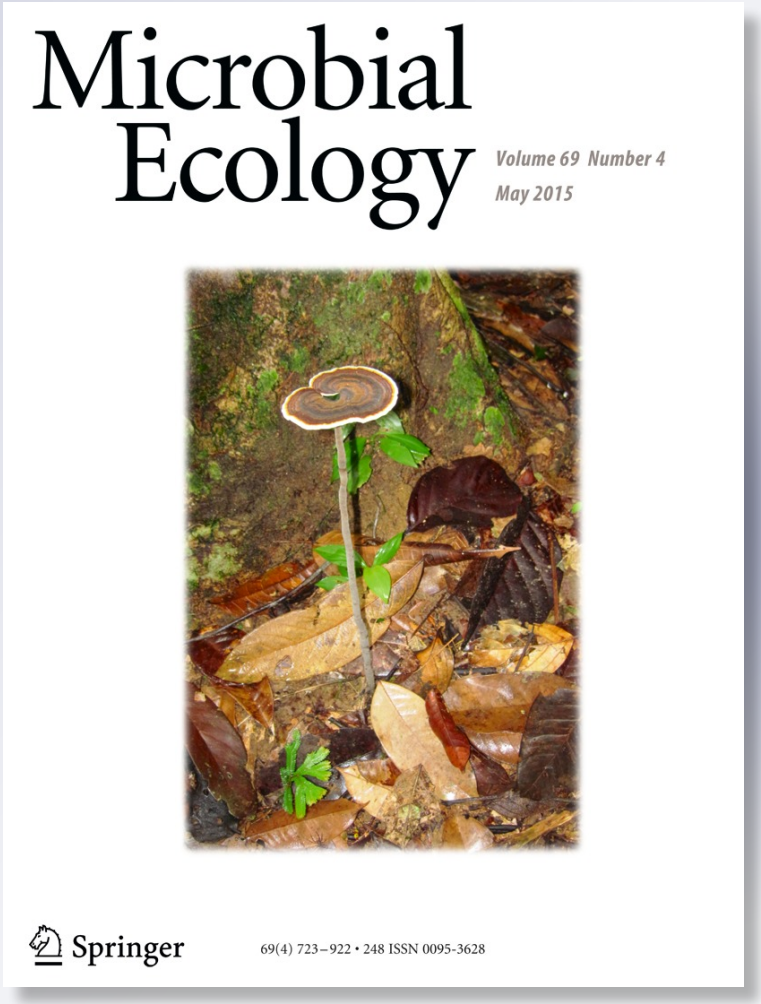
Metagenomic Assessment of the Potential Microbial Nitrogen Pathways in the Rhizosphere of a Mediterranean Forest After a Wildfire

José F. Cobo-Díaz, Antonio J. Fernández-González, Pablo J. Villadas, Ana B. Robles, Nicolás Toro & Manuel Fernández-López

Microbial Ecology

ISSN 0095-3628
Volume 69
Number 4

Microb Ecol (2015) 69:895-904
DOI 10.1007/s00248-015-0586-7



Springer

Microb Ecol (2015) 69:895–904
DOI 10.1007/s00248-015-0586-7

ENVIRONMENTAL MICROBIOLOGY

Metagenomic Assessment of the Potential Microbial Nitrogen Pathways in the Rhizosphere of a Mediterranean Forest After a Wildfire

José F. Cobo-Díaz · Antonio J. Fernández-González · Pablo J. Villadas · Ana B. Robles · Nicolás Toro · Manuel Fernández-López

Received: 29 October 2014 / Accepted: 16 February 2015 / Published online: 3 March 2015

Abstract Wildfires are frequent in the forests of the Mediterranean Basin and have greatly influenced this ecosystem. Changes to the physical and chemical properties of the soil, due to fire and post-fire conditions, result in alterations of both the bacterial communities and the nitrogen cycle. We explored the effects of a holm oak forest wildfire on the rhizospheric bacterial communities involved in the nitrogen cycle. Metagenomic data of the genes involved in the nitrogen cycle showed that both the undisturbed and burned rhizospheres had a conservative nitrogen cycle with a larger number of sequences related to the nitrogen incorporation pathways and a lower number for nitrogen output. However, the burned rhizosphere showed a statistically significant increase in the number of sequences for nitrogen incorporation (allantoin utilization and nitrogen fixation) and a significantly lower number of sequences for denitrification and dissimilatory nitrite reductase subsystems, possibly in order to compensate for nitrogen loss from the soil after burning. The genetic potential for nitrogen incorporation into the ecosystem was assessed through the diversity of the nitrogenase reductase enzyme, which is encoded by the *nif*H gene. We found that *nif*H gene diversity and richness were lower in burned than in undisturbed rhizospheric soils. The structure of the bacterial communities involved in the nitrogen cycle showed a statistically significant increase of *Actinobacteria* and *Firmicutes* phyla after the wildfire. Both approaches showed the important role of gram-positive bacteria in the ecosystem after a wildfire.

Keywords Metagenomics · Microbial communities · Nitrogen cycle · Rhizosphere · Wildfire · Mediterranean forest

Introduction

As occasional natural events, wildfires probably influenced the vegetation of the Mediterranean Basin before the arrival of humans [1]. Their effect, in combination with regular drought, high temperatures, and grazing over thousands of years, shaped the development of a specific type of adapted vegetation [2]. This is the case for the holm oak (*Quercus ilex* subsp. ballota), which displays regrowth after fires. Holm oak formations cover a total area of 9.66×10^6 ha in Spain, but they rarely form entire forests, due to their management as a pastoral woodland called *dehesa* [3], and the effects of fire and clear-cutting. The area affected by wildfires in Spain reached an annual mean of 127,209 ha in the period 2000–2009, and the affected landscapes included shrubs, forests, and herbaceous formations [http://www.magrama.gob.es/es/biodiversidad/temas/defensa-contra-incendios-forestales/estadisticas-de-incendios-forestales/]. These fires included a wildfire in the Sierra Nevada National Park in South East

Electronic supplementary material The online version of this article (doi:10.1007/s00248-015-0586-7) contains supplementary material, which is available to authorized users.

J. F. Cobo-Díaz · A. J. Fernández-González · P. J. Villadas · N. Toro · M. Fernández-López (✉)
Grupo de Ecología Genética de la Rizosfera, Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, calle Profesor Albareda 1, E-18008 Granada, Spain
e-mail: manuel.fernandez@eez.csic.es

A. B. Robles
Grupo de Pastos y Sistemas Silvopastorales Mediterráneos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, calle Profesor Albareda 1, E-18008 Granada, Spain

Spain in September 2005, which affected 3416.74 ha [4], including 412 ha dominated by holm oaks.

The high temperatures reached during the wildfire cause immediate changes in the physical and chemical properties of the soil, the magnitude of which depends on the fire severity and soil type [5, 6]. These changes, together with the environmental conditions prevailing after the fire, may cause alterations in the soil's biological characteristics, such as microbial biomass and activity [5, 7, 8]. They may also modify the taxonomic structure of the soil's microbial communities, with an increasing proportion of spore-forming bacteria [9–11]. Understanding the processes that contribute to the recovery of the soil and the microbial communities could help to re-establish the plant formations of the burnt area.

Available nitrogen (N) and water are the most common limiting factors in both natural and agricultural ecosystems [11, 12]. Remarkably, after wildfires, an ephemeral flush in mineral N can often be observed [5, 8]. In a recent meta-analysis, Wang et al. [8] concluded that wildfires in Mediterranean forests typically have a positive effect on soil organic carbon (C) and N pools, but a negative effect on N mineralization, increasing the likelihood of N losses from forest ecosystems due to leaching and decreases in microbial activity. The soil bacteria are essential components of the biogeochemical N cycle, but there is only little study on their presence in post-fire environments [13].

The biological incorporation of molecular nitrogen (N_2) in the ecosystem is only possible by means of its fixation by diazotrophic prokaryotes, a reaction carried out by the nitrogenase enzyme that is composed of two subunits: the MoFe protein and the Fe protein. The *nif*H gene encodes the Fe protein, which acts as the nitrogenase reductase, and the high level of conservation of this gene and its presence in all diazotrophic bacteria make it an ideal molecular marker [14]. The molecular analysis of this gene has been used to study nitrogen-fixing bacteria after a forest fire [11], where, despite the overall decrease in microbial biomass, including that of nitrogen-cycling bacteria, the nitrogen-fixing community actually became more diverse within a month of the fire. However, other authors have concluded that the relative richness and evenness of these communities decreases 16 months after various types of fire [15]. Other genes playing a key role in the N cycle have been used as markers for different stages of this biogeochemical cycle. For example, the *nos*Z gene has been used to study denitrification, and the *amo*A gene has been used to study ammonia oxidation [16]. Nevertheless, to our knowledge, no study of the entire N cycle after a wildfire has been carried out before, despite the need to understand the reaction of the ecosystem in terms of the strength of N incorporation pathways after a catastrophic event. However, high-throughput shotgun sequencing has been applied to different environmental samples, in order to characterize the nitrogen metabolism [17–19].

We investigated the dynamics of microbial communities and their potential role in the N cycle within the holm oak rhizosphere 3 years after a wildfire. Bacteria are early indicators of environmental changes, due to the important role they play in various biogeochemical cycles and their sensitivity to environmental changes. Therefore, we carried out a metagenomic analysis of nitrogen metabolism, following the direct pyrosequencing of total soil DNA extracted 3 years after the wildfire. Since we did not detect any structural genes of the nitrogenase enzyme with this technique, we used clone libraries to assess the diversity of the *nif*H gene, to investigate changes in potential nitrogen fixation during the recovery of the ecosystem.

Methods

Experimental Sites

The study area is located in the Sierra Nevada Natural and National Park (SE Spain), where a wildfire in September 2005 burned 3426.74 ha, including 412 ha of evergreen holm oaks (*Q. ilex* subsp. ballota). Soil samples were collected in the valley of the Lanjarón River where three sites were selected: two in areas directly affected by the wildfire (burned forest containing holm oak trees [BOF] and burned bulk soil [BBS] covered by grasses and shrubs) and a nearby site in evergreen, undisturbed oak forest (UOF, Fig. S1).

Three sampling plots were randomly chosen within each study site along transects of 1.0 km length. At the BOF and UOF sites, we sampled the rhizosphere of three trees per plot, each with a diameter of at least 15 cm at breast height and separated by at least 5 m. In the BBS plots, we took an equivalent number of samples from bulk soil. For all sites and plots, sampling took place on April 29, 2008 (3 years after the wildfire). The BBS site was on a terraced slope, whereas the BOF and UOF sites were on a steep slope, all of them south-facing. The soil sampling points and the sampled trees were marked, and the positions of all sites were registered with the global positioning system (GPS, Fig. S1).

Sample Collection and Soil Chemical Analysis

The rhizospheric samples were collected by following the tree's main roots until young; cork-free roots were found at a distance of less than 50 cm from the trunk, where we took soil that was attached to the roots. The soils from the sampling areas were loams, with the exception of the one from the BBS site, which was sandy loam (Table 1). All these soils are classified as haplic phaeozems of siliceous origin. At each sample point, we collected soil at depths of 5 to 25 cm, which we stored immediately at 4 °C until processing. The elimination of the first 5 cm of the soil allowed us to discard minor

Table 1 Soil chemical and physical properties at 5–25 cm depth within sampled areas of unburned holm oak forest (UOF), burned holm oak forest (BOF), and burned bulk shrubland soil (BBS) 3 years after wildfire in Sierra Nevada National Park, South Eastern Spain

Parameter	UOF	BOF	BBS
Clay (%)	21.00	20.50	12.05
Sand (%)	45.74	49.54	56.08
Silt (%)	33.26	29.96	31.87
Type of soil	Loam	Loam	Sandy loam
pH (H_2O)	6.1	7.6	6.5
pH (KCl)	5.7	7.0	6.0
Available water (%)	17.11	16.43	15.20
Salinity (mS/cm)	0.14	0.22	0.08
Organic matter (%)	7.61	4.54	4.54
Total N (%)	0.366	0.233	0.250
C/N ratio	11.95	11.19	10.44
Assimilable Phosphorous (mg/kg)	8	5.2	20
Magnesium (mg/kg)	4.36	7.20	1.48
Calcium (%)	12.65	17.97	8.65

roots from herbaceous plants, which ensured the influence of the tree rhizosphere on the microbial communities. We sieved the soil samples through a 2 mm mesh. Then, we processed 2 kg of soil from each site for physicochemical analysis, including soil type, pH, available water, salinity, total nitrogen, organic matter, assimilable phosphorous, magnesium, and calcium. All physicochemical analyses were carried out using standardized procedures at the Food and Agriculture Laboratory of the Andalusian regional government at Atarfe (Granada, Spain) [http://www.juntadeandalucia.es/agriculturaypesca/portal/agenciaagrariaypesquera/centros/laboratorios/red-de-laboratorios-agroalimentarios-y-estaciones-enologicas/index.html] which methodology is listed in the supplementary material.

DNA Extraction and Deep-Sequencing

Within 24 h after sample collection, we extracted DNA from each soil sample with the PowerSoil DNA Isolation kit (MoBio Laboratories Inc.), following the manufacturer's recommendations. Aliquots of total community DNA were then used for PCR-based analyses of diazotrophic communities. For each sampling site, DNA from the rhizosphere of nine trees of the three plots was pooled in equal amounts to obtain 5 μg. The DNA from BOF and UOF samples was subjected to pyrosequencing with the Roche 454 GS FLX Titanium platform at LifeSequencing SL (Valencia, Spain), on a quarter of a plate in order to obtain *c.* 100 Mb of information from each sample. The raw metagenomic reads were filtered for replicated sequences with the 454 Replicate Filter web tool [20], with a 0.9 sequence identity cutoff, a 0 length difference requirement, and 10 starting base pairs to be checked. The SeqTrim pipeline with default parameters [21] was then used to filter out sequences of low complexity and quality. The trimmed metagenomic sequences for the UOF and BOF sites, with an average length of 387 and 393 bp, respectively, were assigned to SEED subsystem categories and KEGG orthology database on the MG-RAST web server [22], with a maximum e-value cutoff of e^{-10}, a minimum identity cutoff of 60 %, and a minimum alignment length of 50 bp. SEED subsystems and KEGG orthology are databases with a categorization system that classifies gene functional categories into a hierarchy with four levels of resolution, in which the finest level of resolution is the function or orthology protein group, respectively. The sequences from this study are publicly accessible from MG-RAST server under the codes 4465556.3 for UOF and 4465558.3 for BOF. The MG-RAST table format of sequences associated with nitrogen metabolism on SEED subsystems database was transformed to STAMP [23] format, and the metabolic features of the two samples were statistically compared on STAMP software by two-tailed Fisher's exact tests with Newcombe-Wilson confidence intervals and Storey FDR correction, with a minimum p value cutoff of 0.05. Metagenomic sequences associated with the nitrogen metabolism on the KEGG orthology database (ko 00910) were phylogenetically classified by BlastX against the NCBI non-redundant protein database (nr), with a maximum e-value cutoff of e^{-10}, a minimum identity cutoff of 60 %, and a minimum alignment length of 50 bp, taking the closest affiliations with known genera on the basis of sequence similarity. Taxonomical abundances were compared, using the METASTAT web server [24].

*nif*H Gene Amplification, Libraries Construction and Sequence Analysis

Since no sequences of the *nif*H gene were obtained in the metagenomic analysis, we decided to explore the nitrogen fixation pathway by the construction of gene libraries. In this approach, the BBS was used as control, since 3 years after the wildfire, it was covered by the nitrogen-fixing leguminous shrub *Adenocarpus decorticans*. The *nif*H gene was amplified by PCR with the primers described by Widmer et al. [25], by a nested PCR as described by Villadas et al. [26] to obtain a 370 bp amplicon. Gene libraries were generated from an equimolecular mix of the PCR products for the three trees from each plot. In this way, we obtained a representative sample for the plot, thereby minimizing the potential bias associated with the use of a single sample from a single tree. The pooled PCR products were cleaned by centrifugation on Illustra MicroSpin™ S-300 HR columns (GE Healthcare) according to the manufacturer's instructions. These *nif*H fragments were then ligated into the pGEM-T Easy vector (Promega) and used to transform *Escherichia coli* strain

DH5α, according to Villadas et al. [26]. Positive bacterial colonies were selected (at least 50 from each sampling site) on appropriate LB agar plates, and the sizes of the inserts were checked by PCR with T7 and SP6 primers. PCR products of the correct size were sequenced after cleaning by centrifugation on Illustra MicroSpin™ S-300 HR columns. The integrity, size, and quantity of DNA were checked by gel electrophoresis, according to standard procedures. Clones were sequenced by the Sanger method, with an ABI Prism 3130XL Genetic Analyzer, according to the manufacturer's instructions.

Raw sequence data was processed in Sequence Scanner version 1.0. Sequence alignments were generated on the MAFFT 6 server [27], and the distance matrix was obtained with the Phylip dnadist program. MOTHUR [28] software was used to determine the structure of microbial communities and to obtain the number of operational taxonomic units (OTUs; *S*, observed richness) with a sequence similarity threshold of 93 %, the Chao1 index (Chao1, expected richness), and the Shannon index (*H*′, diversity), according to default parameters of MOTHUR software. Pielou index (*J*′, evenness) was calculated using the formula $J'=H'/\ln(S)$, and coverage (*G*) was obtained according to the Good's coverage index ($G=1-n/N$; n=number of singletons, N=number of sequences).

BlastX comparison with the NCBI non-redundant protein database (nr) was performed for each of the *nif*H sequences for the taxonomic assignment, taking the closest affiliations with known diazotrophs based on the sequence similarity with a maximum cutoff of e^{-10}.

Comparative studies, analyzing differences between sequences of the various gene libraries were performed with ∫-LIBSHUFF [29], which can compare more than two libraries simultaneously. We used the statistical tool Ginkgo [30] to compare libraries by unweighted pair group method with arithmetic mean (UPGMA) clustering, with the Euclidean distance matrix of the relative abundance of each OTU in each sample. All phylogenetic assignments and abundances of bacterial groups presented in the results are based on the similarity of the proteins involved in the nitrogen cycle.

The sequences of the partial *nif*H clone libraries were deposited in the NCBI database under accession numbers KC667152 to KC667559, with the exception of the BOF library whose accession numbers are KC551480 to KC551532.

Results

Physicochemical Properties of the Soils

One of the first impacts fire has on the ecosystem is the modification of the physical and chemical characteristics of the soil, leading to other long-term changes. Our study was conducted 3 years after the wildfire; therefore, the intensity of the impact on the soils could have been modified by other environmental factors. However, even after 3 years, the soil was more alkaline at the BOF site (pH 7.6) than at the other sites, which were slightly acidic (pH 6.1 at UOF and pH 6.5 at BBS; Table 1). Moreover, the area of burned holm oaks (BOF) had the highest salinity (0.22 mS/cm), the highest contents of magnesium (7.2 mg/kg) and calcium (17.97 %), and the lowest assimilable phosphorus concentration (5.2 mg/kg), whereas the other soil affected by the wildfire (BBS) had the lowest salinity (0.08 mS/cm) and the highest assimilable phosphorus concentration (20 mg/kg). Total nitrogen and organic matter contents were lower in the soils affected by the wildfire (BOF and BBS) than in the unburned soil (UOF); however, the C/N ratios were similar in the three soils (Table 1).

Metagenomic Analysis of the Nitrogen Cycle

The DNA obtained from the rhizospheres of burned (BOF) and non-burned (UOF) holm oak in the spring of 2008, 3 years after the wildfire, was pyrosequenced. In total, 316,973 reads were obtained from the BOF soil and 520,430 from the UOF soil, yielding 257,697 and 412,302 sequences after trimming, respectively. The mean length of the trimmed sequences for the BOF soil was 393 nucleotides, yielding 80 Mb of information. For the UOF soil, the mean length of the trimmed sequences was 387 nucleotides, yielding 160 Mb of information. Using the MG-RAST web server, we obtained 115,406 features assigned to identified functional categories for the BOF soil and 171,602 for the UOF soil. A heatmap comparison of both metagenomes showed no statistically significant differences at the level of metabolic groups (data not shown). At the subsystem level 1 (functional groups), only 1007 of the BOF sequences and 1515 of the UOF sequences were related to proteins involved in N metabolism. An analysis of the various functions involved in this process showed that at subsystem level 2 (N metabolism), more than 52 % of the sequences were related to ammonia assimilation (Fig. 1) at both sites, with sequences related to nitrate and nitrite ammonification the next most frequent (around 16 %). The functions classified by MG-RAST in the SEED subsystem level 3 database included nitric oxide synthetase and nitrosative stress, which comprised around 9.1 and 0.65 % of the total number of sequences per sample, respectively. Nitrogen fixation accounted for 20 sequences (1.33 %) in the UOF and 23 sequences (2.36 %) in the BOF. Remarkably, none of the identified N_2 fixation proteins displayed any similarity to NifH in either of the two rhizosphere types. Instead, all the identified sequences displayed similarity with regulators, such as those encoded by *nif*A, *vnf*A, or *ntr*C. There were no statistically significant differences between sites in the aforementioned functions; in contrast, the proportion of sequences involved in denitrification differed significantly between sites, accounting for

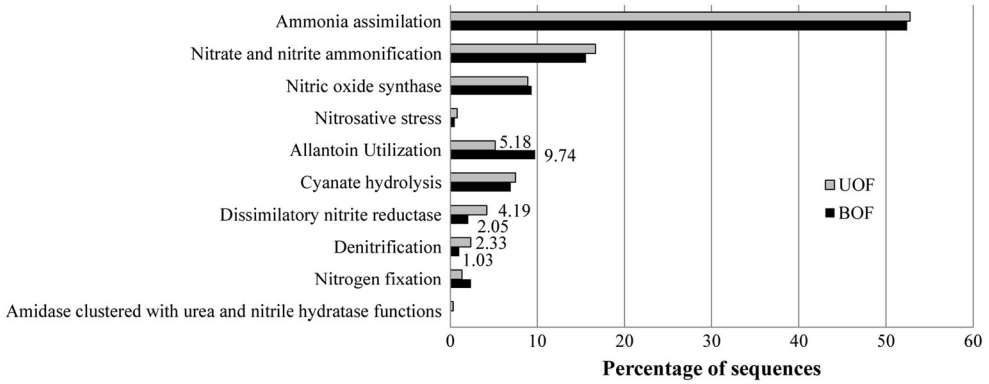


Fig. 1 Percentage of nitrogen metabolism sequences, by function, according to the subsystem classification of the MG-RAST web server. *Black bars* correspond to the metagenome of the burned holm oak rhizosphere (BOF) and *gray bars* to the metagenome of undisturbed (UOF) rhizosphere. *Numbers beside bars* indicate the percentage of proteins corresponding to the function in each sample when significantly ($p<0.05$) different between burned and non-burned rhizospheres

2.33 % of the sequences at the UOF and only 1.03 % at the BOF site (Fig. 1). Dissimilatory nitrite reductase was represented in a higher proportion of sequences in the UOF (4.19 %) compared to the BOF soil (2.05 %; $p<0.01$), whereas allantoin utilization was better represented in the BOF (9.74 %) compared to the UOF soil (5.18 %; $p<0.01$; Fig. 1).

The phylogenetic assignment of the bacterial communities based on the similarity of the proteins involved in the nitrogen cycle showed that more than 80 % of all proteins were related to bacteria of the phyla *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (Fig. 2a). When the phyla *Acidobacteria*, *Firmicutes*, and *Planctomycetes* were included, the percentage of the sequences reached around 90 %, with only 5 % of proteins unassigned to any bacterial taxa. Of these phyla, only *Actinobacteria* and *Firmicutes* were more abundant in the BOF rhizosphere than in the UOF ($p<0.05$) 3 years after the wildfire (Fig. 2a). The phyla *Bacteroidetes* and *Proteobacteria* were the more abundant in the unburned UOF site than in the burned site, although the difference between the UOF and BOF sites was not statistically significant. The analysis at the genus level, with a cutoff above 2 % of the total sequences, showed that there were four genera, which significantly increased in abundance after the wildfire: *Arthrobacter*, *Bacillus*, *Blastococcus*, and *Spirosoma* (Fig. 2b).

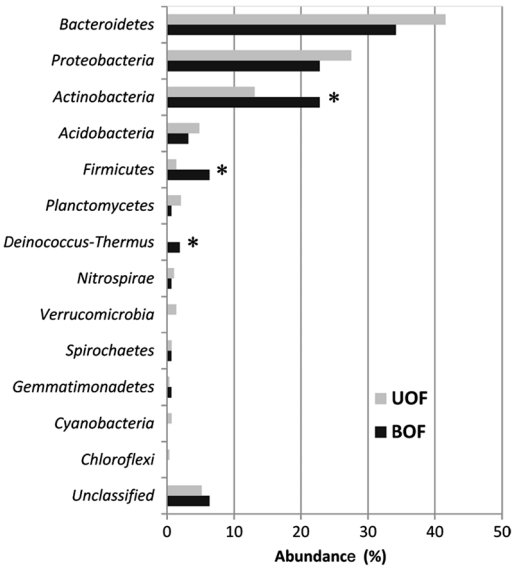
Analysis of Diazotrophic Communities

In total, 154 partial sequences (370 bp) of the *nif*H gene were obtained, with a minimum number of 50 sequences per sample. This number of sequenced clones was sufficient to obtain a representative assessment of *nif*H gene diversity based on rarefaction curves (Fig. S2) and a coverage of between 84 % for the UOF soil and 94 % for the BBS soil (Fig. S2). The diazotrophic community of BBS was more similar to that of UOF, whereas the presence of burned trees in the BOF resulted in a more differentiated diazotrophic community. Agglomerative hierarchical clustering analysis of the abundances of each OTU showed that BBS and UOF communities were separated by a distance of 46 % while the BOF community was joined, to the BBS and UOF branch, at a distance of 62 % (Fig. S3). ∫-LIBSHUFF analysis showed that all samples differed from one another, although the diazotrophic community in the BOF soil could be regarded as a subsample of the UOF community (p value=0.0176).

The UOF soils had the highest number of OTUs, with a richness of 17 OTUs at 93 % similarity, whereas richness was lowest for BOF ($S=9$) and intermediate for BBS ($S=13$, Fig. 3a). The value of the Chao1 richness estimator was higher for the UOF rhizosphere (26) than for the samples from the burned sites (14). The diversity, as measured by Shannon's index, was higher for the unburned than for the burned soils, but the diversity in the BBS soil ($H'=2.24$) was similar to the UOF soil ($H'=2.49$), whereas BOF showed the lowest diversity ($H'=1.23$; Fig. 3b). Pielou's evenness index also was equal for UOF and BBS soils ($J'=0.87$ for both sites) and lowest ($J'=0.56$) at the BOF site where the effect of the fire was most intense (Fig. 3b).

Three years after the wildfire, the diazotrophic community at the rhizosphere of burned holm oaks was dominated by *Azospirillum*, the burned soil with the legume *A. decorticans* was dominated by the genus *Rhizobium*, whereas in the unburned rhizosphere, there was a mixture of five nitrogen-fixing genera of the *Proteobacteria*. Protein sequence analysis showed that most of the NifH sequences were from phyla *Proteobacteria* and *Firmicutes* in all the sites. These two phyla accounted for 100 % of the sequences from the BOF site (Fig. 3c) while at UOF and BBS sites around 2 % of the sequences belong to other phyla. The main difference among sites was the high proportion of *Firmicutes* phylum (15.09 %) in the BOF soil compared with the 7.84 % in the unburned soil and 4.00 % in the UOF soil. Detailed NifH sequence comparisons at the genus level showed that at the BBS site, where a leguminous shrub was predominant, the most abundant genera were *Rhizobium*, *Methylobacterium*, and *Bradyrhizobium* (Table 2); these genera of the

a



b

Phylum	Genus	UOF	BOF
Bacteroidetes	*Niastella*	11.34	10.13
Proteobacteria	*Bradyrhizobium* *	7.22	1.27
Bacteroidetes	*Flavobacterium*	5.84	2.53
Actinobacteria	*Arthrobacter* *	0.00	6.96
Bacteroidetes	*Chitinophaga*	1.72	4.43
Bacteroidetes	*Mucilaginibacter*	2.75	2.53
Actinobacteria	*Mycobacterium*	1.37	3.16
Bacteroidetes	*Pedobacter* *	3.44	0.63
Bacteroidetes	*Solitalea*	2.75	1.27
Proteobacteria	*Burkholderia*	2.41	1.27
Bacteroidetes	*Niabella*	2.41	1.27
Firmicutes	*Bacillus* *	0.34	3.16
Actinobacteria	*Streptomyces*	2.06	1.27
Actinobacteria	*Blastococcus* *	0.00	2.53
Bacteroidetes	*Spirosoma* *	0.00	2.53

Fig. 2 Bacterial taxa involved in the metabolism of the nitrogen cycle (metagenomic sequences associated to ko00910, nitrogen metabolism, of KEGG orthology database). **a** Percentage of the bacterial phyla in the burned (BOF, *black bars*) and in the undisturbed (UOF, *gray bars*) rhizospheres. **b** Relative abundance of those bacterial genera with a proportion higher than 2 % of the nitrogen metabolism sequences of its respective metagenome. *Asterisks* represent a statistically significant ($p<0.05$) difference between burned and undisturbed rhizospheres

Proteobacteria are well-known nitrogen fixers in symbiosis with legume plants. The abundance of *Rhizobium* was significantly higher ($p=0.01$) at the BBS site, compared to BOF and UOF sites where its abundance was very low, whereas the genus *Bradyrhizobium* was present in the UOF and BBS but absent in the BOF site. In the soils from the BOF site, the characteristic genera were *Azospirillum* (*Proteobacteria*) and *Paenibacillus* of the *Firmicutes*, but only the first was significantly more abundant compared to the other sites. The unburned UOF site had the highest diversity, but there was no clear predominance of one genus, with a roughly equal presence of *Methylobacterium*, *Azospirillum*, *Bradyrizobium*, *Rhodopseudomonas*, and *Geobacter*.

Discussion

Although the ratios of carbon to nitrogen (C/N) of the soils were similar at 5–25 cm depth, the effect of the wildfire on the soil was observed in the reduced concentrations of total nitrogen (N) and C in the soils (Table 1) from the burned sites (BOF and BBS). The similar C/N ratios of burned and unburned soils contrasts with other studies reporting an increase [31, 32] or decrease [33, 34] in the C/N ratio of the soil after burning. Nevertheless, since our analysis was made 3 years after the fire, this similar C/N ratio could be an indication of ecosystem recovery. Moreover, our results are consistent with those of previous studies reporting decreases in organic matter content and total N in burned soils in the years following the fire [7, 8, 35]. However, the effect of the fire on the other analyzed parameters is not so clear, since pH in BBS (non-rhizospheric burned bulk soil) is similar to that of UOF but with an alkalinization at the BOF site (Table 1). This increase of pH may be due to the presence of burned holm oak trees, according to other authors [15, 35]. Therefore, 3 years after the wildfire, the main soil factors that influenced the holm oak rhizospheric communities are pH and the decreased content of C and N.

The metagenomic analyses, as an indicator of the potential pathways of the nitrogen cycle, showed no differences between the burned and the unburned rhizospheres at the level of metabolic groups, including that of nitrogen metabolism. However, the detailed examination of this function showed that at the subsystem level 3 (functions of the nitrogen cycle), there were some statistically significant differences among sites. Thus, in the burned rhizosphere, with 0.233 % total N concentration, there were relative decreases of the dissimilatory nitrite reductase and the denitrification functions and an increase of allantoin utilization (Fig. 1), compared to the unburned rhizosphere, with 0.366 % total N. The main functions identified in both rhizospheres were ammonia assimilation, and the ammonification of nitrate and nitrite, which, together with cyanate hydrolysis, allantoin utilization, and N_2 fixation, reflect the pathways of N incorporation to the ecosystem by the bacterial communities. However, the potential N cycle in the BOF rhizosphere can be seen as conservative because the percentage of sequences related to allantoin utilization (N influx to the metabolic pathways) was higher than in UOF; moreover, the percentage of sequences for denitrification and dissimilatory nitrite reductase functions (N efflux) was lower in BOF than in UOF (Fig. 1). Our data shows that at the BOF site, the N metabolism of the rhizospheric community is likely to limit losses of nitrogen with an increased potential pathway of N incorporation from plant origin, such

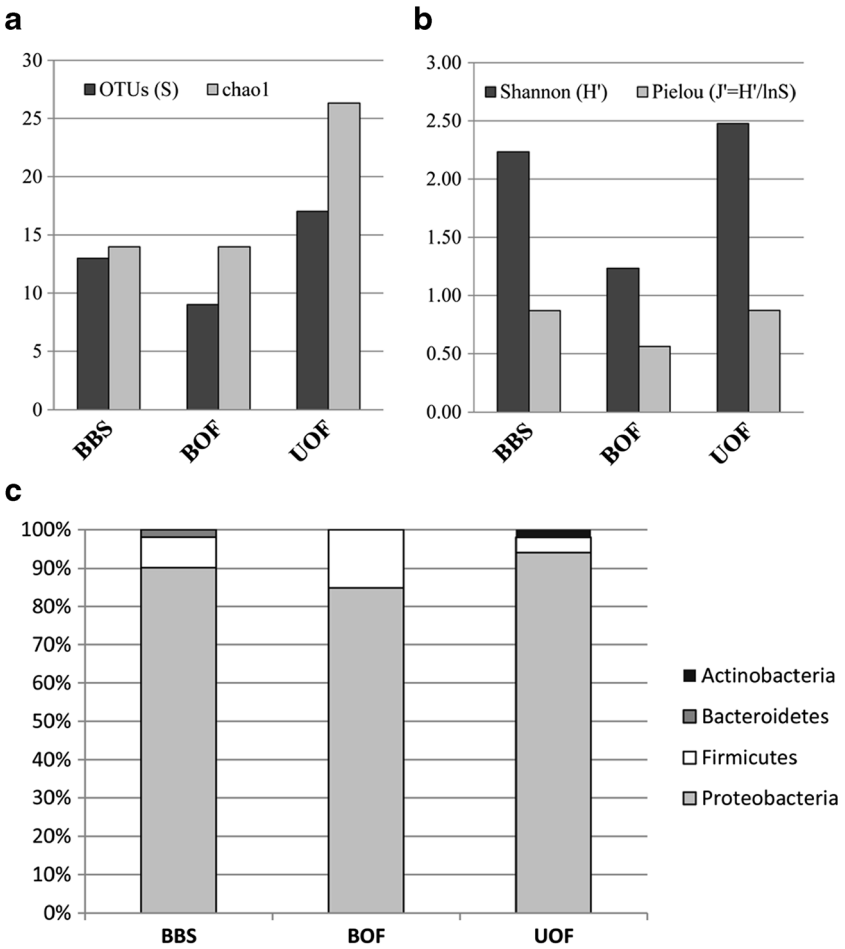


Fig. 3 Ecological indices and proportion of bacterial phyla for the *nif*H clone libraries. The *names below the bars* correspond to the sampled rhizosphere undisturbed holm oak forest (UOF), rhizosphere of burned holm oak forest (BOF), burned bulk soil (BBS). **a** Observed OTUs and Chao1 index of estimated richness. **b** Shannon-Wiener index of diversity and Pielou index of evenness. **c** Relative abundance of NifH sequences from phyla detected in the sampled sites. The phyla considered are *Proteobacteria* (*light gray bar*), *Firmicutes* (*white bar*), *Bacteroidetes* (*dark gray bar*), and *Actinobacteria* (*black bar*). *OTUs* operational taxonomic units defined by a 93 % DNA similarity cutoff for the *nif*H gene

as allantoin. The most obvious functions involved in N losses from the rhizosphere of unburned holm oak are the higher levels of denitrification pathway and the dissimilatory nitrite reductase activity, together with the nitrosative stress and nitric oxide synthase. Nitric oxide (NO) is involved in all of these pathways of N losses, since it is formed not only as an intermediate step during denitrification but also as a signaling and defense molecule of major importance [36]. These functions show that large losses of N are likely to occur via gaseous emissions. Goberna et al. [37] concluded that immediately after a prescribed fire, the biogeochemical cycling in Mediterranean shrublands becomes less conservative, due to an increase in microbial biomass and activity and changes in the structure of the bacterial community. In our study, the analysis was performed 3 years after the wildfire, in a context of possible N loss by volatilization and leaching. Thus, the microbial communities of burned rhizospheres tend to incorporate N from alternative N sources, as shown by the higher abundance of sequences related to allantoin utilization and nitrogen fixation. It is important to note that allantoin in soil is derived from eukaryotes, possibly from the burned wood, which would stimulate bacteria versus fungi and gram-positive versus gram-negative bacteria [38]. In general, the number of sequences of the different N functions showed a conservative ecosystem for the nitrogen cycle in burned and unburned soils. However, the unburned rhizosphere is a wild, developed system, with a consistent resource use and therefore may display more nitrogen loss through denitrification and dissmilatory nitrite reductase activity.

In spite of the deep sequencing effort, no sequences of the nitrogenase reductase gene (*nif*H) were obtained. Since this is the marker gene for the N-fixation pathway, we employed *nif*H clone libraries to study the fire effect on this pathway. Burning has frequently been reported to have a detrimental effect on N-fixing bacteria in the soil, decreasing their diversity, richness, and biomass [11, 15, 39]. The presence of *Proteobacteria* and *Firmicutes* in the NifH libraries (Fig. 3c) are consistent with the results of Yeager et al. [11], who found NifH sequences from these phyla plus *Cyanobacteria*, on soils of a ponderosa pine forest between 1 and 14 months after a fire. Moreover, our results indicate that the species richness and the diversity of NifH sequences were lower in burned (BOF and BBS) than in UOF soils (Fig. 3), with the lowest values obtained at the site most severely affected by the fire (BOF). The results of Kennedy and Egger [15] suggested that the presence of living trees during the fire influences the soil

Table 2 Phylogenetic assignment of *nif*H gene sequences at phylum and genus levels

		BBS	BOF	UOF	Total
Proteobacteria	*Gluconacetobacter*	2	0	0	2
	Azospirillum	1	33	9	43
	Bradyrhizobium	6	0	7	13
	Rhodopseudomonas	2	1	7	10
	Rhizobium	22	2	0	24
	Ensifer	2	1	0	3
	Methylobacterium	7	0	12	19
	Zymomonas	0	0	1	1
	Azorhizobium	0	3	1	4
	Skermanella	3	2	2	7
	Methylocystis	0	1	0	1
	Methylovirgula	0	1	0	1
	Rhodocista	1	0	0	1
	Cupriavidus	0	1	1	2
	Geobacter	0	0	7	7
Firmicutes	*Paenibacillus*	4	7	1	12
	Clostridium	0	0	1	1
	Pelosinus	0	1	0	1
Actinobacteria	*Arthrobacter*	0	0	1	1
Bacteroidetes/Chlorobi	*Dysgonomonas*	1	0	0	1
		51	53	50	154

The numbers indicates the numbers of sequences detected within sampled areas of burned bulk soil (BBS), burned holm oak forest (BOF), and unburned holm oak forest (UOF) 3 years after wildfire in Sierra Nevada National Park, South Eastern Spain

microbial communities more than an intact forest floor, and this observation was corroborated by Switzer et al. [35] who demonstrated that soils from burned living trees have smaller bacterial populations than cut trees or stacked wood. Thus, after the fire, the rhizosphere of holm oak (BOF) was more affected than soils with herbaceous vegetation (BBS), as shown by the diversity indices, the number of OTUs, and the rarefaction curves (Fig. 3, Fig. S2). On the other hand, the very similar values of diversity between UOF and BBS sites could be due to the proliferation of a nitrogen-fixing shrub in BBS after the fire.

The phyla *Bacteroidetes* and *Actinobacteria* were two of the most abundant at all sites, according to the similarity of the proteins of the N cycle in the metagenomic analysis (Fig. 2), but only one sequence of each phylum was obtained in the analysis of the NifH diversity. This discrepancy could reflect the lower proportion of diazotrophic microorganisms of these taxa compared to the *Proteobacteria* [40]; only the phylum *Firmicutes* shows similar proportional abundances using both approaches (gene libraries and metagenomics) in the BOF rhizosphere. The increase of both gram-positive phyla (*Firmicutes* and *Actinobacteria*) after the wildfire is in agreement with the results of other authors on wildfires [9, 11] and the presence of functional genes associated with allantoin utilization in soil [38]. The observed differences in the structure and the composition of the *Actinobacteria* communities probably reflect the ability of many of the bacteria of this group to form spores and proliferate after adverse events via sporulation; at the same time, specific genera like *Arthrobacter* are adapted to oligotrophic conditions. Gram-positive bacteria can withstand high temperatures and proliferate on partially sterile burned soils in the form of spores [9, 11, 41]. These results are consistent with those of other studies reporting an increase in the relative abundance and richness of bacteria within the phylum *Actinobacteria* in biochar-treated soils after 6 months of incubation [42] or an increase in actinobacterial colony-forming units on soils 32 months after a fire [9]. Within the phylum *Actinobacteria*, the largest differences among sites in the phylogeny related to the N cycle was due to increased abundance of the genus *Arthrobacter* in the BOF rhizosphere (Fig. 2b). This genus has been related to the degradation of aromatic compounds [43], which appear in the soil after a wildfire; therefore, it could play a key role in the recovery of the microbial community facilitating the pass from oligotrophic to a copiotrophic conditions. Similarly, the presence of the genus *Azospirillum* as a diazotrophic microorganism in the BOF rhizosphere (Table 2) could reflect its importance at the onset of ecosystem recovery, since it has been described as a plant growth-promoting rhizobacterium increasing root development [44]. The increase of the *Arthrobacter* genus after wildfire is important, as it was the second-most abundant in the BOF rhizosphere, and the genus with strongest significant differences between unburned and burned holm oak rhizospheres (Fig. 2b).

In combination, these results show a modification of microbial community structure, with an increase of the gram-positive phyla, as a consequence of a wildfire. These bacteria fulfil a range of potential functions in the N cycle, with a major role for the phylum *Firmicutes* in nitrogen fixation and for the phylum *Actinobacteria* in other potential pathways of the nitrogen cycle associated with the holm oak rhizosphere. After a wildfire, these pathways showed a greater tendency toward the conservation of nitrogen in the ecosystem, with an increase in functions associated with N retention (allantoin utilization) and a decrease in functions associated with N losses (denitrification and dissimilatory nitrite reductase) in the burned rhizosphere. Due to its high abundance and potential important role in N cycling, the phylum *Actinobacteria* merits further attention as a biomarker for ecosystem recovery after wildfire.

Acknowledgments We would like to thank the authorities of the Sierra Nevada National Park for the access, facilities, and soil sampling. This work was funded by the following grants: P08-CVI-03549 from the Consejería de Innovación, Ciencia y Empresa of the Junta de Andalucía, OAPN 021/2007 and OAPN 748/2012 from the Organismo Autónomo Parques Nacionales (Ministry of the Environment), including ERDF

(European Regional Development Fund). JFCD was awarded a predoctoral fellowship from the Junta de Andalucia, and AJFG was awarded a predoctoral fellowship (FPU) from the Spanish Ministry of Education.

References

1. Clark SC (1996) Mediterranean ecology and an ecological synthesis of the field sites. In: Brandt CJ, Thornes JB (eds.) Mediterranean desertification and land use. John Wiley and sons, Ltd. pp. 271-301
2. Pausas JG (2006) Simulating Mediterranean landscape pattern and vegetation dynamics under different fire regimes. Plant Ecol 187: 249–259
3. Felicísimo ÁM, Muñoz J, Villalba CJ, Mateo RG (2011) Impactos, vulnerabilidad y adaptación al cambio climático de la biodiversidad española. 1. Flora y vegetación. Ministerio de Medio Ambiente y Medio Rural y Marino, Madrid
4. Gómez-Zotano J, Moreno-Sánchez JJ, Rodríguez-Martínez F (2005) El incendio de Sierra Nevada (22-24 de septiembre de 2005). Una catástrofe ecológica. Cuadernos Geográficos 37:205–214
5. Certini G (2005) Effects of fire on properties of forest soils: a review. Oecologia 143:1–10
6. Choromanska U, DeLuca TH (2002) Microbial activity and nitrogen mineralization in forest mineral soils following heating: evaluation of post-fire effects. Soil Biol Biochem 34:263–271
7. Prieto-Fernández A, Acea MJ, Carballas T (1998) Soil microbial and extractable C and N after wildfire. Biol Fertil Soils 27:132–142
8. Wang Q, Zhong M, Wang S (2012) A meta-analysis on the response of microbial biomass, dissolved organic matter, respiration, and N mineralization in mineral soil to fire in forest ecosystems. For Ecol Manag 271:91–97
9. Bárcenas-Moreno G, García-Orenes F, Mataix-Solera J, Mataix-Beneyto J, Bååth E (2011) Soil microbial recolonisation after a fire in a Mediterranean forest. Biol Fertil Soils 47:261–272
10. Smith NR, Kishchuk BE, Mohn WW (2008) Effects of wildfire and harvest disturbances on forest soil bacterial communities. Appl Environ Microbiol 74:216–224
11. Yeager CM, Northup DE, Grow CC, Barns SM, Kuske CR (2005) Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. Appl Environ Microbiol 71:2713–2722
12. Allen CD, Savage M, Falk DA, Suckling KF, Swetnam TW, Schulke T, Stacey PM, Hoffman M, Klingelm JT (2002) Ecological restoration of Southwestern ponderosa pine ecosystems: a broad perspective. Ecol Appl 12:1418–1433
13. Goodale CL, Aber JD (2001) The long-term effects of land-use history on nitrogen cycling in northern hardwood forests. Ecol Appl 11: 253–267
14. Zehr JP, Jenkins BD, Short SM, Steward GF (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol 5:539–554
15. Kennedy N, Egger KN (2010) Impact of wildfire intensity and logging on fungal and nitrogen-cycling bacterial communities in British Columbia forest soils. For Ecol Manag 260:787–794
16. Wallenstein MD, Vilgalys RJ (2005) Quantitative analyses of nitrogen cycling genes in soils. Pedobiologia 49:665–672
17. Andreote FD, Jiménez DJ, Chaves D, Dias ACF, Luvizotto DM, Dini-Andreote F, Fasanella CC, Lopez MV, Baena S, Taketani RG, de Melo IS (2012) The microbiome of Brazilian mangrove sediments as revealed by metagenomics. PLoS ONE 7(6):e38600
18. Tringe SG, von Mering C, Kobayashi A, Salamov AA, Chen K, Chang HW, Podar M, Short JM, Mathur EJ, Detter JC, Bork P, Hugenholtz P, Rubin EM (2005) Comparative metagenomics of microbial communities. Science 308:554–557
19. Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS, Banfield JF (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43
20. Gomez-Alvarez V, Teal TK, Schmidt TM (2009) Systematic artifacts in metagenomes from complex microbial communities. ISME J 3: 1314–1317
21. Falgueras J, Lara AJ, Fernández-Pozo N, Cantón FR, Pérez-Trabado G, Claros MG (2010) SeqTrim: a high-throughput pipeline for preprocessing any type of sequence read. BMC Bioinforma 11:38
22. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA (2008) The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinforma 9:386
23. Parks DH, Beiko RG (2010) Identifying biologically relevant differences between metagenomic communities. Bioinformatics 26:715–721
24. White JR, Nagarajan N, Pop M (2009) Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput Biol 5:e1000352. doi:10.1371/journal.pcbi.1000352
25. Widmer F, Shaffer BT, Porteus LA, Seidler RJ (1999) Analysis of *nif*H gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. Appl Environ Microbiol 65: 374–380
26. Villadas PJ, Fernández-López M, Ramírez-Saad H, Toro N (2007) Rhizosphere-bacterial community in *Eperua falcata* (Caesalpiniaceae) a putative nitrogen-fixing tree from French Guiana rainforest. Microb Ecol 53:317–327
27. Katoh K, Standley DM (2013) MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780
28. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541
29. Schloss PD, Larget BR, Handelsman J (2004) Integration of microbial ecology and statistics: a test to compare gene libraries. Appl Environ Microbiol 70:5485–5492
30. De Cáceres M, Font X, Oliva F, Vives S (2007) GINKGO, a program for non-standard multivariate fuzzy analysis. Adv Fuzzy Sets Syst 2: 41–56
31. Marañón-Jiménez S, Castro J (2013) Effect of decomposing post-fire coarse woody debris on soil fertility and nutrient availability in a Mediterranean ecosystem. Biogeochemistry 112:519–535
32. Williams RJ, Hallgren SW, Wilson GWT (2012) Frequency of prescribed burning in an upland oak forest determines soil and litter properties and alters the soil microbial community. For Ecol Manag 265:241–247
33. Jiménez-Esquilín AE, Stromberger ME, Shepperd WD (2008) Soil scarification and wildfire interactions and effects on microbial communities and carbon. Soil Sci Soc Am J 72:111–118
34. Parker JL, Fernández IJ, Rustad LE, Norton SA (2001) Effects of nitrogen enrichment, wildfire, and harvesting on forest-soil carbon and nitrogen. Soil Sci Soc Am J 65:1248–1255
35. Switzer JM, Hope GD, Grayston SJ, Prescott CE (2012) Changes in soil chemical and biological properties after thinning and prescribed fire for ecosystem restoration in a Rocky Mountain Douglas-fir forest. For Ecol Manag 275:1–13
36. Poole RK (2005) Nitric oxide and nitrosative stress tolerance in bacteria. Biochem Soc Trans 33:176–180
37. Goberna M, García C, Insam H, Hernández MT, Verdú M (2012) Burning fire-prone Mediterranean shrublands: immediate changes

in soil microbial community structure and ecosystem functions. Microb Ecol 64:242–255
38. Wang P, Kong C, Sun B, Xu X (2010) Allantoin-induced changes of microbial diversity and community in rice soil. Plant Soil 332:357–368
39. Shaffer BT, Widmer F, Porteous LA, Seidler RJ (2000) Temporal and spatial distribution of the nifH gene of N2 fixing bacteria in forests and clear cuts in western Oregon. Microb Ecol 39:12–21
40. Raymond J, Siefert JL, Staples CR, Blankenship RE (2004) The natural history of nitrogen fixation. Mol Biol Evol 21(3):541–554
41. Moseby AH, Burgos J, Reed J, Tobin-Janzen T (2000) Isolation and identification of soil bacteria from the Centralia mine fire area. J Pennsylvania Acad Sci 73:150
42. Khodadad CLM, Zimmerman AR, Green SJ, Uthandi S, Foster JS (2011) Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. Soil Biol Biochem 43:385–392
43. Westerberg K, Elvang AM, Stackebrandt E, Jansson JK (2000) *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol. Int J Syst Evol Microbiol 50:2083–2092
44. Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556