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Structure and function of bacterial communities in ageing soils: Insights from the Mendocino ecological staircase

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ABSTRACT

The ecological staircase of Mendocino (California, USA) is characterized by a succession of uplifted marine terraces that are derived from the same mineralogical parent material but have different ages, levels of fertility, and types of vegetation, from grassland in the youngest and most fertile terrace to a pygmy forest in the older terraces. Such conditions present a unique opportunity to determine how the structure, abundance, and function of bacterial communities vary with soil fertility along this natural chronosequence. Pyrosequencing analysis of 16S rRNA gene amplicons revealed that Acidobacteria, Proteobacteria, Actinobacteria, and Bacteroidetes were the most abundantly represented phyla. Bacteroidetes, Firmicutes, Verrucomicrobia were significantly enriched in the grasslands, while the less fertile forested terraces showed higher abundance of Acidobacteria Gp2 and Alphaproteobacteria. The pygmy forest soil harboured significantly more Actinobacteria and OP10 than the non-pygmy forest. Between samples from different terraces, the structure of the bacterial community clearly correlated with soil characteristics. Notably, the number of operational taxonomic units was greater in the fertile terrace, as was the density of culturable bacterial populations. Functional characterization of the soil culturable bacteria from the pygmy and non-pygmy forest terraces revealed that the soil bacteria from the non-pygmy terrace were significantly more effective in solubilizing minerals and more abundant than in the pygmy terrace. Our results provide new information on bacterial community structure as a function of soil age, land cover and fertility, which improve our understanding of soil evolution.

1. Introduction

Chronosequences such as marine terraces, glaciers, volcanic lands or abandoned fields are ideal environments to study biological colonization and succession in relation to geological time scale and nutrient availability (Huggett, 1998; Kuramae et al., 2011; Izquierdo et al., 2013; Moore et al., 2010; Philippot et al., 2011; White et al., 2008). A shared characteristic of soil chronosequences is that they feature a set of sites that are derived from the same mineralogical parent material. Typically, the older sites in the sequence are increasingly depleted for nutrients, especially phosphorus, as a result of mineral weathering and leaching (Izquierdo et al., 2013). This phenomenon of 'ecosystem retrogression' (Peltzer et al., 2010; Wardle et al., 2004) is a topic of much interest but is still poorly understood in terms of the structure and function of microbial communities associated with these soils or their impacts on soil chemistry, nutrient availability, and vegetation (Wardle et al., 2004; Chadwick et al., 1999).

Long-term chronosequences can be found in various parts of the world (Jenny et al., 1969; Thompson, 1981; Wardle et al., 1997), but they remain relatively rare due to the impact of land management and natural events such as fires or earthquakes. Among the chronosequences that have been described along the coast of California (White et al., 2008; Jenny et al., 1969; Moore et al., 2010; Westman and Whittaker, 1975), the ecological staircase of Mendocino is one of the best studied (Jenny et al., 1969). It is located in the Jug Handle State Natural Reserve and characterized by a succession of five uplifted marine terraces formed from the same parent material, with each terrace different in age from the next by approximately 100,000 years (Merritts et al., 1991). Soils range from young and relatively nutrient-rich near the coast to old and weathered podzols defined as spodosol at the top of the chronosequence, thus forming a natural soil fertility gradient (Izquierdo et al., 2013; White et al., 2008; Westman and Whittaker, 1975). The Mendocino terraces

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are populated with evergreen tree species, except the youngest terrace, which is a grassland adapted to the coastal influences. In the older terraces, nutrient limitation has led to the development of a pygmy forest with dwarfed trees adapted to such soil conditions. Compared to non-pygmy trees of the same age (60–70 m high), pygmy *Pinus muricata* are smaller (only 3–5 m high) and their root system is restricted to the first soil horizon where it grows in a dense mat (Jenny et al., 1969). They also present increased leaf longevity, a lower rate of leaf production and growth (Westman, 1978; Yu et al., 1999; Eckert et al., 2012). Dwarfing represents an adaptation to oligotrophic conditions, which allows more effective assimilation and recycling of nutritional cations (Westman, 1978; Yu et al., 1999). The trees in the Mendocino pygmy forest are notably enriched in polyphenol, a metabolite known to increase nutrient availability, detoxify soluble aluminium, and regulate nitrogen availability (Northup et al., 1995a,b; Yu et al., 1999). Bolander pine trees colonizing the older soils of the Mendocino ecological staircase have evolved specific aluminium and inorganic phosphate transporters permitting them to grow in these extremely podzolic soils (Eckert et al., 2012). On the contrary, the intermediate terraces are colonized by the same tree species but presenting a 'normal' (i.e. non-pygmy) phenotype.

The relationships between the soil physico-chemical characteristics and the development of plant and microbial successions have been subject of investigation for a long time. Parameters such as pH and nutrient availability are well known as important drivers of the structure, the diversity and the functioning of both plant and microbial communities (Chapin, 1980; Grayston et al., 2004; Lauber et al., 2009; Marschner et al., 2004). With respect to nutrient deficiency, plants are known to employ strategies based on the selection of particular genotypes or ecotypes adapted to access soil nutrients, on the activation of specific root mechanisms to enhance the release and the utilization of nutrients, and on the selection of specific microbial communities with beneficial properties (Chapin, 1980; Rengel and Marschner, 2005). However, demonstration of the effect of nutrient availability on the structure, abundance and functional potential of the microbial communities in natural environment remains limited due to the difficulty to find natural gradients of fertility, characterized by the same mineralogical parent material.

In the Mendocino ecological staircase, there is a good understanding of how the composition and adaptation of vegetation varies with soil conditions (Jenny et al., 1969; Westman and Whittaker, 1975; Westman, 1978; Yu et al., 1999; Northup et al., 1995a,b). However, little is known still about the microbiology of the soils along this fertility gradient. Soil microbial communities are important drivers of nutrient cycling, including organic matter decomposition and mineral weathering. Their activities strongly impact nutrient availability and consequently plant development (Calvaruso et al., 2006; Rengel and Marschner, 2005; Uroz et al., 2009a, 2011; Smits et al., 2012). It is also well established that nutrient availability impacts the structure and the function of soil microbial communities such as according to the land management or the seasonal variations (Collignon et al., 2011; Grayston et al., 2004). The only study we are aware off that addresses the microbiology of the Mendocino ecological staircase is by Wurzburger and colleagues (Wurzburger and Bledsoe, 2001; Wurzburger et al., 2001), who analysed the distribution of pine-symbiotic fungi to reveal that ectomycorrhizal fungi were less abundant in the pygmy forest than in the non-pygmy forest, and that certain ectomycorrhizal species were only associated with pygmy trees, suggesting a selective impact of the pygmy forest or an adaptation to nutrient-poor conditions (Wurzburger and Bledsoe, 2001; Wurzburger et al., 2001). Additionally, Yu et al. (2003) measured the global mineralization, nitrification and respiration along the soil chronosequence.

Our objective for this study was to determine the composition and functional abilities of bacterial communities in the soils of the Mendocino chronosequence and to assess how community structure and function change in relation to soil age and fertility. We collected soil samples from the first three terraces T1, T2, and T3, as defined by Jenny et al. (1969). The youngest terrace (T1) consists of coastal prairie, dominated by grasses, wildflowers, and blackberries. The second terrace (T2) is a forested area dominated by pines. The third terrace (T3) features the most weathered soil and a pygmy forest with dwarfed vegetation. To assess community structure, we performed 16S rRNA gene amplicon pyrosequencing on DNA isolated from the organo-mineral soil horizons in order to uncover the relationship between bacterial diversity and species richness on the one hand and various soil characteristics, such as edaphic quality, bacterial population size, and bacterial culturable fraction, on the other. For the functional assessment of bacteria in these soils, we took a two-pronged approach. In the first, we recovered bacterial isolates and tested them for the ability to solubilise phosphorus and mobilise iron. These are common functions among bacteria from forest soils (Uroz et al., 2007, 2011), and can be expected to be more prevalent in nutrient-poor soils. Our second approach involved culture-independent quantification of a bacterial guild specifically adapted to oligotrophic soils. For this, we chose the bacterial genus *Collimonas*, representatives of which have been demonstrated to be common in undisturbed soil environments and to be efficient weathering bacteria (de Boer et al., 2001; Leveau and Preston, 2008; Leveau et al., 2010; Uroz et al., 2009b).

2. Materials and methods

2.1. Study site

The Jug Handle State Natural Reserve (39° 22′ 31′ N, 123° 48′ 37′ W) (Fig. 1) is located in Mendocino county, California, about 160 miles north of San Francisco. It stretches 5 km from the Pacific coast in the West to Jackson State Forest in the East. The park features five terraces that have been uplifted from sea level by glacier, ocean, and tectonic activity. Each terrace differs in age from the next by about 100,000 years (Jenny et al., 1969; Jenny, 1973; Merritts et al., 1991) so that each represents a different level of weathering of the same graywacke sandstone parent material (Jenny et al., 1969; Jenny, 1973). The soils of the ecological staircase are spodosols classified as ustic humitropept in the first terrace to a mix of Typic Albaquult and Tropaquod soils in the second and third terraces (Yu et al., 1999, 2003; Northup et al., 1995b). The site is characterized by a Mediterranean climate with frequent fog in summer and an annual precipitation of 983 mm and a mean annual temperature of 12.5 °C (Northup et al., 1995b). The plant communities on these terraces reflect the soil conditions and represent the sequential stages of succession. For this study, we considered only the first three staircases T1, T2, and T3, as explained in the Introduction. Native Bishop Pine (*P. muricata*) grows in both T2 and T3 and was used as a reference tree species for our soil sampling.

2.2. Soil sampling and soil analyses

A permit to sample soil from the Jug Handle State Natural Reserve was obtained from the California State Department of Parks and Recreation. On August 31, 2010, a total of $n = 15$ soil cores with 20 × 20 × 20-cm dimensions were taken from terraces T1 (youngest terrace colonized by coastal grassland; $n = 3$), T2 (forest terrace; $n = 6$), and T3 (older terrace colonized by pygmy vegetation; $n = 6$). From T1 (39°22′ 31″ N, 123° 49′ 11″ W), three distant soil samples (ten meters apart from each other) were collected. For both forest terraces T2 (39° 22′ 30″ N, 123° 47′ 48‘‘ W) and T3 (39°

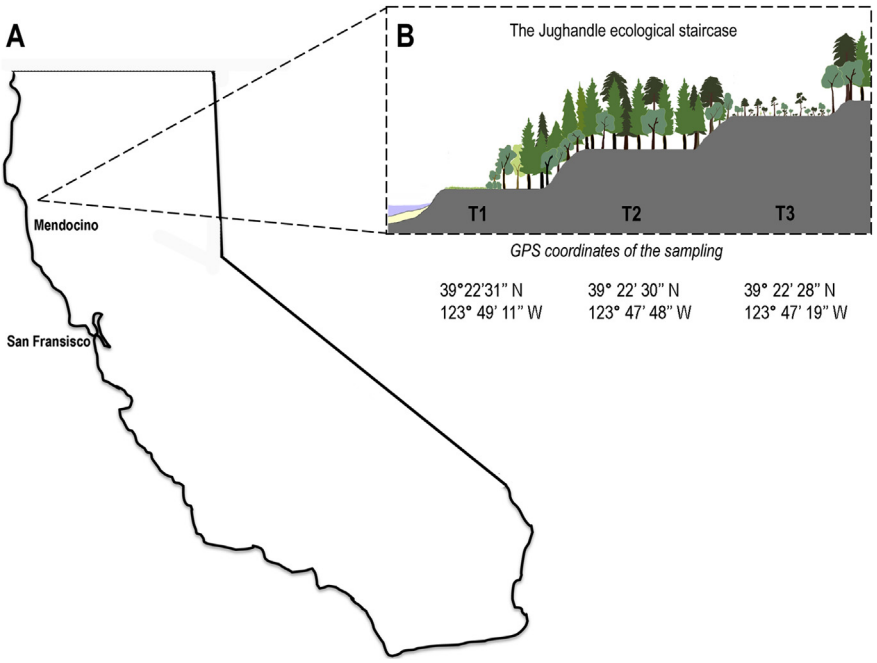

Fig. 1. Map presenting the ecological staircase of Mendocino and its location.

22′ 28″ N, 123° 47′ 19" W), three soil samples were collected from the foot of each one of two *P. muricata* trees, for a total of 6 cores per terrace. The two trees on each terrace were separated by 10 m, and the 3 soil samples per tree by 3 m. During sampling, we noticed that the soil developed in the terrace T3 (colonized by the pygmy forest) was mainly composed of quartz giving a white/ash aspect to the soil and by an iron-concreted harpan around 60 cm to 1 m of depth. On the contrary, the soil developed in the second terrace presented a classical forest soil aspect. Soil samples were transported back to the lab, stored overnight at 4 °C, after which the mineral horizon (5–15 cm) was separated from the rest of each core, sieved through a 2-mm mesh, and homogenised using a blender prior to microbiological, molecular and soil analyses. Soil analyses were carried out by the Laboratoire d'Analyse des Sols d'Arras (http://www5.lille.inra.fr/las). The cation exchange capacity (CEC) was determined according to the Metson method (Metson, 1956; Extraction with ammonium acetate 1 M, pH = 7), the pH (water method; ratio 1/5 w/v), total carbon (C) and total nitrogen (N) contents (both obtained after combustion at 1000 °C) and phosphorus (P) content according to Duchaufour and Bonneau (1959) and Duval (1963). Exchangeable cations (Ca, Mg, Na, K, Fe, Mn and Al) were extracted using cobaltihexamine and determined by inductively coupled plasma spectrometry–atomic emission spectrometry (ICP–AES). Soil analysis are presented in detail in Table 1

2.3. Bacterial collection and taxonomic and functional characterization

For each soil sample ($n = 15$), one gram of soil was suspended in 10 mL of 1× Basic Salt Solution (de boer et al., 1998) and vortexed at maximum speed for 45 s. Serial dilutions of each original soil suspension were spread on a nutrient-rich culture medium, King's B agar with 50 mg natacid (Caglificio Clerici, Italy; active ingredient is the antifungal compound natamycin at 50%) per liter, and on nutrient-poor culture medium 0.1× tryptic soy agar (TSA) with 50 mg natacid per liter. Plates were incubated at 20 °C for 5 days, then analysed for colony-forming units (CFUs) which were normalized per gram of soil. A total of 112 bacterial isolates growing on 0.1× TSA ($n = 12$, 49, and 51 from T1, T2, and T3, respectively) were purified by three successive transfers on 0.1× TSA. Nearly full-length PCR amplicons of the 16S rRNA gene were produced using primers pA and 1492r (Edwards et al., 1989; Lane, 1991), purified using the UltraClean PCR Clean-up DNA Purification Kit (MOBIO Laboratories, Inc., Carlsbad, CA), and sequenced using primer 1492r at the UC Davis DNA Sequencing Facility (Davis, CA). DNA sequences were analysed using Lasergene software (DNASTAR, Madison, WI). The ability of bacterial strains from terraces T2 ($n = 49$) and T3 ($n = 51$) to solubilize inorganic phosphorous and to mobilize

Table 1
Soil analyses and bacterial quantification.

Terrace	pH	Total C	Total N	P_2O_5	C/N	CEC	Ca	Mg	Na	K	Fe	Mn	Al	Total**	Total	Collimonads
		g/kg				cmol/kg								Culturable	qPCR	
T1*	5.41^a	44.00^a	3.14^a	0.12^a	14.01^a	14.03^a	2.62^a	2.31^a	0.74^a	0.39^a	0.02^a	0.06^a	1.07^a	6.75^a	9.22^a	5.97^a
T2	4.05^b	40.27^a	0.74^b	0.04^b	52.19^b	8.41^{ab}	0.78^b	0.71^b	0.14^b	0.14^b	0.06^b	0.01^b	0.99^a	4.97^b	9.01^a	3.97^b
T3	4.24^c	39.22^a	0.68^b	0.03^b	57.04^b	7.23^b	0.31^b	0.54^b	0.11^b	0.12^b	0.03^a	0.01^b	0.62^a	4.64^b	9.02^a	3.68^b

Soil analyses were performed by the INRA Soil Analyses Laboratory of Arras (France, http://www5.lille.inra.fr/las). The analyses performed were referenced as follow: Total C and N (SOL-0406), pH in water (SOL-0501), P_2O_5 (SOL-0603), CEC (SOL-0701), cations (Ca, Mg, Na, K, Fe, Mn, Al; SOL-0719).
*T1: grassland (3 spatially independent samples); T2: Non-pygmy forest (3 spatially independent samples) and T3: pygmy forest (6 spatially independent samples). Different letters between terraces (a, b or c) indicate significant differences, according to a one-factor ANOVA and a Bonferroni–Dunn test ($P < 0.05$).
** 'Total' means total bacterial population. Values (culturable or qPCR) are expressed as log of bacteria per gram of soil.

iron was assessed on solid tricalcium orthophosphate (TCP) and chrome azurol S (CAS) media, respectively, following the protocol of Frey-Klett et al. (2005). Briefly, bacteria were grown on 0.1× TSA at 25 °C for 48 h, then grown on liquid LB medium, for 48 h at 25 °C, collected by centrifugation, and re-suspended in sterile water to an optical density OD_{595nm} of 0.8 (approximately 10^9 cells/mL). For each bacterial isolate, 5 μL of this suspension was dropped in triplicate on TCP or CAS plates. After incubation at 25 °C for 7 days (all 100 isolates produced biomass), clearing of the TCP or CAS media indicated phosphate solubilization or iron mobilization, respectively. The diameters of the cleared haloes around the bacteria were measured and averaged between triplicates.

2.4. Enrichment for and identification of Collimonas isolates

For one of the T1 samples, two of the T2 samples (each representing one of the two trees), and two of the T3 samples (each representing one of the two trees), one gram of soil was resuspended in 10 mL of 1× Basic Salt Solution (de boer et al., 1998) by vortexing at maximum speed for 45 s. One milliliter of each suspension ($n = 5$) was inoculated into 50 ml of tryptic soy broth (Oxoid) with 50 mg natacid and 30 mg nalidixic acid (Sigma) per liter and incubated at 20 °C for 3 days at 200 rpm, after which a 100-ul aliquot was transferred to 50 ml of chitin broth (modified from de Boer et al., 2004; excluding the agar) supplemented with 50 mg natacid and 30 mg nalidixic acid per liter for an additional incubation at 20 °C for 7 days at 200 rpm. In parallel, one milliliter of the original soil suspension was inoculated directly into 50 ml of chitin broth supplemented with 50 mg natacid and 30 mg nalidixic acid per liter and incubated at 20 °C for 7 days at 200 rpm. Ten microliters of the chitin broth enrichments were spread in duplicate onto chitin agar plates (de Boer et al., 2004), and incubated at 20 °C for 7 days. Colonies with a halo of cleared chitin were selected and analysed using a *Collimonas*-specific probe assay (Höppener-Ogawa et al., 2007). Positive isolates were further tested by a Restriction Fragment Length Polymorphism (RFLP) assay on amplified 16S rRNA genes using the *Bst*BI restriction enzyme, which is specific for the identification of *Collimonas* (Höppener-Ogawa et al., 2007). Positive isolates were confirmed to be *Collimonas* by DNA sequencing of PCR amplified 16S rRNA genes using primers pA (Edwards et al., 1989) and 1492r (Lane, 1991). These *Collimonas* strains were then tested for their ability to solubilize inorganic phosphate and mobilize iron.

2.5. DNA extraction and quantitative PCR

Total DNA was extracted from soil samples using the PowerSoil DNA isolation kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA). One microliter of 1/10 diluted DNA extracts was used as template in a quantitative real-time PCR assay using the Bio-Rad CFX96 instrument and Bio-Rad CFX Manager software (version 1.5.534.0511). Quantification of total bacteria was performed according to Rastogi and colleagues (Rastogi et al., 2012). For the quantification of *Collimonas*, we used the *Collimonas*-specific Taqman assay developed by Höppener-Ogawa et al. (2007). Briefly, the PCR was performed with initial denaturation at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 65 °C for 45 s. The 10-μl PCR reactions contained 1× iQ Supermix (Bio-Rad, Hercules, CA), 480 nM each of Eddy3for and Eddy3rev primers, and 100 nM Sophie probe (Höppener-Ogawa et al., 2007). Population sizes of total bacteria or *Collimonas* were expressed as log [number of 16S rRNA gene copies per gram soil].

2.6. Barcoded pyrosequencing, DNA sequence processing and taxonomic analysis

For analysis of the 15 soil DNA samples by 16S rRNA gene amplicon pyrosequencing, we used the bacterial primers PYRO-799f (Rastogi et al., 2012) and 1492r (Lane, 1991). Primer PYRO799f (5′-ccatctcatccctgcgtgtctccgactcagnnnnnnnnnnnAACMGGATTAGA-TACCCKG-3′) is a derivative of primer 799f (Chelius and Triplett, 2001) containing a 16S rRNA gene conserved region (AACMGGAT-TAGATACCCKG), a unique barcode (nnnnnnnnnn), and pyrosequencing adaptator sequence (ccatctcatccctgcgtgtctccgactcag). Four microliters of 1/10 diluted metagenomic DNA extract was used in a PCR reaction containing 3 U TaKaRa Ex Taq polymerase, 1X Ex Taq Buffer, 200 μM dNTP, 500 nM PYRO-799f primer, and 500 nM 1492r primer in a final volume of 100 μl. PCR was performed with initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min, with a final extension of 72 °C for 10 min. Amplicon pyrosequencing was performed on the GS-FLX 454 Titanium platform (Roche, Basel, Switzerland) at the Core for Applied Genomics and Ecology (CAGE) at the University of Nebraska (Lincoln, NE, USA) generating a total of 64,452 reads. Sequences were parsed through the custom length and quality filters at CAGE, trimmed to 280 bp, and analysed through the Ribosomal Database Project (RDP) pyrosequencing pipeline (Cole et al., 2009). From each one of the 15 samples, 2600 sequences (corresponding to the smaller set of sequences after RDP processing), encompassing the V5 and V6 hypervariable regions, were randomly selected, for a total of 39,000 high-quality FASTA-formatted sequences, which were passed through RDP Classifier (Wang et al., 2007). The phylogenetic hierarchy of each sample was determined at an 80% confidence threshold (Leveau and Tech, 2011). Complete linkage clustering was performed using RDP's pyrosequencing pipeline (Cole et al., 2009).

2.7. Statistical analyses

For the 16S rRNA gene sequence data set, relative abundances of taxa were transformed by arcsin sqrt as done before (Uroz et al., 2010, 2013). Analysis of variance (ANOVA, $p < 0.05$), Pearson correlation and multivariate analyses were done using XLstat2011 (Addinsoft, Paris, France). For the functional assays (phosphorous solubilisation and iron mobilisation), the bacterial isolates were binned into three classes according to halo size: 'non effective', (<0.1 cm halo); 'effective' (>0.1 but <1 cm) and 'very effective' (>1 cm). These bin classes were chosen to avoid classes with zero bacterial isolates. The proportions of bacterial isolates per class were compared using a χ^2 test ($P < 0.05$). Real-time PCR data were analysed using a Student-t test.

2.8. Nucleotide sequence accession numbers

The 454 pyrosequencing data generated for this study were submitted to the Sequence Read Archive (SRA) and are available under project SRP013944: Forest Soil Metagenome, accessions SRX282017 through SRX282031. The near-complete 16S rRNA gene sequences of 100 bacterial isolates from T1, T2 and T3 soils have been deposited into GenBank under the accession numbers KC987362 through KC987473.

3. Results

3.1. Soil characteristics and bacterial population sizes along the Jug Handle soil chronosequence

Chemical characteristics of the mineral horizons of these soils are summarized in Table 1, and differences between soil samples

are visualized in Fig. 2. Soil pH differed significantly ($P < 0.05$) between the three terraces: with a pH of 5.41, T1 soil was the least acidic, compared to a pH of 4.05 for T2 and pH = 4.24 for T3. The T2 and T3 soils contained significantly ($P < 0.05$) less nitrogen, phosphate, calcium, magnesium, sodium, potassium, and manganese than those from T1. A significant decrease of the cation-exchange capacity (CEC) was observed between the T1 and T3 terraces. The Fe concentration was correlated negatively with soil pH, and was highest in T2 soils. We found no significant differences between T1, T2, and T3 in terms of total carbon (C), and aluminium (Al), although averages decreased with increased age of the soils. The C/N ratio was 3–4 times higher in T2 and T3 than in T1 soils.

At an average of 9.06 ± 0.04, total bacterial population sizes (expressed as log[16S rRNA gene copies per gram of soil]) did not differ significantly ($P = 0.066$) between the 15 soil samples (Table 1). In contrast, numbers of total culturable bacteria (expressed as log[CFUs per gram of soil]) were on average 1 to 2 orders of magnitude higher in T1 soil samples than in the T2 and T3 samples ($P < 0.0001$) (Table 1). We observed a positive and significant correlation between the density of culturable bacteria and most of the soil characteristics measured, except C and N (Pearson correlation tests $P < 0.05$; data not shown). Total *Collimonas* population sizes (expressed as log[16S rRNA gene copies per gram of soil) fluctuated around the detection limit of 3.67 ± 0.21 in the T2 and T3 soil samples (representing only 0.0004% of the total bacterial population). In contrast, *Collimonas* were 2 orders of magnitude more abundant in the T1 samples, where they represented about 0.08% of the total bacterial population.

3.2. Comparison of bacterial community composition in Jug Handle soil samples

Taxonomic assignment showed that the most abundant bacterial phyla in the soil samples were Acidobacteria (39.5%), Proteobacteria (26.4%), Actinobacteria (10.0%), Bacteroidetes (4.1%) and Verrucomicrobia (2.0%), together representing 82.0% of all reads. The most abundant classes were Acidobacteria Gp1 (22.5%), Alphaproteobacteria (14.9%), Acidobacteria Gp2 (11.9%), Actinobacteria (10.0%) and Gammaproteobacteria (4.3%). The most abundantly represented known bacterial genera were *Mycobacterium* (1.3%), *Bradyrhizobium* (0.9%), *Burkholderia* (0.8%), *Mucilaginibacter* (0.5%), *Methylosinus* (0.5%), *Ferruginibacter* (0.4%), *Phenylobacterium* (0.4%), *Steroidobacter* (0.3%), *Acidocella* (0.3%) and *Conexibacter* (0.2%). Notably, we found only 2 *Collimonas* sequences (0.005%) in our data set, both in the same T1 soil sample.

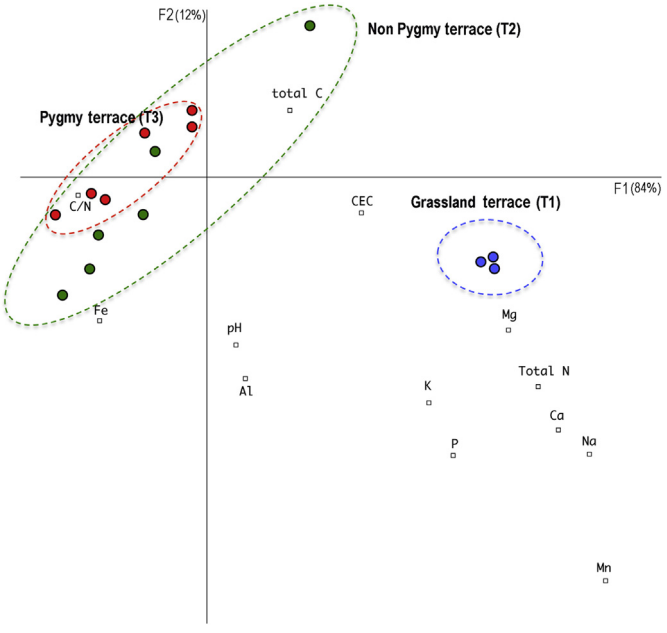

Fig. 2. Multifactorial analysis of the soil characteristics. In this analysis, principal component axis 1 and 2 explain most of the variance in the data cumulatively ($F1 = 84\%$ and $F2 = 12\%$). Treatments are presented as follow: blue dots, grassland soil samples; green dots, non-pygmy *P. muricata* soil samples and red dots, pygmy *P. muricata* soil samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bacterial community composition was significantly different between soil samples from the three Jug Handle terraces. The T1 soil samples harboured a higher number of operational taxonomic units (OTUs) (885 ± 28) than the T2 and T3 soil samples (579 ± 40 and 530 ± 34, respectively). Also, Chao1 and Shannon estimates for bacterial diversity were significantly higher for the grassland than the forest soils (T1 > T2 > T3; $P < 0.0001$) (Table S1). Phyla (Fig. 3), classes, orders and genera were differently represented across the different terraces (Table 2), such that not only did the younger terrace (T1) separate from the older terraces (T2 and T3), but also the terrace colonized by the pygmy forest (T3) from terrace colonized by the non-pygmy (T2) forest. For example, T1 soils were significantly enriched in representatives from the phyla Bacteroidetes, Firmicutes, and Verrucomicrobia and featured more unclassified bacterial sequences. In general, abundances of Betaproteobacteria, Deltaproteobacteria and Acidobacteria Gp1/Gp3 were higher in T1 compared to T2 and T3. On the other hand, the T2 and T3 soil samples were enriched in sequences belonging to the classes Acidobacteria Gp2 and Alphaproteobacteria. Compared to the soil under the influence of the non-pygmy forest (T2), the pygmy forest soil samples (T3) were significantly enriched in sequences related to the phyla Actinobacteria and OP10. In contrast, T2 samples showed greater abundances of Acidobacteria Gp2 sequences and of the genera *Acidocella* and *Aquicella*. These trends were confirmed by a multivariate analysis (Fig. 4), which revealed differences not only between the grassland terrace (T1) and the forest environments (T2 and T3), but also between the two forest terraces (T2 and T3).

3.3. Correlation between bacterial community composition and physicochemical soil characteristics

Comparison of the three terraces revealed that there was a strong correlation between most soil characteristics and bacterial community structure across the Jug Handle staircase. This correlation was confirmed by a Mantel test (Pearson score $r = 0.351$; $P = 0.001$) and by regression analysis of the F1 axis scores (Fig. 4) and the soil characteristics (Table 3). Considering only T2 and T3, the bacterial community structure correlated significantly with C/N ratio and the Al and Fe concentrations (Table 3). Regression analysis comparing the OTU richness and the chemical characteristics of each soil sample collected along the staircase revealed that this richness was significantly positively correlated with pH, N, P, CEC, Ca, Mg, K and negatively with the C/N ratio (data not shown).

3.4. Functional and taxonomic characterization of culturable bacterial isolates from the forest terraces

From T2 and T3 soils, we isolated a total of 100 bacterial strains ($n = 49$ and 51 from T2 and T3, respectively). On the basis of their 16S rRNA gene sequences, isolates were identified as members of the Betaproteobacteria, Gammaproteobacteria, Actinobacteria, or Firmicutes, representing the genera *Arthrobacter*, *Burkholderia*, *Cobetia*, *Dyella*, *Erwinia*, *Leifsonia*, *Microbacterium*, *Paenibacilllus* and

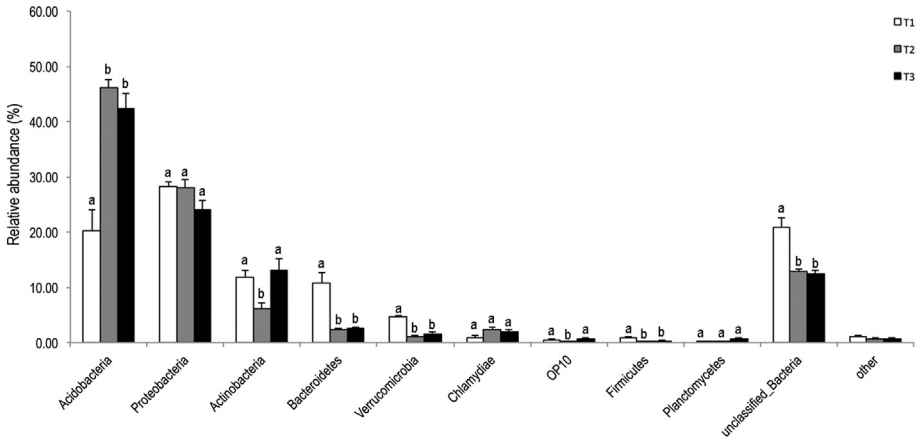

Fig. 3. Relative distribution of the major phyla detected along the soil chronosequence of Mendocino. Treatments are presented as follow: T1, grassland soil samples from terrace 1; T2, non-pygmy *P. muricata* soil samples from terrace 2 and T3, pygmy *P. muricata* soil samples from terrace 3.

Rhodanobacter, with *Burkholderia* being the most abundantly represented genus ($n = 89$) in the collection. These genera were also identified by our 16S rRNA gene amplicon pyrosequencing analysis, and represent the most readily culturable representatives in these soils.

Analysis of the mineral weathering ability of these bacteria revealed that T2 isolates were significantly ($P = 0.028$) more effective at solubilizing inorganic phosphorus than T3 isolates (Fig. 5). In addition, the frequency of 'very effective' P-solubilizing bacterial isolates was greater in T2 than T3 soils, according to Chi2 analysis ($P = 0.04$). T2 isolates were not significantly more effective at mobilising iron ($P = 0.7$) than T3 isolates (Fig. 5). Among the tested strains, those assigned as *Burkholderia* were the most effective in solubilising phosphorous, with T2 isolate 2E1 showing the highest efficacy.

None of the 100 bacterial isolates from the T2 and T3 soils were identified as belonging to the *Collimonas* genus, confirming the culture-independent conclusion that they are not very abundant in these soils. However, using an enrichment strategy in combination with a *Collimonas*-specific Taqman and RFLP assay (see Materials and Methods), we were able to retrieve 12 confirmed *Collimonas* strains from the T1, T2 and T3 soil samples. Sequencing of their 16S rRNA gene revealed that they represented the three of the recognized *Collimonas* species to date: *pratensis*, *arenae*, and *fungivorans*. In assays on TCP and CAS plates respectively, *Collimonas* strains from T3 and T2 performed significantly better than those from T1, suggesting that *Collimonas* from forest soils are more efficient in P-solubilization and Fe mobilisation. Between T2 and T3 *Collimonas*, there was no significant difference in P solubilisation, but T3 isolates performed better than T2 isolates in terms of iron mobilization.

4. Discussion

Our results revealed that bacterial community structure and abundance were significantly correlated to soil parameters. Although it is difficult to separate the land cover effect from the soil characteristics, significant differences related to pH, cationic-exchange capacity or iron concentrations were observed between the different terraces. A focused analysis comparing the soil bacterial communities inhabiting the terraces under the influence of the pygmy and non-pygmy forests revealed significant differences in terms of structure and functional potential.

All studies so far on the ecological staircase of Mendocino distinguished the most nutrient-rich terrace (T1) from the other more weathered terraces (Jenny et al., 1969; Jenny, 1973; Izquierdo

Table 2
Comparison of terraces at the class, order and genus levels. The relative distribution of the sequences in the different taxonomic levels considered was analysed by one-factor ANOVA (and a Bonferroni–Dunn test, $P < 0.05$). The symbols '>' mean significantly more abundant and '=' not significantly different. Bold indicates significant difference between the normal forest terrace (T2) and the pygmy terrace (T3).

Class	Order	Genus
***Acidobacteria Gp1* (T1 > T3 > T2)**	***Acidobacteria Gp1* (T1 > T3 > T2)**	***Acidobacteria Gp1* (T1 > T3 > T2)**
***Acidobacteria Gp2* (T2 > T3 > T1)**	***Acidobacteria Gp2* (T2 > T3 > T1)**	***Acidobacteria Gp2* (T2 > T1 and T2 > T3)**
Acidobacteria Gp3 (T1 > T2 = T3)	Acidobacteria Gp3 (T1 > T2 = T3)	*Acidobacteria* Gp3 (T1 > T2)
***Actinobacteria* (T3 > T1 > T2)**	*Rhodospirillales* (T2 = T3 > T1)	***Acidocella* (T2 > T3 > T1)**
Alphaproteobacteria (T2 = T3 > T1)	*Sphingobacteriales* (T1 > T2 = T3)	*Acidisoma* (T2 > T1)
Betaproteobacteria (T1 > T2 = T3)	***Solirubrobacterales* (T1 > T3 > T2)**	***Aquicella* (T2 > T3 > T1)**
Deltaproteobacteria (T1 > T2 = T3)	*Pseudomonadales* (T1 > T2 = T3)	*Bacillus* (T1 > T3 = T2)
Bacilli (T1 > T2 = T3)	*Sphingomonadales* (T1 > T2 = T3)	*Chitinophaga* (T1 > T2 = T3)
Flavobacteria (T1 > T2 = T3)	*Rhodocyclales* (T1 > T2 = T3)	*Conexibacter* (T2 = T3 > T1)
Sphingobacteria (T1 > T2 = T3)	*Rhodobacterales* (T1 > T2 = T3)	*Flavobacterium* (T1 > T2 = T3)
OP10 (T3 > T2)	*Subdivision 3* (T1 > T2 = T3)	*Methylosinus* (T3 = T2 > T1)
Subdivision 3 (T1 > T2 = T3)	*Burkholderiales* (T1 > T3)	*Mucilaginibacter* (T1 > T2 = T3)
	Myxococcales (T1 > T2 = T3)	***Mycobacterium* (T3 > T2 = T1)**
	OP10 (T3 > T2)	***Solirubrobacter* (T1 = T3 > T2)**
	Bacilliales (T1 > T2 = T3)	*Sphingomonas* (T1 > T2 = T3)
	Acidimicrobiales (T1 > T2)	*Subdivision* 3 (T1 > T2 = T3)
		Terrimonas (T1 > T2 = T3)

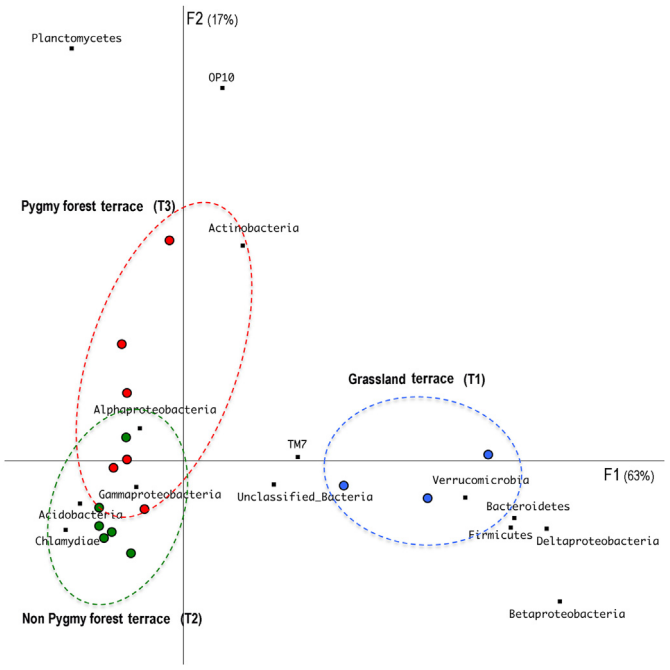

Fig. 4. Multifactorial analysis of the relative proportion of the major phyla present along the soil chronosequence. In this analysis, principal component axis 1 and 2 explain most of the variance in the data cumulatively (*F*1 = 63% and *F*2 = 17%). Treatments are presented as follow: blue dots, grassland soil samples; green dots, non-pygmy *P. muricata* soil samples and red dots, pygmy *P. muricata* soil samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

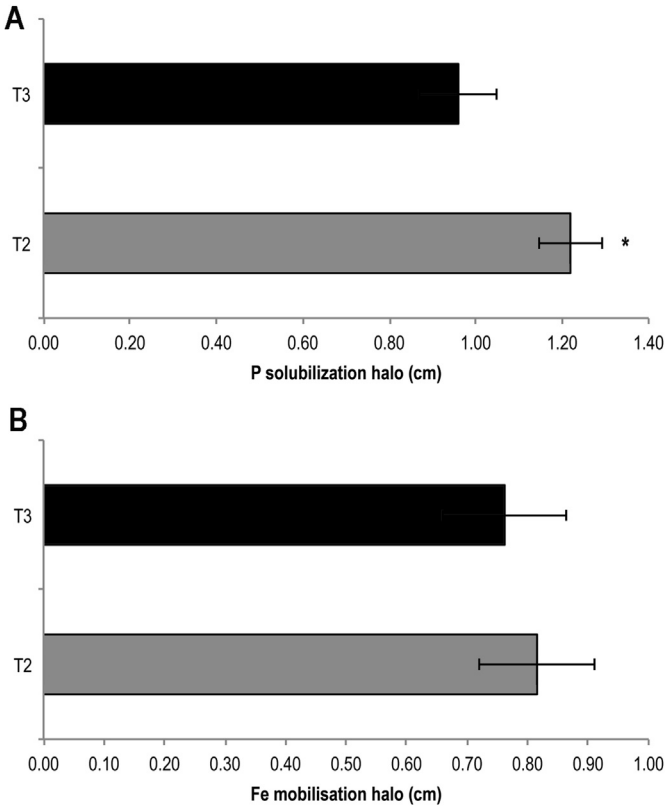

Fig. 5. Mineral weathering potential of the bacterial isolates as assessed by measurement of the diameter halo on the phosphorous and iron assays. Treatments are presented as follow: grey, average value obtained for the bacterial isolates coming from the non-pygmy *P. muricata* soil samples and black, average value obtained for the bacterial isolates coming from the pygmy *P. muricata* soil samples. **A.** Phosphorous solbilization assay. **B.** Iron mobilization assay.

et al., 2013; Merritts et al., 1991; Yu et al., 2003; this study). However, the strong differentiation of the bacterial communities in term of size and structure we observed on this first terrace as compared to the forest terraces can not be exclusively attributed to soil fertility as this first terrace is colonized by grassland. Indeed, the forest terraces are characterized by higher C/N ratio, revealing the strong influence of the forest, and high amount of recalcitrant organic matter, which decomposes slowly compared to the grassland terrace. However, in term of pedogenesis, the soil characteristics measured along the first three terraces fit very well with their soil age. Notably, several studies have revealed that soil characteristics rather than plant species are major controllers of the bacterial communities (Kuramae et al., 2011; Lauber et al., 2008). Other studies reported that different factors including the type of soil and plant species contributed to shape the soil bacterial communities (Grayston et al., 2004; Marschner et al., 2004). Indeed, plants impact soil chemistry and soil affect plant diversity and development. Consequently, both of these parameters influence the structure and function of the soil bacterial communities. However, the site of Mendocino has the unique property to be developed on the same mineralogical parent material with different ages. In the Mendocino gradient, young soils are characterized by higher pH, nutrient concentrations and availability, parameters that decrease along the T1–T2–T3 chronosequence (Jenny et al., 1969; Jenny, 1973; Merritts et al., 1991; Yu et al., 2003). Our analysis confirmed the existence of this fertility gradient and revealed significant correlation between the density of culturable bacteria, the OTU richness, the structure of the bacterial communities and the soil parameters. Among these parameters, pH, N, P, C/N ratio but also inorganic nutrients such as Ca, Mg, Na, K and Fe have strong influence of the structure of the bacterial communities across the soil chronosequence of Mendocino.

In term of bacterial community structure, the grassland (T1) terrace appeared dominated by Proteobacteria, Actinobacteria, Bacteroidetes and Verrucomicrobia contrary to the forest terraces dominated by Acidobacteria (Table 2). Interestingly, the dominant phyla of the grassland (T1) terrace were also those described in the literature as adapted to easily accessible carbon substrates and nutrient rich environments (Bergmann et al., 2011; Uroz et al.,

Table 3
Pearson correlation tests highlight the relationships shared between soil characteristics and community composition. Pearson correlation tests have been performed on the soil analyses of each terrace (grassland terrace (T1); normal forest terrace (T2) and the pygmy terrace (T3)) and the coordinate values obtained on the F1 and F2 axis of the multivariate analysis presented in Fig. 4.

	F1 axis		F2 axis	
	T1, T2 and T3	T2, T3	T1, T2 and T3	T2, T3
pH	**0.967*****	0.260	−0.057	0.206
C	0.084	−0.008	0.186	0.196
N	**0.916*****	−0.129	−0.084	0.062
C/N	**−0.880*****	**0.659***	0.359	**0.768****
P_2O_5	**0.944*****	−0.536	−0.187	−0.454
CEC	0.515	−0.089	−0.044	0.026
Ca	**0.884*****	−0.238	−0.186	−0.306
Mg	**0.854*****	−0.097	0	0.192
Na	**0.953*****	0.247	0.007	0.453
K	**0.899*****	−0.23	−0.151	−0.114
Fe	**−0.521***	−0.517	−0.464	**−0.604***
Al	0.123	−0.415	**−0.557***	**−0.590***

* Indicates *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$.
Bold indicate that the correlation is significant.

2013; Will et al., 2010). Notably, we did not observe a significant enrichment of nitrogen-fixing taxa in the grassland terrace. The shift in the relative abundance of phyla such as Acidobacteria and Bacteroidetes along a pH gradient corroborates previous studies (Lauber et al., 2009; Rousk et al., 2010) and suggests that these phyla may be good indicators of soil characteristics. Interestingly, we observed a variable representation of subgroups in the Acidobacteria phylum across the chronosequence. Acidobacteria subgroups gp1 and gp3 appeared significantly enriched in the first terrace (pH = 5.4) contrary to subgroup gp2, which was significantly enriched in the T2–T3 terraces (pH = 4). Moreover, Alphaproteobacteria appeared more abundant in the T2–T3 terraces.

Apart from the direct pH effect, nutritive cations, N and C availability may also be important drivers of the soil bacterial communities (Lauber et al., 2009; Uroz et al., 2013; Yu et al., 2003). In our study, the significant C/N increase across the chronosequence correlates with a significant decrease of the abundance of Beta- Deltaproteobacteria and Flavobacteria confirming that C availability may be an important parameter. Such correlations fit very well with the proposition of classification of the soil bacteria according to nutrient availability done by Fierer et al. (2007), which differentiated the copiotroph from the oligotrophs communities. The ecological staircase of Mendocino seems to be a good model ecosystem to observe transition/succession of specialised communities from a copiotroph to oligotroph behaviour. Together, these data suggest a strong interaction between the structure of the soil bacterial communities and the quality and availability of nutrients.

Previous studies have revealed that the pygmy forest development was an adaptation of the tree species colonizing the neighbour terraces to the nutrient depleted upper terraces (Westman, 1978). In this study, we addressed the question of the possible relationship between such an adaptation and the taxonomic and functional structure of the soil bacterial communities colonizing soil under influence of pygmy and non-pygmy *P. muricata* trees. Although our soil analysis did not highlight significant differences between the two forest (T2, T3) terraces (only a non significant decrease of the CEC), previous analyses performed on the Mendocino site have clearly demonstrated that the intense podzolisation process has led to the development of very nutrient-poor soil mainly composed of quartz and to the formation a hardpan limiting root growth in the T3 terrace (Izquierdo et al., 2013; Jenny et al., 1969; Westman, 1978). In terms of microbiology, Yu et al. (2003) revealed on the same site a significant decrease of the global respiration, mineralization and nitrification activities along the fertility gradient. Previous studies have also demonstrated that the pygmy trees carry smaller and less diverse ectomycorrhizal communities on their root system compared to non-pygmy trees (Wurzbuger and Bledsoe, 2001; Wurzbuger et al., 2001). In addition, Northup et al. (1995a) proposed that the pygmy-associated mycorrhizal species were specifically adapted to nitrogen limitation and able to recover nitrogen directly from the polyphenol rich litter of the pygmy trees. The pyrosequencing survey of the bacterial communities performed in this study revealed that the oldest terrace considered in the study (T3), colonized by a pygmy forest, presented a significant lower diversity (OTU richness) compared to the T1 and T2 terraces and a higher abundance of Actinobacteria and OP10 phyla (T3 > T2) and of the subgroup gp1 of Acidobacteria. We revealed that genera such as *Mycobacterium* or *Solirubrobacter* were significantly enriched in the pygmy terrace (T3) contrary to *Acidisoma*, *Acidocella* and *Aquicella*, which were more abundant in the non-pygmy terrace (T2). Although our knowledge on these genera remains limited, all the bacterial genera enriched in the T2 terrace are described as acidophilic organisms, which may explain their enrichment in the more acidic terrace. For the T3 soil samples, the enrichment of *Mycobacterium* may be related to the high amount of polyphenol produced by the pygmy trees to detoxify aluminium from their environment and control nitrogen cycling.

Although the pyrosequencing approach gave a very comprehensive view of the structure and abundance of bacterial communities, it gives no information on the functional potential of these communities. The presence along the chronosequence of a common mineralogical parent material characterized by a decreasing nutrient availability raises the question: are soils under the influence of the non-pygmy trees more or less selective than pygmy trees for bacteria that effectively release nutritive cations from the soil minerals? The ability of bacterial communities to release nutritive cations from soil minerals was reported in other acidic forest soils (Calvaruso et al., 2010; Uroz et al., 2007) and several studies suggest that this ability correlates with nutrient availability (Calvaruso et al., 2006; Lepleux et al., 2012; Uroz et al., 2011; Uroz and Frey-Klett, 2011). Some bacterial genera such as *Burkholderia* and *Collimonas* have even been proposed as bioindicators of bacterial weathering potential in soil (Uroz et al., 2011; Leveau et al., 2010). Here, we demonstrated by a culture-dependent approach that the bacterial communities from the younger soil under influence of the non-pygmy trees (T2) were significantly more effective at solubilizing minerals than in the older soil under influence of the pygmy trees (T3). This would suggest that the younger soil (T2), still containing weatherable minerals, allowed selection of effective bacterial communities permitting a continuous supply of nutritive cations and a potential improvement of the tree nutrition. On the contrary, the older soil (T3), characterized by a quartz bed depleted of nutrients (Jenny et al., 1969; Merritts et al., 1991), allowed to the development of pygmy trees adapted to recycle nutrients and to preferentially utilize atmospheric deposits (Yu et al., 1999). This hypothesis fits very well with our knowledge of the pygmy tree physiology (Chapin, 1980; Westman, 1978; Yu et al., 1999) and with the recent results of Eckert et al. (2012), which showed that pygmy tree roots have evolved specific aluminium and inorganic phosphate transporters permitting the tree growth in these extreme podzolic soils.

5. Conclusion

In conclusion, the ecological staircase of Mendocino proves to be particularly useful to address the question of the possible relationship between nutrient availability and taxonomic and functional structure of the soil bacterial communities. To our knowledge, our study presents the first comprehensive view of the structure, abundance and functional potential of the bacterial communities colonizing the soil of the ecological staircase of Mendocino. Analysis of the first three terraces of the chronosequence showed a decreasing gradient of soil fertility and a significant correlation between this fertility and the complexity of the bacterial communities. These new data and especially the significant enrichment of specific taxa in the different terraces considered open new questions regarding the relationships between the soil bacterial communities and their environment, as well as their role in nutrient cycling, fertility and plant development. Additional studies are required to determine if these taxa are relevant bioindicators of the fertility status of the soils. At last, the ecosystem retrogression stage observed in the pygmy terrace due to the intensive podzolisation of the soil gave us one of the unique opportunities to test the possible relationships between soil evolution and the taxonomic and functional structuration of bacterial communities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.11.002.

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