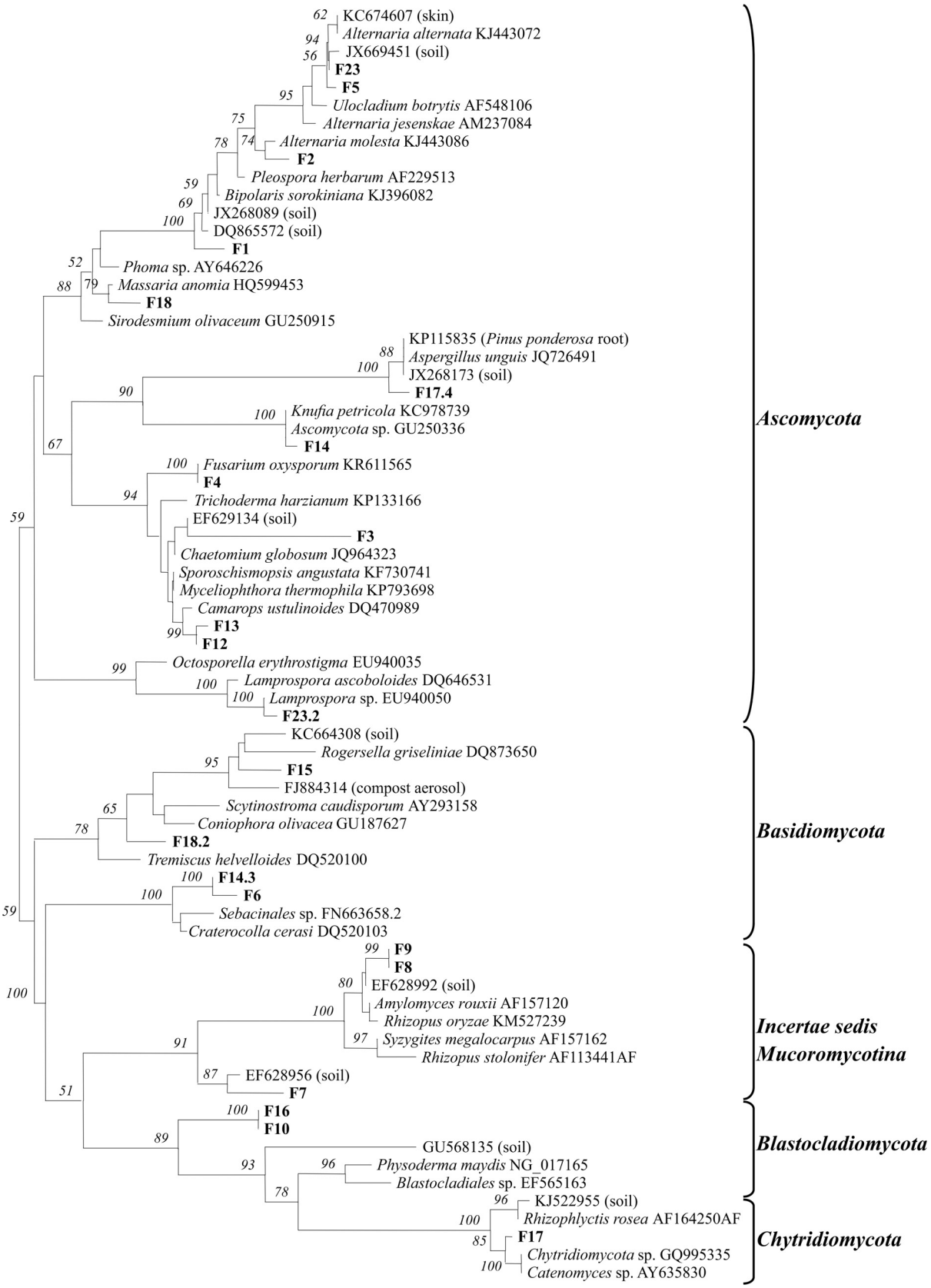


**Fig. 5.** Neighbour-joining tree of 18S rDNA PCR products sequenced. Sequences found in this study are shown in boldface. Sequences of 11 uncultured soil fungi, 2 other uncultured environmental fungi and 36 reference strains of *Ascomycota* (21), *Basidiomycota* (6), *Blastocladiomycota* (2), *Chytridiomycota* (3) and *Mucoromycotina* (4) available in the GeneBank database are also included, and their accession numbers are given. Numbers at nodes represent bootstrap values after 1000 replicates. Values below 50% are omitted.

*Ascomycota* and *Basidiomycota* phyla, suggest potential high taxonomic diversity in the microbial soil communities. The majority of bacterial clones were related to *Acidobacteria* subgroup 4, whereas sequences related to *Ascomycota* were the most abundant in the 18S-DGGE bands sequenced. These two lineages, along with *Proteobacteria* and *Actinobacteria*, are considered dominant in soils and are also widely described in soil with low nutritional content and in semiarid climates (Neilson et al., 2012; Wang et al., 2012; Bastida et al., 2013b). In particular, *Acidobacterium* has been suggested to be correlated to a low nutritional soil status or a strong presence of recalcitrant substrates (Torsvik and Øvreås, 2002). *Acidobacteria*, almost unknown prior to rDNA sequence-based surveys, is now considered a vast group of bacteria, presumably ubiquitous in soil environments (Barns et al., 1999). Moreover, the *Acidobacteria* subgroups have been shown to be strongly affected by soil pH in different ways, and subgroup 4 is confirmed to be positively correlated to high pH (Jones et al., 2009; Rousk et al., 2010).

## 5. Conclusions

This study provides a snapshot of relevant microbiological and biochemical soil properties five years after the beginning of a restoration process, confirming the benefits of low-cost restoration strategies, such as adding organic amendments (compost and sewage sludge) and mulches (gravel and woodchip) in quarrying areas under a semiarid climate. As expected, organic amendments combined or not combined with mulches improved soil functionality and microbial biomass, particularly the compost treatment, which showed the closest values to those of the surrounding reference soils. However, increases in soil functionality and microbial biomass were not associated with changes in microbial diversity. Genetic fingerprinting showed different bacterial and fungal community compositions in every restoration treatment and in surrounding undisturbed soils. Despite the proven advantages of these soil restoration strategies, the results indicate that five years after the restoration setup is still not enough for a compositional and functional convergence in microbial communities between restored and undisturbed soil. Further studies are still needed to draw rigorous conclusions. In particular, because soil microbial communities are strongly affected by environmental changes and climate fluctuations (Fierer and Jackson, 2006), much effort should be dedicated to using replicated plots in a larger area and to periodical monitoring of both microbiological and biochemical soil properties.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.05.006.

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