FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Microbial community structure in vineyard soils across altitudinal gradients and in different seasons

Paola E. Corneo[1,2], Alberto Pellegrini[1], Luca Cappellin[1], Marco Roncador[3], Marco Chierici[3], Cesare Gessler[2] & Ilaria Pertot[1]

[1]Department of Sustainable Agro-ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), San Michele all'Adige, Italy; [2]Department of Environmental Systems (D-USYS), Plant Pathology group, Institute of Integrative Biology (IBZ), Swiss Federal Institute of Technology (ETHZ), Zurich, Switzerland; and [3]Department of Predictive Models for Biomedicine and Environment, Fondazione Bruno Kessler (FBK), Trento, Italy

Correspondence: Ilaria Pertot, Department of Sustainable Agro-ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM),Via E. Mach 1, 38010 San Michele all'Adige, Italy. Tel.: +39 0461 615515; fax: +39 0461 615500; e-mail: ilaria.pertot@fmach.it

Received 29 October 2012; revised 30 January 2013; accepted 31 January 2013. Final version published online 27 February 2013.

DOI: 10.1111/1574-6941.12087

Editor: Angela Sessitsch

Keywords
ARISA; soil microbial structure; altitude; vineyard; seasonality.

Abstract

Microbial communities living in nine vineyards distributed over three altitudinal transects were studied over 2 years. Fungal and bacterial community dynamics were explored using automated ribosomal intergenic spacer analysis (ARISA) and by determining bacterial cells and fungal colony-forming units (CFUs). Moreover, extensive chemical and physical analyses of the soils were carried out. Multivariate analyses demonstrated that bacterial and fungal communities are affected by altitude, which acts as a complex physicochemical gradient. In fact, soil moisture, Al, Mg, Mn and clay content are changing with altitude and influencing the bacterial genetic structure, while in the case of fungi, soil moisture, B and clay content are found to be the main drivers of the community. Moreover, other exchangeable cations and heavy metals, not correlating with altitude, are involved in the ordination of the sites, especially Cu. Qualitative ARISA revealed the presence of a stable core microbiome of operational taxonomic units (OTUs) within each transect, which ranged between 57% and 68% of total OTUs in the case of fungi and between 63% and 72% for bacteria. No seasonal effect on the composition of microbial communities was found, demonstrating that bacterial and fungal communities in vineyards are mostly stable over the considered seasons.

Introduction

Soil is an essential component of the ecosystem (Kennedy & Smith, 1995), and understanding the biological processes that take place in the soil is crucial for correct soil use and to preserve soil quality (Lavelle *et al.*, 2006). Soil quality is determined by its chemical, physical and biological components and how they interact (Kennedy & Smith, 1995). The biological component of the soil is mainly represented by microorganisms, which carry out important functions and play a key role in the food web chain (Wardle *et al.*, 2004; van der Heijden *et al.*, 2008; Pritchard, 2011). The study of microbial diversity and how it varies across space and time is therefore of great interest to ecologists (Stres & Tiedje, 2006). Moreover, the preservation of soil microbial diversity is crucial for a balanced agro-ecosystem, especially under increasing agricultural intensification (Vandermeer *et al.*, 1998). The protection and conservation of soil biodiversity has therefore economic as well as ecological implications (Gardi *et al.*, 2009), hence the importance of monitoring microbial diversity. Microorganisms can be affected by abiotic factors, such as temperature, moisture and soil nutrients, or by biotic factors, namely interactions with other microorganisms. Although many microorganisms in the soil are redundant (Vandermeer *et al.*, 1998; Nannipieri *et al.*, 2003) as their functions could be carried out by other microorganisms, it is important to understand how the environment affects communities of microorganisms. The effect of season on soil microorganisms has been addressed by several researchers, and it has been suggested that season-dependent abiotic parameters, such as soil temperature and moisture, could influence the microbial community structure. In addition, vegetation

cover undergoes significant changes throughout the season (Lavelle & Spain, 2001), and its contribution to organic matter and the nitrogen content of the soil could affect the composition of microorganism communities (Lejon *et al.*, 2007). The effect of season, either alone or combined with other biotic or abiotic parameters, has therefore been investigated in a wide range of environments, in conventional and organic farming systems, along fertilisation gradients of grasslands, in tundra soils, oak canopies, alpine meadows and subalpine forests. The seasonal effects observed in all these studies are highly dependent on the type of climate characterising the various environments, and, on the whole, the role season plays in microbial communities dynamics remains unclear (Bossio *et al.*, 1998; Bardgett *et al.*, 1999; Schadt *et al.*, 2003; Waldrop & Firestone, 2006; Lipson, 2007)

In most previous studies, phospholipid fatty acid (PLFA) was used to measure the effect of season on the bacterial and fungal biomass to assess changes in the soil community structure (Bossio *et al.*, 1998; Bardgett *et al.*, 1999). Although PLFA has been shown to be a useful method, it has clear limitations when it comes to determining the structure of communities (Pettersson & Baath, 2003). Molecular fingerprinting techniques to investigate the role of seasonal dynamics and environmental parameters have been rarely used (Smit *et al.*, 2001; Griffiths *et al.*, 2003; Kennedy *et al.*, 2005; Pereira e Silva *et al.*, 2011). Recently, a study based on next-generation sequencing (NGS) enabled a deep investigation of the impact of seasons in forest soil (Kuffner *et al.*, 2012).

Automated ribosomal intergenic spacer analysis (ARISA) has been previously shown to be a valuable and sensitive method for investigating overall changes in microbial genetic structure of communities consisting of unknown members and a powerful cultivation-independent technique, especially in the study of soil community dynamics (Lejon *et al.*, 2005; Savazzini *et al.*, 2008; Popa *et al.*, 2009), highly standardised (Hewson & Fuhrman, 2006) and suitable when dealing with big amounts of data compared with sequencing of rRNA genes (Ramette, 2009).

Most studies on soil biodiversity have been carried out on grassland soils, while only a few have been carried out in rural areas, a far more important environment with regard to agricultural production (Gardi *et al.*, 2009). A few studies have explored the total microbial community in woody-perennial agro-ecosystems such as vineyards (Steenwerth *et al.*, 2008; Fernandez-Calvino *et al.*, 2010), although without taking seasonal effect into account, so that the impact of seasonality, altitude and its connection with chemical parameters on the total microbial community in vineyard soils is still unknown. Vines are long-lived woody-perennial crops that are normally cultivated at different altitudes, and for this reason, the effects of altitude and chemical parameters on the grape ripening and on the wine produced have been previously investigated (Mateus *et al.*, 2001; de Andres-de Prado *et al.*, 2007). Our aim was to understand the effect of altitude, which may be viewed as a chemical, temperature and moisture gradient (Smith *et al.*, 2002), on the dynamics of total soil fungal and bacterial communities in different seasons. The study was carried out on soil samples collected in nine vineyards located along three altitudinal transects. The sites were selected on the basis of the same soil origin, texture and pH, and similar weather conditions. The impact of altitude, seasonality and physicochemical parameters on the microbial communities was evaluated at three different altitudinal levels. The total bacterial cells and fungal CFUs were measured at different sampling times in the various vineyards. The genetic structure of the bacterial and fungal communities was then assessed by ARISA. Comparison of microbial communities in a field experiment makes it possible to evaluate the effects of different factors simultaneously and to clarify the role of climatic and physicochemical parameters driving microbial community structure in vineyard soils.

Materials and methods

Study sites and sampling

The study area is located in northern Italy (Trentino region), which has a humid, temperate, oceanic climate. Precipitation is usually distributed over two maxima: in autumn and in spring. Viticulture is widespread in the region with Chardonnay the prevalent cultivar, accounting for about one-third of production (Caffarra & Eccel, 2011), and therefore, this variety was selected for this study. The study area comprised three altitudinal transects (T1, T2, T3) of vineyards managed according to integrated pest management (IPM) principles (http://www.fmach.it/Centro-Trasferimento-Tecnologico/Pubblicazioni/Iasma-Notizie/IASMA-NOTIZIE-VITICOLTURA-n.-1-dd.-22.03.2011). All vines were grafted onto Kober 5BB rootstock, and plants were between ten and fifteen years old. In each of the three transects, three sampling sites were selected within a radius of about 2 km, at 200, 450 and 700 m a.s.l. (S200, S450, S700). The first transect (T1) is located in the area from San Michele all'Adige up to Faedo, the second transect (T2) is located in the area from Rovereto up to Lenzima, and the third (T3) is located in the area from Trento south up to Vigolo Vattaro (Table 1). The selected sites have a chalky soil (Pinamonti *et al.*, 1997) with similar textures. The sites at the lowest and highest altitudes are monitored by automatic meteorological stations (http://meteo.iasma.it/meteo/)

Table 1. Location of the study sites and altitudinal level expressed as metres a.s.l. For each site, transects (T1-T2-T3) at the corresponding level of altitude (S200-S450-S700) are indicated.

Site	Location	Altitude	Latitude	Longitude
T1S200	S. Michele a/A	205	46° 11′ 32.38″N	11° 8′ 10.46″ E
T1S450	Villa Piccola	439	46° 11′ 48.36″N	11° 9′ 3.59″ E
T1S700	Faedo-Maso Togn	727	46° 11′ 48.99″N	11° 10′ 18.03″ E
T2S200	Rovereto	167	45° 52′ 30.48″N	11° 1′ 7.83″ E
T2S450	Isera	383	45° 53′ 17.23″N	11° 0′ 5.91″ E
T2S700	Lenzima	663	45° 52′ 26.50″N	10° 59′ 22.29″ E
T3S200	Trento south	219	46° 0′ 46.98″N	11° 8′ 8.65″ E
T3S450	Val Sorda	458	46° 0′ 44.09″N	11° 8′ 47.82″ E
T3S700	Vigolo Vattaro	659	46° 0′ 23.10″N	11°10′ 16.26″ E

which record soil temperatures (at 0–10 and 10–20 cm) and rainfall hourly. The sampling sites were chosen on the basis of their soil temperature profiles. Analysis of soil temperature profiles from a 10-year period (2000–2009) showed the soil temperature at the 200 m a.s.l. sites to be on average about 2 °C higher than at the 700 m a.s.l. sites. Average annual rainfall is 930–1030 mm at the 200 m a.s.l. sites and 1090–1330 mm at the 700 m a.s.l. sites.

Soil samples were collected in February and July in two consecutive years, 2010 and 2011, for a total of four sampling times (February 2010, July 2010, February 2011, July 2011). These sampling times were chosen because they represent the two extremes of soil temperature (−0.2 to 2.1 °C in winter, 18.1–23.1 °C in summer). In each of the nine sites, a W-shaped sampling design was used to gather composite samples (van Elsas & Smalla, 1997), with each 'W' covering an area of 250 m^2. Five composite samples per field, collected between two rows of grapevines, were obtained, each of them comprised five subsamples consisting of soil cores collected from the topsoil (2–15 cm) within an area of 2 m^2 using a sterile 50-mL falcon tube (Sarstedt, Germany). The first 2 cm of organic layer was removed. Soil cores were sieved separately to 2 mm particle size, and five biological replicates were created after pooling the five cores in equal amounts. Three replicates of 1 g of fresh soil for each composite sample were used for the microbiological analysis, and the remainder was then lyophilised and stored at −80 °C for subsequent molecular analysis. Gravimetric analysis was carried out to measure soil moisture content and to standardise the amount of fresh soil used for the microbiological analysis. The sampling at each of the four time points was carried out in the same area following the same sampling design.

Physicochemical analysis

A soil sample (1000 g) was collected from each of the nine vineyards at each of the four sampling times, for a total of 36 samples, for the chemical and physical analyses. Physicochemical analyses of each of the five composite samples were carried out individually after the first sampling in February 2010, but considering there were no significant differences in soil parameters between the five replicates, they were pooled at the subsequent sampling times.

The following physicochemical parameters were measured: three major groups of soil separates – total sand (2.0–0.050 mm), silt (0.050–0.002 mm) and clay (< 0.002 mm) – were determined by measuring the volumetric mass of the water–soil suspension and the distribution of the elementary particles by wet sieving and hydrometer; total soil organic matter (SOM) and total nitrogen content (N), determined by elemental analysis using the Dumas method; carbon/nitrogen ratio (C/N), calculated from total C and N; pH in water (1 : 2.5 soil/water ratio); total $CaCO_3$ by gas-volumetric determination of CO_2 after HCl treatment; Ca, Mg, K, exchangeable cations by extraction with ammonium acetate 1 M at pH 7; P using the Olsen method; total Fe, Al, Cu, Mn, Ni, Pb and Zn, quantified in aqua regia; soluble B by extraction with $MgCl_2$ (2 g L^{-1}). The analyses were carried out in accordance with Italian ministerial decrees (DM 13/9/99 and DM 11/5/92) concerning official methods for soil chemical analysis. pH values were classified according to the study by Bruce & Rayment (1983).

Microbiological analysis

Total cultivable bacterial and fungal CFUs were measured using classical microbiological methods. Triplicates of fresh soil (1 g) of each of the five composites were diluted in 10 mL of 0.9% NaCl solution, vortexed for 4 min and then agitated for 20 min at 200 r.p.m. Serial dilutions were made in falcon tubes (Sarstedt, Germany) containing 9 mL of saline solution. Total fungi were grown on potato dextrose agar (PDA, Oxoid, UK) with chloramphenicol (0.035 g L^{-1}, Sigma, MO) and streptomycin (0.018 g L^{-1}, Sigma), kept at 24 ± 0.5 °C and counted from the second day until the seventh day. For total bacteria growth, serial dilutions of four replicates of 20 μL of each composite were serially diluted (1 : 10) in a 96-well microplate (Sterilin Ltd, UK) filled with 180 μL of tryptic soy broth (TSB, Sigma) plus cycloheximide (0.1 g L^{-1}, Oxoid, UK). Microtiter plates were sealed with sterile tape (Sarstedt) to avoid evaporation and agitated at 27 ± 0.5 °C in the dark until no further growth was detected; a blank broth was used as control.

Bacterial growth was estimated visually, and the highest dilution showing growth was used to calculate the total bacterial cells size of a sample by the most probable number technique (MPN) (Briones & Reichardt, 1999). Cell numbers per gram of dry weight soil were calculated.

Soil DNA extraction and PCR amplification

DNA was extracted from 0.25 g of lyophilised soil from each of the five composite samples using a PowerSoil-htp™96-well Soil DNA isolation kit (MO BIO Laboratories, CA), following the manufacturer's instructions. For DNA quantification, 50 μL of the 50-fold diluted total DNA was added to 50 μL of a 200-fold dilution of Quant-iT™ PicoGreen (Invitrogen, CA) and agitated at 100 r.p.m. for 5 min at room temperature. Fluorescence was measured using a Synergy 2 Multi-Mode microplate reader (BioTek, VT) at 485-nm excitation and 516-nm emission. The amount of DNA in the soil was determined using serial dilutions of lambda DNA standard, provided with the PicoGreen probe (Invitrogen). The 18S rRNA – 28S rRNA internal spacer (ITS) of the fungal rRNA was amplified using the primer set FAM (carboxy-fluorescein)-labelled 2234C (5′-GTTTCCGTAGGTGAACCTGC-3′) and 3126T (5′-ATATGCTTAAGTTCAGCGGGT-3′), annealing, respectively, to the 3′ end of the 18S rRNA genes and to the 5′ end of the 28S rRNA genes (Sequerra *et al.*, 1997). Bacterial-specific primer ITSF (5′-GTCG TAACAAGGTAGCCGTA-3′) and the FAM (carboxy-fluorescein)-labelled ITSReub (5′-GCCAAGGCATCCACC-3′) (Cardinale *et al.*, 2004), annealing, respectively, to the 3′ of the 16S rRNA gene and to the 5′ of the 23S rRNA gene, were used to amplify the bacterial ITS region. The PCR mixture was prepared in a final volume of 25 μL containing 10 ng of template DNA, 2.5 μL of 10 × Taq buffer (Dream Taq DNA polymerase, Fermentas, containing 20 mM of $MgCl_2$), 0.2 mM of each dNTP (Fermentas, Canada, USA), 0.2 μM of each primer, 0.0006 g mL^{-1} bovine serum albumin (BSA) (New England Biolab, Beverly, MA) and 1.5 U of Taq DNA Polymerase. Cycling was carried out in a Biometra 96 TProfessional (Biometra, Germany) with an initial denaturation step at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s, and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 10 min for fungal ITS. For bacterial amplification, cycling was carried out as described (Cardinale *et al.*, 2004). PCR products were quantified (MassRuler™ Low Range DNA Ladder, ready-to-use, Fermentas) by electrophoresis on 1% agarose gel in TBE supplemented with ethidium bromide (0.5 μL mL^{-1}) (Sigma), and the bands visualised under UV light by Bio-Rad (Life Science Group, Italy).

Automated ribosomal intergenic spacer analysis (ARISA)

For this analysis, 1 μL of each PCR amplicon was mixed with 8.8 μL of Hi-Di™ formamide (Applied Biosystems, CA) and 0.2 μL of GeneScan™ 1200 LIZ™ size standard (Applied Biosystems), denatured for 5 min at 95 °C then cooled on ice before loading. The denatured amplicons were loaded on an ABI Prism 3130 xl Genetic Analyzer (Applied Biosystems) equipped with 50-cm capillaries filled with POP 7™ polymer (Applied Biosystems). Run conditions were set to 8.5 kV and 60 °C with a run time of 6700 s, as previously described (Pancher *et al.*, 2012). Size standard profiles were checked, and ARISA data were analysed using GeneMapper® 4.0 software (Applied Biosystems). The software converted fluorescence data to an electropherogram, which consists of a series of peaks, each representing a different length of the ITS region, and each characterised by a specific length, height and area. Fluorescence height and area were assigned in a normalised way. Presence–absence of each OTU provides qualitative information, while fluorescence and the area associated with each OTU provide information regarding the relative amount associated with each peak. The best-fit size calling curves were built according to the second-order least-squares method and the local southern method (Ramette, 2009). Original files obtained from GeneMapper® 4.0 were converted using custom Python (v. 2.7.1) scripts to obtain tables fulfilling the available R script for binning. Binning was performed in R 2.14 using automatic-binner script (Ramette, 2009). Only fragments larger than 0.5% of total fluorescence ranging from 100 and 1200 bp were considered. A binning window of 3 bp (± 1 bp) for fragments up to 700 bp, bins of 5 bp for fragments between 700 and 1000 bp in length and bins of 10 bp for fragments above 1000 bp were used to minimise inaccuracies in the ARISA profiles (Brown *et al.*, 2005). An operational taxonomic unit (OTU) is therefore a collection of amplicons within a range of ITS lengths, so each OTU represents more than one ribotype.

Statistical analysis

Principal component analysis (PCA) was performed using PAST 2.16 (Hammer *et al.*, 2001) on the physicochemical profiles of the nine sites, to visualise their ordination. The effect of altitude, sampling time and their interaction on the physicochemical parameters was tested by two-way nonparametric MANOVA (NPMANOVA) (Anderson, 2001). One-way ANOVA was carried out on the logarithm of each chemical parameter separately to assess the effect of altitude, and pairwise multiple comparisons were made using the Tukey's test at $\alpha = 0.05$, by Statistica 9 software

package (Statsoft; Tulsa, OK). Furthermore, a nonparametric Kendall rank test (KyPlot v. 2.0 Beta 15, Koichi Yoshioka 1997–2001) was carried out to assess the correlation between each chemical parameter and altitude. The effects of altitude and sampling time on the amount of cultivable bacterial cells/g soil and fungal CFUs were assessed by Kruskal–Wallis nonpararametric test using Statistica 9 and significance difference was set at $P < 0.05$.

A Kendall rank correlation test (KyPlot v. 2.0 Beta 15, Koichi Yoshioka 1997–2001) was carried out to assess the correlation between total fungal and total bacterial cells. The same test was used to assess correlations between total fungal and bacterial CFUs with soil moisture and between each physicochemical parameter and the total fungal CFUs and bacterial cells.

Relative quantity matrices of the bacterial and fungal profiles were firstly explored by PCA to assess effects of altitude and sampling time. Canonical correspondence analysis (CCA) was carried out on the same matrices obtained, to summarise and graphically represent the nine different sites and to correlate their ordination with the ecological patterns. Sites with similar community structures are close on the plot. CCA plots and correlation coefficients were generated using PAST 2.16.

Analysis of similarity (ANOSIM), based on 9999 permutations runs, was used to make multivariate comparisons on groups obtained with PCA and CCA. ANOSIM tests differences among defined groups in multivariate data sets, and it is a nonparametric test for the analysis of variance (Clarke, 1993). The ARISA matrix is firstly converted to a similarity matrix by a chosen similarity index (in the present study, Bray–curtis was chosen), and differences among groups are then calculated on this matrix by ANOSIM. Significance of *P*-values was corrected with Bonferroni correction (Ramette, 2007). A Kendall rank correlation test was carried out between scores on the first and second axis of each site at the four sampling times obtained by CCA and each physicochemical parameter, to estimate the significance of each parameter on the ordination of the samples.

VENNY software (Oliveros, 2007) was used to build a list for each site, consisting of the OTUs present at least one sampling time; each list was then compared with the lists for all the other sites in the same transect to determine shared OTUs. To assess the overall core of the three altitudinal levels, the OTUs in the three sites at the same altitude were merged and compared with the lists for the other altitudes. The same procedure was followed for the lists consisting of the OTUs present at all four sampling times, to determine the core microbiome of OTUs in each transect.

Results

Soil physicochemical characteristics

A first exploratory analysis on the physical and chemical data is provided by the PCA (Fig. 1a,b). PCA was carried out on all physicochemical parameters measured at the four sampling times to visualise the ordination of the nine sites. Samples corresponding to the same site and different sampling times cluster consistently, while there is a clear separation between different sites.

The nine sites had similar textures: medium-loam, silty-loam soil (29–45% sand, 45–65% silt and 6–13% clay) at T1, medium-loam, sandy-loam soil (40–57% sand, 34–49% silt and 5–12% of clay) at T2 and T3. The pH was similar in all vineyards and at all sampling times, ranging from a minimum of 7.3 to a maximum of 8, and classified as mild–moderate alkaline. Further details of the physicochemical analysis are listed in Supporting Information, Table S1 and Table 2.

The two-way NP-MANOVA test, which was used to assess the effect of altitude and sampling time on the physicochemical parameters, showed the presence of a highly significant altitude effect on the physicochemical parameters ($P = 0.0001$), while there was no significant effect of sampling time ($P = 0.9132$) and of the interaction of the two factors ($P = 1$). It is worth looking in details at each physicochemical parameter to assess how it was affected by altitude (Table 3). It is evident that most parameters significantly varied with altitude, except C/N, Ca, Fe, Pb, $CaCO_3$, sand and silt (Table 3). Interestingly, as suggested by a closer look at Table S1, SOM, N, B, P, Cu, Zn, K displayed a significantly higher level in the sites at 450 m a.s.l. than in those at 200 and 700 a.s.l., and this was confirmed by the Tukey's pairwise comparison. Hence, such parameters showed a non-linear response to altitude. Other parameters, for instance Mg and Mn, showed a general trend of increasing with increasing altitude, and therefore, a linear response to altitude was expected. In fact, a significant positive correlation of Mg and Mn with altitude was highlighted by a Kendall rank correlation test, the correlation coefficient being 0.52 ($P < 0.001$) and 0.43 ($P < 0.001$), respectively (Table 3). A significant negative correlation with altitude was instead found for clay (correlation coefficient -0.67, $P < 0.001$).

Bacterial cells and fungal CFU quantification

The average number of bacterial cells is ranging from a minimum of 1.48×10^7 cells g^{-1} dry soil in T2S700 in February 2010 to a maximum of 2.52×10^8 cells g^{-1} dry soil in T1S450 in July 2010 (Table S2). In the case of

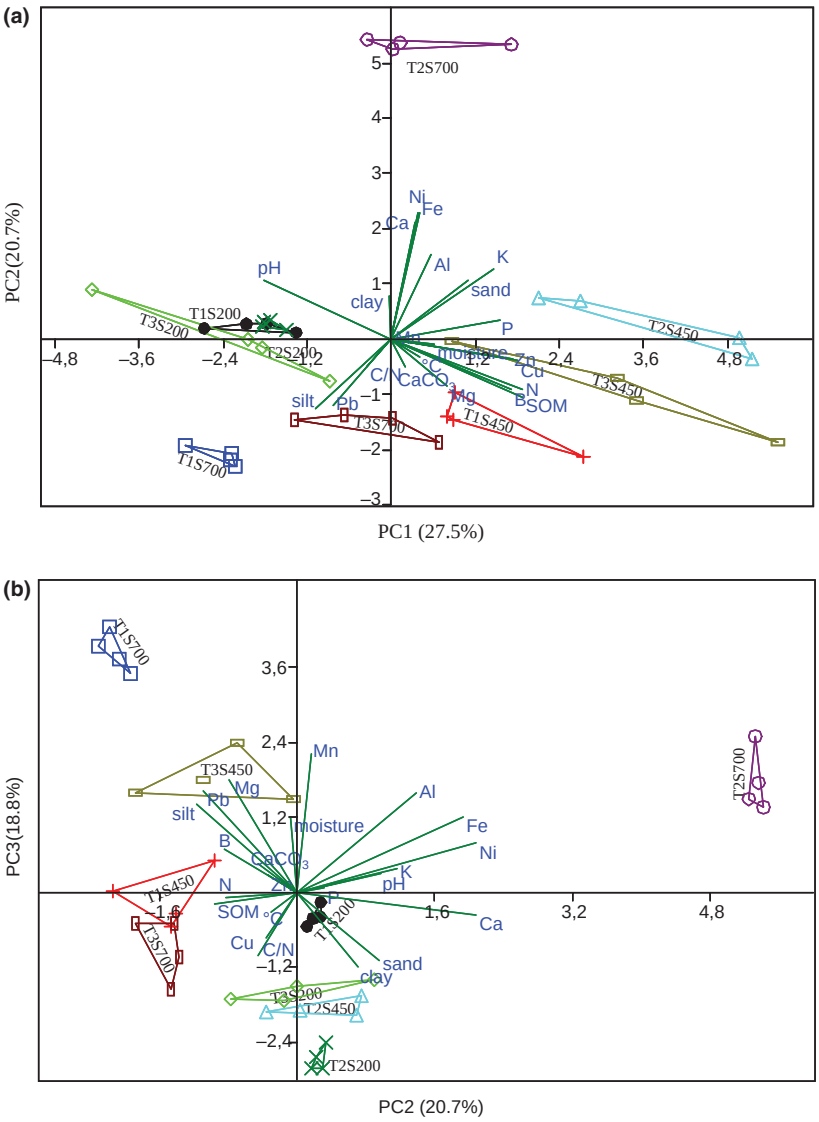

Fig. 1. PCA ordination of physicochemical parameters of the nine sites at the four sampling times. Convex hulls were used to connect the physicochemical profile of each site at the four sampling times, and the name of the sites is indicated inside each hull. Vectors are indicating the importance of each single parameter. Plot of PC1 and PC2 (a). Plot of PC2 and PC3 (b).

fungi, the minimum number was 2.5×10^4 CFUs g^{-1} dry soil, measured in T1S700 in February 2010, and the highest was 1.89×10^5 CFUs g^{-1} dry soil in T2S450 in July 2011. In July 2011, almost all sites present a higher number of fungal CFUs compared with all the other sampling times (Table S2).

The effects of altitude and sampling time were evaluated by Kruskal–Wallis test. For bacteria, there is no effect of altitude, but an effect of sampling time is present (Table 4), with total number of bacterial cells being higher in July 2011 compared with February 2010 (data not shown). Also, in the case of fungi, an effect of altitude was not detected, while a strong significant effect of sampling time was measured with fungal CFUs at July 2011 being significantly higher than all the other sampling times (data not shown). A positive correlation was found between fungal CFUs and moisture ($P = 0.017$, $\tau = 0.28$) by Kendall correlation test.

The Kendall correlation test showed also a positive correlations between bacterial cells and fungal CFUs ($P = 0.0000214$, $\tau = 0.49$). No correlation between the number of bacterial cells and moisture was found. Among all the physicochemical parameters, there was only a slight negative correlation between fungal CFUs and Ni content ($P = 0.049$, $\tau = -0.23$) (data not shown).

Qualitative ARISA profile of the microbial community

PCR amplicons loaded onto the capillary gel yielded electropherograms ranging from 180 to 1200 bp. After binning, the total number of unique OTUs detected in

Table 2. Values of soil moisture (expressed as percentage of water on grams of dry soil) and of soil temperature (°C) measured at the moment of sampling

	Moisture (%)				Soil temperature (°C)			
	February 2010	July 2010	February 2011	July 2011	February 2010	July 2010	February 2011	July 2011
T1S200	7.0	8.0	15.3	10.3	3.3	22.4	3.7	19.7
T1S450	39.0	11.9	23.5	21.2	2.8	21.5	3.2	19.4
T1S700	28.0	17.1	20.7	24.0	0.4	19.6	0.4	19.7
T2S200	22.0	5.4	19.0	8.9	3.8	23.1	4.3	17.0
T2S450	15.0	6.5	17.7	22.1	3.5	21.2	4.8	17.9
T2S700	31.0	7.4	23.5	22.3	3.3	19.2	1.8	16.0
T3S200	15.0	8.0	9.2	17.2	3.5	22.9	5.0	18.4
T3S450	37.0	17.0	14.1	23.7	3.8	19.5	5.1	20.5
T3S700	18.0	7.0	14.2	22.2	3.5	21.1	4.1	19.2

Values are indicated for each site at the four sampling dates. Transects (T1-T2-T3) at the corresponding level of altitude (S200-S450-S700) are indicated.

Table 3. Result of the one-way ANOVA performed on each chemical parameter considered separately to determine the effect of altitude (A).

Parameter	One-way ANOVA	Correlation *P*-value	Tau Kendall
SOM	0.000***	0.2307	0.1597
N	0.000***	0.1204	0.2072
C/N	0.13	0.0381*	−0.2756
B	0.000***	0.0381*	0.2733
P	0.000***	0.6093	0.0691
Ca	0.568	0.1044	−0.2131
Mg	0.000***	0.0001***	0.518
K	0.01*	0.8072	0.0326
Al	0.03*	0.0162*	0.3204
Fe	0.076	0.0552	0.2534
Ni	0.04*	0.1144	0.2131
Cu	0.000***	0.216	−0.1648
Mn	0.012*	0.0007***	0.4332
Zn	0.000***	0.4961	−0.092
Pb	0.803	0.8343	−0.0269
$CaCO_3$	0.621	0.818	−0.0321
Sand	0.231	0.7323	−0.06175
Silt	0.352	0.4468	0.13636
Clay	0.000***	0.0001***	−0.67365
Moisture	0.000***	0.0126*	0.3288
pH	0.000***	0.9109	-0.0154
Soil Temperature		0.2465	-0.1543

Probabilities of *F* values from one-way ANOVA significant differences are indicated: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Values of correlation (*P*-value) and coefficients of correlation (τ) calculated by Kendall rank correlation test. Significance levels are indicated: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Positive values of tau indicate a positive correlation, contrarily negative values represent a negative correlation. Values of tau = 0 indicate no correlation, while values of $\tau = 1$ represent a perfect correlation.

Table 4. Result of the Kruskal–Wallis performed on the total amount of bacterial cells and fungal CFUs to determine altitude and sampling time

Effect	Bacteria *P*-value	Fungi *P*-value
Altitude (A)	0.4204	0.5961
Sampling time (S)	0.0068**	0.000***

Significant differences are indicated as follow: $*P < 0.05$, $**P < 0.01$ $***P < 0.001$.

all nine sites analysed were 220 for fungi and 265 for bacteria. Fungal profiles displayed a predominance of peaks between 450 and 650 bp and between 700 and 850 bp, while the bacterial soil profile was characterised mainly by OTUs between 500 and 850 bp (data not shown).

With respect to presence–absence of OTUs, Venn diagrams evidenced very high numbers of conserved OTUs inside each transect (Fig. 2). In particular, analysing all the OTUs that were present at least once at a given site and at a given sampling time, the common bacterial OTU inside each transect ranged between 63% and 72% of the total 254 OTUs (Fig. 2a), and the common fungal OTUs ranged between 57% and 68% of the total 192–204 OTUs (Fig. 2b). The core OTUs of the three altitudinal levels were always higher in T3 than in the other transects. Merging the OTUs of sites at the same altitudinal level to investigate the global effect of altitude considering the sites at the same altitudinal level as replicates, it was found that 89.4% of bacterial OTUs (Fig. 3a) and 78.2% of fungal OTUs (Fig. 3b) were present at least once and were conserved across the three altitudes.

Fungal OTUs present at all the four sampling times accounted for 15.7% of the total in T1, 12% in T2 and 11% in T3, while in the case of the bacterial OTUs, 13.8% were persistent in T1, 16.1% in T2 and 18.1% in T3 (data not shown).

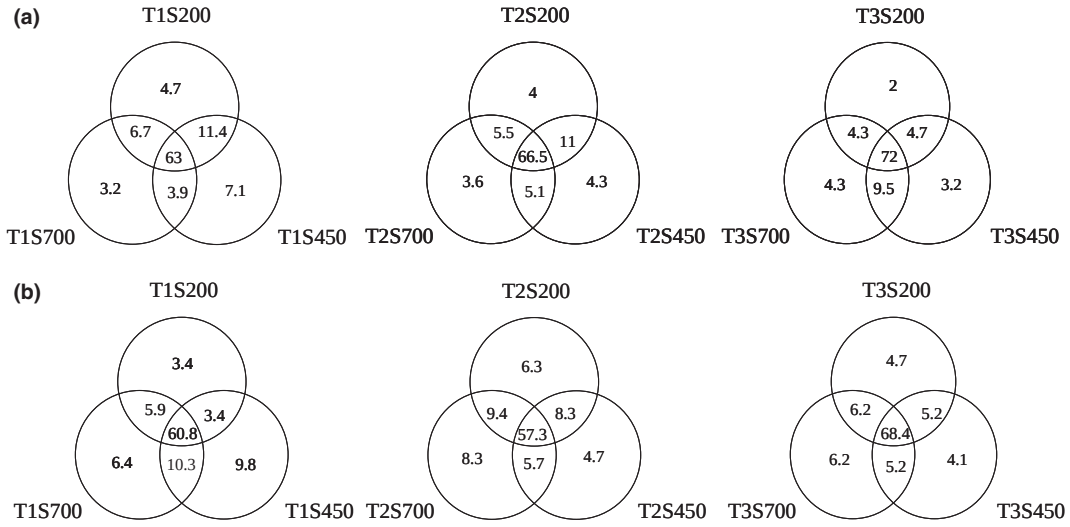

Fig. 2. Percentages of bacterial (a) and fungal (b) OTUs common to each of the three altitudes (200-450-700 m a.s.l.) within each transect (T1-T2-T3), common to two altitudinal levels within each transect or unique to each altitudinal level within each transect. All the OTUs present at least once in the four sampling times at each site were considered and plotted in the Venn diagrams.

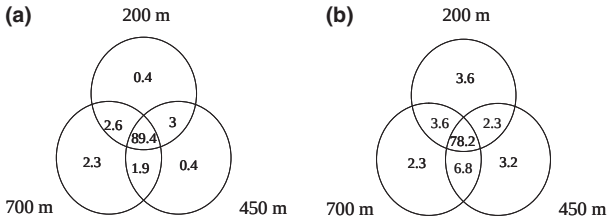

Fig. 3. Percentages of bacterial (a) and fungal (b) OTUs, common to each of the three altitudes (200-450-700 m a.s.l.), common to two altitudinal levels (T1-T2-T3) or unique to each altitudinal level. All OTUs present at least once in the four sampling times at each site were considered; those at the same altitudinal level were merged and then plotted in the Venn diagrams.

Relationship between genetic structure and altitude, sampling time and chemical composition

In the PCA of the soil bacterial (Fig. 4a) and fungal (Fig. 4b) community, each represented sample is the average over five biological replicates in the same site for a given time. PCA plots suggest an effect of altitude for both bacteria and fungi (Fig. 4). In fact, two-way ANOSIM test shows that such effect is significant ($P = 0.0001$) in both cases (Table 5). The same table also shows that no significant effect of sampling time is present as this is clearly highlighted by the PCA plot, where samples related to different sampling dates are overlapping (Fig. 5).

CCA has been used to dissect the influence of environmental factors on ARISA profiles of the microbial communities (Fig. 6). Environmental variables are represented by arrows whose length indicates the relative importance of each environmental factor in explaining variation in bacterial or fungal profiles. Correlation coefficients between sample scores on the first and the second ordination axes were calculated (data not shown). Altitude has a strong influence on bacterial communities (Fig. 6a), consistent with the findings of the two-way ANOSIM test. Altitude has a large positive correlation (0.62, $P = 0.0001$) with the first axis. Positive correlations are also found in the case of Mg, Mn and moisture (0.47, $P = 0.005$, 0.30 $P = 0.008$ and 0.31 $P = 0.008$, respectively). Sampling time is expected to show no influence and in fact its arrow is very short. The arrow related to soil temperature is also very short suggesting no important impact of this factor. Interestingly, a strong negative influence is that of clay (-0.73, $P = 0.0001$). Cu and Zn contents also display negative correlations with the first axis (-0.44, $P = 0.0014$ and -0.25, $P = 0.0089$, respectively). The second axis is correlated (positively or negatively) with Pb (0.48, $P = 0.0001$), silt (0.51, $P = 0.005$), sand (-0.52, $P = 0.0067$), Ca (-0.57, $P = 0.0013$). Such factors are mainly related to the geographical location of the site and not to the altitude, as it was previously pointed out by the analysis carried out on the physicochemical data. Other strong factors of variability of the bacterial communities are represented by Al, Fe, Ni with site T2S700 having a positive orientation in their direction.

In the case of fungi as observed in the case of bacteria, altitude has a strong influence on the ordination (Fig. 6b), showing a strong positive correlation with the

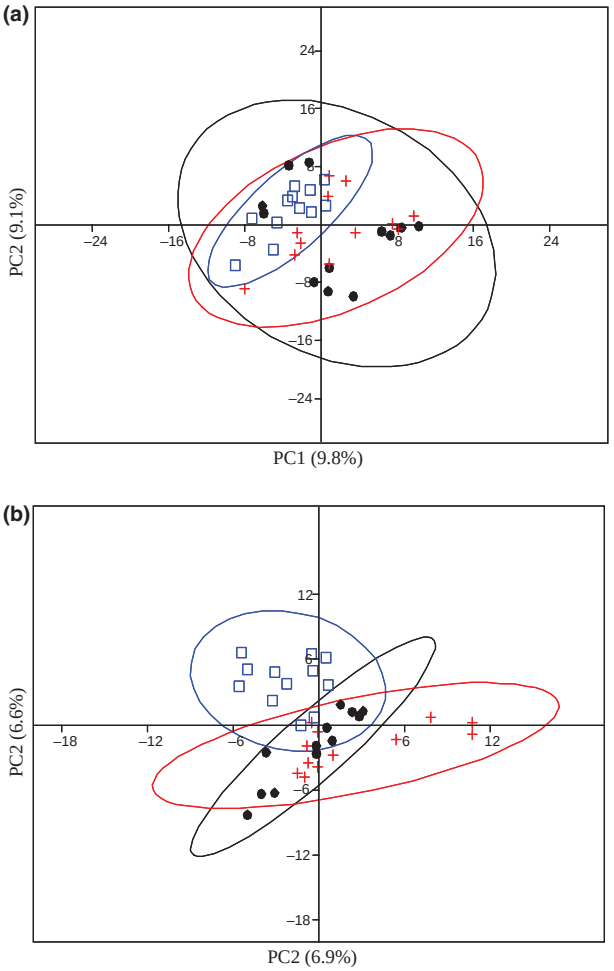

Fig. 4. PCA ordination of the soil bacterial (a) and fungal (b) community structure of replicates from each altitude (200-450-700 m a.s.l.) at the four sampling times (February 2010, July 2010, February 2011, July 2011) to visualise the altitude effect. Blue squares represent three sites at 700 m a.s.l., red crosses three 450 m a.s.l. sites and black dots three 200 m a.s.l. sites.

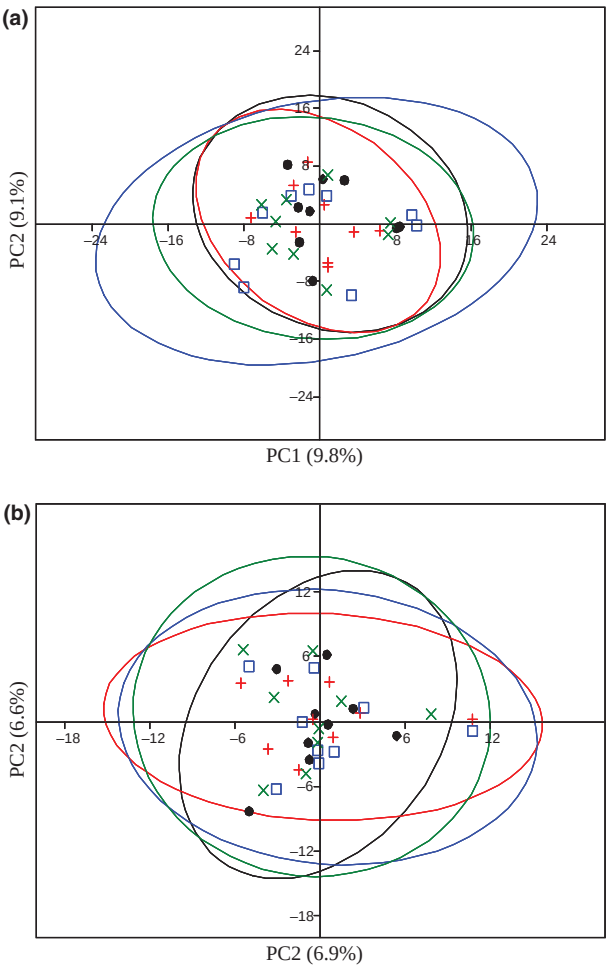

Fig. 5. PCA ordination of the soil bacterial (a) and fungal (b) community structure of replicates from each altitude (200-450-700 m a.s.l.) at the four sampling times (February 2010, July 2010, February 2011, July 2011) to visualise the (absent) sampling time effect. Black dots represent February 2010, red crosses represent July 2010, blue squares represent February 2011 and green crosses represent July 2011.

Table 5. Results of the two-way ANOSIM test. Altitude, sampling time and their interactive effect were tested on bacterial and fungal relative quantity matrices obtained by ARISA, to see the significance difference of the groups visualised by PCA and CCA ordination

Effect	Bacteria *P*-value	Fungi *P*-value
Altitude (A)	0.0001***	0.0001***
Sampling time (S)	0.5454	0.1358
A*S	1	1

Significant differences are indicated as follow:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

first axis ($P = 0.0005$). Positive correlations were also found for SOM, N and B (0.48, $P = 0.0004$, 0.50, $P = 0.0001$ and 0.49, $P = 0.0007$). As observed in the case of bacteria, sampling time and temperature do not sort any effect, while clay is exerting a strong effect, negatively correlating with the first axis (−0.47, $P = 0.0004$). Interestingly, pH is negatively correlated with the first axis, affecting the ordination (−0.43, $P = 0.0007$). The second axis is correlated positively with Cu (0.74, $P = 0.0001$), K (0.50, $P = 0.0001$), Zn (0.55, $P = 0.0001$), P (0.48, $P = 0.0001$), Ca (0.33, $P = 0.0009$) and clay (0.64, $P = 0.0001$) and negatively with Mg (−0.36, $P = 0.0008$).

Discussion

Our study demonstrated that altitude, behaving as a complex climatic and physicochemical gradient, has a

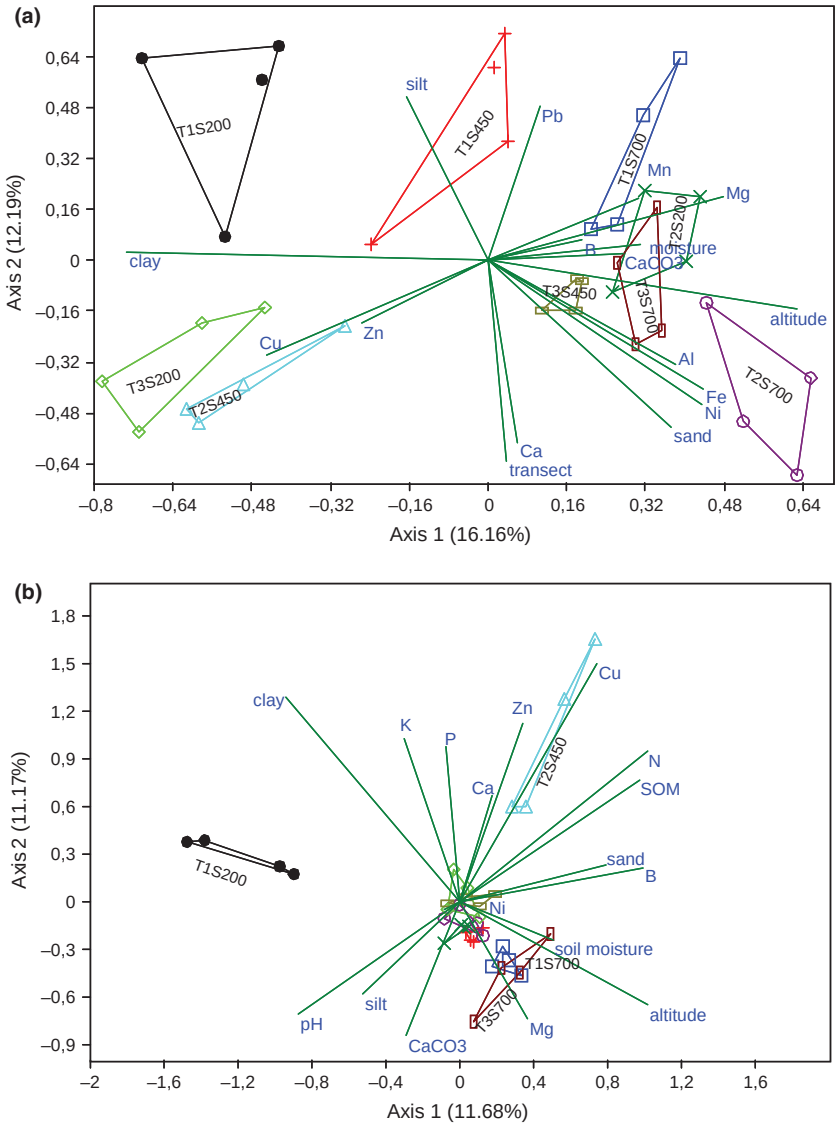

Fig. 6. CCA ordination plot of the soil bacterial and fungal community based on the relative quantity matrices of the bacterial and fungal profiles to summarise and graphically represent the nine different sites and to correlate their ordination with the ecological patterns. Different colours of the convex hulls were used to indicate the nine sites at the four sampling times (February 2010, July 2010, February 2011, July 2011). Only the vectors that were significant for the distribution of the soil microbial community of the nine sites were indicated. In the CCA plot of the soil fungal community, the sites (T2S200, T3S200, T1S450, T3S450, T2S700) were overlapping in the middle of the plot.

strong separating effect on the genetic structure of soil microbial communities and that, in our system, bacterial and fungal soil communities have different compositions at higher altitudes compared with lower elevations and respond differently to environmental parameters. In a previous study, altitudinal transect was used to investigate the effect of climatic factors on soil properties and on microbial activity in a semi-arid environment, and it proved to be a useful approach for shedding light on the role of temperature in a field study (Smith *et al.*, 2002). It was found that microbial biomass and respiration were not affected by elevation, while chemical parameters such as pH, electrical conductivity (EC), and total C and N were affected (Smith *et al.*, 2002); the relation between soil organic carbon (SOC) and altitude was previously shown to increase linearly with altitude in grassland soil (Leifeld *et al.*, 2005). In other studies, the effect of altitude and climate change on soil processes and on physicochemical properties was investigated without considering the effect on the soil microbial components (Dahlgren *et al.*, 1997; Riebe *et al.*, 2004). The effect of altitude of the physicochemical parameters was often dependent on the type of environment investigated and on the climate of the study site.

In our study, we first investigated the effect of altitude, simply considered as climatic and physicochemical gradient, on the total amount of cultivable fungi and bacterial cells and an effect of altitude was not found. Furthermore, microbiological approaches are cultivation dependent, and it has been shown in the past (Ranjard *et al.*, 2000; Kirk *et al.*, 2004; Savazzini *et al.*, 2008) that they are suitable for investigating only a small percentage of

the soil microbial community; thus, we also used a fingerprinting approach to gain deeper knowledge of altitude effect on microbial dynamics. Given its high resolution, ARISA has proved to be a more suitable method than other available fingerprinting techniques, such as DGGE and T-RFLP (Okubo & Sugiyama, 2009), for studying microbial genetic structure where communities consist of unknown members. It is especially suitable to compare microbial communities in different samples, considering the high level of standardisation of the method (Hewson & Fuhrman, 2006).

For a better comprehension of the altitude effect, we first analysed the qualitative output of the fingerprinting analysis, highlighting that the number of OTUs unique to each altitudinal level was very low, yet we found a highly conserved core microbiome consisting of a temporally and spatially stable group of OTUs. This means that, in qualitative terms, the microorganisms in the vineyard environment were conserved, even across a broad spectrum of sites under different abiotic conditions, confirming the result obtained by cultivation-dependent approach. This could be related to the effect of similar monoculture systems, which has been previously reported to negatively affect the quantity of DNA isolated in vineyards (Dequiedt *et al.*, 2011). A similar result also emerged from a previous study carried out on different soil types, where using denaturant gradient gel electrophoresis (DGGE), the authors displayed the presence of a set of well-conserved bands and changes in minor bands (Gelsomino *et al.*, 1999).

The use of OTUs as a measure of structure and function should be supported by further analysis, as it takes only qualitative data into account (Shade & Handelsman, 2012). It was for this reason that we supported our results with multivariate analyses of the relative quantitative data associated with each OTU, thus providing information on OTU evenness in the community.

A first exploitation of the relative quantitative data from the ARISA by PCA revealed the presence of a strong altitudinal effect, with the bacterial and fungal communities at the lowest altitude separating from those at the highest altitude, indirectly leading to consider the possibility of an effect due to abiotic parameters. Climate change is expected to raise temperatures, and consequently soil temperatures, and to modify rainfalls (Solomon *et al.*, 2007). Through the study of the impact of altitude, we aimed to obtain information about the impact of climatic parameters (e.g. temperature and moisture) on the microbial community living in vineyard soils.

The understanding of the impact of altitude, as climatic gradient, is of particular importance in vineyard environment, where vines, which represent one of the longest-lived woody-perennial plants, are normally cropped at different altitudes. The studied altitudinal transects represent a natural gradient of temperature and moisture. In fact, soil temperature is approximately 2 °C higher at 200 m a.s.l. sites than at 700 m a.s.l. sites throughout the year and soil moisture is positively correlated with altitude.

However, a deeper investigation of the same data by CCA, to understand their relationship with physicochemical parameters and not only the effect of soil temperature and moisture, led to identify chemical parameters as the main drivers in the separation of the communities at higher altitudes from those at lower altitudes.

The observed altitude effect is, in fact, the result of a complex physicochemical gradient that is differently affecting fungi and bacteria, although some parameters are both influencing the bacterial and fungal community structure. While moisture that is positively correlating with altitude is slightly affecting the soil bacterial and fungal communities, temperature did not sort any effect.

Moisture can indirectly affect pH, O_2, CO_2 contents (Barros *et al.*, 1995) or N release (Agehara & Warncke, 2005) or directly affect some classes of bacteria, such as nitrifying bacteria and ammonia oxidising bacteria (Stark & Firestone, 1995; Horz *et al.*, 2004) and fungal germination and growth (McLean & Huhta, 2000), thus having an effect on both bacterial and fungal soil communities.

On its hand, soil temperature is also known to affect bacterial and fungal behaviour (Lavelle & Spain, 2001), and for this reason, we expected an effect on the structure of the soil microbial communities. However, seasonal temperature shifts (summer-winter) and sampling time (February 2010, July 2010, February 2011, July 2011) did not sort any effect on the ordination of the nine sites as demonstrated by both NP-MANOVA and CCA. Passing from summer to winter, the soil temperature is gradually going from about 20 °C in summer to about 0 °C in winter, and we expected a change in the structure of the soil microbial communities; instead, within each of the nine sites, the genetic structure was relatively conserved in different seasons. In our study, sampling time affects only the quantity of cultivable fungi and not the viable bacterial cells, while no effects are measured on the soil microbial community structure.

The effect of soil temperature may be hidden by the stronger effect of the physicochemical parameters. In fact granulometry, Cu and slightly Mg are affecting both fungal and bacterial community structure. SOM, N, B and pH demonstrated an effect only on fungi, while Al, Fe, Ni and Mn mainly determined the ordination of the bacteria. Among these parameters, clay, B, Mg, Mn and Al are correlating with altitude, therefore helpful to explain the separation of 200 m a.s.l. sites from 700 m a.s.l. sites.

Clay minerals have been previously described for their influence on the soil properties and for their indirect

effect on the microorganisms (Filip, 1973). Clay binds soil particles together creating a more stable soil structure acting as an aggregator, so influencing SOC decomposition and turnover. Clay in particular tends to create closer contacts between particles forming bridges, especially under the effect of wet-dry cycles (Singer *et al.*, 1992), thus affecting soil water movement (Bronick & Lal, 2005). Raising soil temperatures can lead to an increase in the soil clay content as consequence of clay neoformation (Jenny, 1941); this could explain the correlation between clay content and altitude. Boron effect on microorganisms is mainly unknown as it is not an essential element for fungi and bacteria, but normally essential for plants (Nelson & Mele, 2007); however, some studies showed the ability of B to inhibit the growth of fungi (Bowen & Gauch, 1966).

In the case of bacteria, some other factors that are positively correlated to altitude, like Al, Mn and Mg, are some of the main drivers of the bacterial community ordination. Al is considered a toxic metal for microorganisms (Pina & Cervantes, 1996), and in the site T2S700, it was found at higher levels compared with the other sites, strongly influencing the microbial community. These metals should be toxic in conditions of acid pH that causes their solubilisation, this is not the case of our vineyards, which are characterised by a mildly alkaline pH. Anyway, some studies have linked the possible solubilisation of the Al to the presence of acid rains (Pina & Cervantes, 1996), thus giving an explanation of the higher level of these elements at the higher altitudes.

Differences in Mg, as registered in our sites, with positive correlations with altitude were another strong driver; Mg is in fact an essential ion for the bacteria, and so another element expected to influence the community structure (Pina & Cervantes, 1996).

The effect of Cu was found in the case of bacteria but not for fungi (Ranjard *et al.*, 2006). In the case of fungi also, the pH, although it undergoes to subtle changes with mildly alkaline pH in all the nine sites, is always higher at 450 m a.s.l. sites and influencing the fungal community structure. Studies in vineyard environment, where pH and Cu effects were specifically investigated, found that pH seemed mainly to affect the microbial phospholipids profile (Fernandez-Calvino *et al.*, 2010). In fact, pH had previously been highlighted as one of the main factors affecting microbial structure when sampling locations with similar climate and vegetation (Fierer & Jackson, 2006). In particular, fungal growth was found to be negatively correlated with pH values (Rousk *et al.*, 2009). Furthermore, fungi are affected by SOM, N, which were not correlating with altitude, but presented higher amounts in the middle altitude. SOM and organic N are essential elements for fungi, representing a source of energy and nutrients for soil microorganisms (Fontaine *et al.*, 2003; Lejon *et al.*, 2005) and therefore expected to affect the microbial structure.

In conclusion, altitude represents a physicochemical gradient that over time has been differentiating soil microbial communities living at different altitudes. Over time, the different climatic conditions may have affected the structure of the soil, indirectly affecting the microbial community structure. The physicochemical profile did not change over time, and there are greater similarities in the physicochemical patterns found in vineyard sites at the same altitudinal level than in those within a given transect, probably as a result of a complex and gradual process of change in the physicochemical structure. Instead, pH values are mildly alkaline in all sites, probably due to similar vineyard management practices (Fernandez-Calvino *et al.*, 2010) and to the chalky soil (Pinamonti *et al.*, 1997).

The presence of a conserved physicochemical pattern over 2 years of sampling provides further support for the view that seasonality does not affect the soil microbial community profiles and those differences in the individual physicochemical profiles of the nine sites are instead a strong driver.

Temperature does not affect the microbial community structure, probably because microorganisms acclimatise quickly to seasonal temperature shifts but are more sensitive to permanent, stable differences in physicochemical parameters, as occurs in an altitudinal gradient. As is generally recognised, physicochemical characteristics play a determining role in separating communities and help to shed light on bacterial and fungal behaviour. It is therefore important that the evaluation of environmental parameters is always coupled with the analysis of physicochemical profiles when carrying out field studies. Finally, we can conclude that the vineyard environment is a fairly stable ecological niche where monoculture has in the course of time selected a relatively constant microbial structure which is mainly unaffected by considered seasonal abiotic changes.

Acknowledgements

This project was funded by the Autonomous Province of Trento ENVIROCHANGE project. We thank Carmela Sicher for help with the microbiological analysis, Simone Larger and the FEM sequencing platform, Daniela Bertoldi for physicochemical analysis, Valerio Mazzoni and Ron Wehrens for statistical advices, Monica Rinaldi for advice on mapping and Andrew Hegerty for English reviewing.

References

Agehara S & Warncke DD (2005) Soil moisture and temperature effects on nitrogen release from organic nitrogen sources. *Soil Sci Soc Am J* **69**: 1844–1855.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **26**: 32–46.

Bardgett RD, Lovell RD, Hobbs PJ & Jarvis SC (1999) Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. *Soil Biol Biochem* **31**: 1021–1030.

Barros N, Gomezorellana I, Feijoo S & Balsa R (1995) The effect of soil moisture on soil microbial activity studied by microcalorimetry. *Thermochim Acta* **249**: 161–168.

Bossio DA, Scow KM, Gunapala N & Graham KJ (1998) Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microb Ecol* **36**: 1–12.

Bowen JE & Gauch HG (1966) Nonessentiality of boron in fungi and the nature of its toxicity. *Plant Physiol* **41**: 319–324.

Briones AM & Reichardt W (1999) Estimating microbial population counts by 'most probable number' using Microsoft Excel (R). *J Microbiol Meth* **35**: 157–161.

Bronick CJ & Lal R (2005) Soil structure and management: a review. *Geoderma* **124**: 3–22.

Brown MV, Schwalbach MS, Hewson I & Fuhrman JA (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7**: 1466–1479.

Bruce RC & Rayment GE (1983) *Analytical Methods and Interpretations Used by the Agricultural Chemistry Branch for Soil and Land Use Surveys*. Bullettin QB 82004 Queensland Department of Primary Industries, Brisbane.

Caffarra A & Eccel E (2011) Projecting the impacts of climate on the phenology of grapevine in a mountain area. *Aust J Grape Wine* **17**: 52–61.

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C & Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol* **70**: 6147–6156.

Clarke KR (1993) Non parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.

Dahlgren RA, Boettinger JL, Huntington GL & Amundson RG (1997) Soil development along an elevational transect in the western Sierra Nevada, California. *Geoderma* **78**: 207–236.

de Andres-de Prado R, Yuste-Rojas M, Sort X, Andres-Lacueva C, Torres M & Lamuela-Raventos RM (2007) Effect of soil type on wines produced from Vitis vinifera L. Cv. Grenache in commercial vineyards. *J Agric Food Chem* **55**: 779–786.

Dequiedt S, Saby NPA, Lelievre M, Jolivet C, Thioulouse J, Toutain B, Arrouays D, Bispo A, Lemanceau P & Ranjard L (2011) Biogeographical patterns of soil molecular microbial biomass as influenced by soil characteristics and management. *Global Ecol Biogeogr* **20**: 641–652.

Fernandez-Calvino D, Martin A, Arias-Estevez M, Baath E & Diaz-Ravina M (2010) Microbial community structure of vineyard soils with different pH and copper content. *Appl Soil Ecol* **46**: 276–282.

Fierer N & Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *P Natl Acad Sci USA* **103**: 626–631.

Filip Z (1973) Clay minerals as a factor influencing the biochemical activity of soil microorganisms. *Folia Microbiol* **18**: 56–74.

Fontaine S, Mariotti A & Abbadie L (2003) The priming effect of organic matter: a question of microbial competition? *Soil Biol Biochem* **35**: 837–843.

Gardi C, Montanarella L, Arrouays D *et al.* (2009) Soil biodiversity monitoring in Europe: ongoing activities and challenges. *Eur J Soil Sci* **60**: 807–819.

Gelsomino A, Keijzer-Wolters AC, Cacco G & van Elsas JD (1999) Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *J Microbiol Meth* **38**: 1–15.

Griffiths RI, Whiteley AS, O'Donnell AG & Bailey MJ (2003) Influence of depth and sampling time on bacterial community structure in an upland grassland soil. *FEMS Microbiol Ecol* **43**: 35–43.

Hammer Ø, Harper DAT & Ryan PD (2001) PAST: paleontological statistics software for education and data analysis. *Palaeontol Electronica* **4**: 1–9.

Hewson I & Fuhrman JA (2006) Improved strategy for comparing microbial assemblage fingerprints. *Microb Ecol* **51**: 147–153.

Horz HP, Barbrook A, Field CB & Bohannan BJM (2004) Ammonia-oxidizing bacteria respond to multifactorial global change. *P Natl Acad Sci USA* **101**: 15136–15141.

Jenny H (1941) *Factors of soil formation. A system of quantitative pedology*. Mcgraw-Hill, New York, NY, 281.

Kennedy AC & Smith KL (1995) Soil microbial diversity and the sustainability of agricultural soils. *Plant Soil* **170**: 75–86.

Kennedy NM, Gleeson DE, Connolly J & Clipson NJW (2005) Seasonal and management influences on bacterial community structure in an upland grassland soil. *FEMS Microbiol Ecol* **53**: 329–337.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Khironomos JN, Lee H & Trevors JT (2004) Methods of studying soil microbial diversity. *J Microbiol Meth* **58**: 169–188.

Kuffner M, Hai B, Rattei T, Melodelima C, Schloter M, Zechmeister-Boltenstern S, Jandl R, Schindlbacher A & Sessitsch A (2012) Effects of season and experimental warming on the bacterial community in a temperate mountain forest soil assessed by 16S rRNA gene pyrosequencing. *FEMS Microbiol Ecol* **82**: 551–562.

Lavelle P & Spain AV (2001) *Soil Ecology*. Kluwer Academic Publisher, The Netherlands.

Lavelle P, Decaens T, Aubert M, Barot S, Blouin M, Bureau F, Margerie P, Mora P & Rossi JP (2006) Soil invertebrates and ecosystem services. *Eur J Soil Biol* **42**: S3–S15.

Leifeld J, Bassin S & Fuhrer J (2005) Carbon stocks in Swiss agricultural soils predicted by land-use, soil characteristics, and altitude. *Agr Ecosyst Environ* **105**: 255–266.

Lejon DPH, Chaussod R, Ranger J & Ranjard L (2005) Microbial community structure and density under different tree species in an acid forest soil (Morvan, France). *Microb Ecol* **50**: 614–625.

Lejon DPH, Sebastia J, Lamy I, Chaussod R & Ranjard L (2007) Relationships between soil organic status and microbial community density and genetic structure in two agricultural soils submitted to various types of organic management. *Microb Ecol* **53**: 650–663.

Lipson DA (2007) Relationships between temperature responses and bacterial community structure along seasonal and altitudinal gradients. *FEMS Microbiol Ecol* **59**: 418–427.

Mateus N, Marques S, Goncalves AC, Machado JM & De Freitas V (2001) Proanthocyanidin composition of red Vitis vinifera varieties from the Douro valley during ripening: influence of cultivation altitude. *Am J Eno Vitic* **52**: 115–121.

McLean MA & Huhta V (2000) Temporal and spatial fluctuations in moisture affect humus microfungal community structure in microcosms. *Biol Fert Soils* **32**: 114–119.

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G & Renella G (2003) Microbial diversity and soil functions. *Eur J Soil Sci* **54**: 655–670.

Nelson DR & Mele PM (2007) Subtle changes in rhizosphere microbial community structure in response to increased boron and sodium chloride concentrations. *Soil Biol Biochem* **39**: 340–351.

Okubo A & Sugiyama S-i (2009) Comparison of molecular fingerprinting methods for analysis of soil microbial community structure. *Ecol Res* **24**: 1399–1405.

Oliveros JC (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Pancher M, Ceol M, Corneo PE, Longa CMO, Yousaf S, Pertot I & Campisano A (2012) Fungal endophytic communities in grapevines (Vitis vinifera L.) respond to crop management. *Appl Environ Microbiol* **78**: 4308–4317.

Pereira e Silva MC, Semenov AV, van Elsas JD & Salles JF (2011) Seasonal variations in the diversity and abundance of diazotrophic communities across soils. *FEMS Microbiol Ecol* **77**: 57–68.

Pettersson M & Baath E (2003) Temperature-dependent changes in the soil bacterial community in limed and unlimed soil. *FEMS Microbiol Ecol* **45**: 13–21.

Pina RG & Cervantes C (1996) Microbial interactions with aluminium. *Biometals* **9**: 311–316.

Pinamonti F, Stringari G, Gasperi F & Zorzi G (1997) The use of compost: its effects on heavy metal levels in soil and plants. *Res Cons Recycl* **21**: 129–143.

Popa R, Popa R, Mashall MJ, Nguyen H, Tebo BM & Brauer S (2009) Limitations and benefits of ARISA intra-genomic diversity fingerprinting. *J Microbiol Meth* **78**: 111–118.

Pritchard SG (2011) Soil organisms and global climate change. *Plant Pathol* **60**: 82–99.

Ramette A (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* **62**: 142–160.

Ramette A (2009) Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl Environ Microbiol* **75**: 2495–2505.

Ranjard L, Poly F & Nazaret S (2000) Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Res Microbiol* **151**: 167–177.

Ranjard L, Echairi A, Nowak V, Lejon DPH, Nouaim R & Chaussod R (2006) Field and microcosm experiments to evaluate the effects of agricultural Cu treatment on the density and genetic structure of microbial communities in two different soils. *FEMS Microbiol Ecol* **58**: 303–315.

Riebe CS, Kirchner JW & Finkel RC (2004) Sharp decrease in long-term chemical weathering rates along an altitudinal transect. *Earth Planet Sci Lett* **218**: 421–434.

Rousk J, Brookes PC & Baath E (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appl Environ Microbiol* **75**: 1589–1596.

Savazzini F, Longa CMO, Pertot I & Gessler C (2008) Real-time PCR for detection and quantification of the biocontrol agent Trichoderma atroviride strain SC1 in soil. *J Microbiol Meth* **73**: 185–194.

Schadt CW, Martin AP, Lipson DA & Schmidt SK (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science* **301**: 1359–1361.

Sequerra J, Marmeisse R, Valla G, Normand P, Capellano A & Moiroud A (1997) Taxonomic position and intraspecific variability of the nodule forming Penicillium nodositatum inferred from RFLP analysis of the ribosomal intergenic spacer and random amplified polymorphic DNA. *Mycol Res* **101**: 465–472.

Shade A & Handelsman J (2012) Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* **14**: 4–12.

Singer MJ, Southard RJ, Warrington DN & Janitzky P (1992) Stability of synthetic sand clay aggregates after wetting and drying cycles. *Soil Sci Soc Am J* **56**: 1843–1848.

Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S & Wernars K (2001) Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl Environ Microbiol* **67**: 2284–2291.

Smith JL, Halvorson JJ & Bolton H (2002) Soil properties and microbial activity across a 500 m elevation gradient in a semi-arid environment. *Soil Biol Biochem* **34**: 1749–1757.

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M & Miller HL (2007) *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I*

to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Summary for Policymakers.

Stark JM & Firestone MK (1995) Mechanisms for soil moisture effects on activity of nitrifying bacteria. *Appl Environ Microbiol* **61**: 218–221.

Steenwerth KL, Drenovsky RE, Lambert JJ, Kluepfel DA, Scow KM & Smart DR (2008) Soil morphology, depth and grapevine root frequency influence microbial communities in a Pinot noir vineyard. *Soil Biol Biochem* **40**: 1330–1340.

Stres B & Tiedje JM (2006) New frontiers in soil microbiology: how to link structure and function of microbial communities?. *Nucleic Acids and Proteins in Soil, Soil Biology*, Vol. 8 (Nannipieri P, Smalla K, eds), pp. 1–22. Springer-Verlag Berlin Heidelberg, Germany.

van der Heijden MGA, Bardgett RD & van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* **11**: 296–310.

van Elsas JD & Smalla K (1997) Methods for sampling soil microbes. *Manual of environmental microbiology*, (Hurst CJ, Knusden GR, McInerney MJ, Stetzenbach LD & Walter MV, eds), pp. 505–515. Manual on Environmental Microbiology ASM Press, Washington, DC.

Vandermeer J, van Noordwijk M, Anderson J, Ong C & Perfecto I (1998) Global change and multi-species agroecosystems: concepts and issues. *Agr Ecosyst Environ* **67**: 1–22.

Waldrop MP & Firestone MK (2006) Seasonal dynamics of microbial community composition and function in oak canopy and open grassland soils. *Microb Ecol* **52**: 470–479.

Wardle DA, Bardgett RD, Klironomos JN, Setala H, van der Putten WH & Wall DH (2004) Ecological linkages between aboveground and belowground biota. *Science* **304**: 1629–1633.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Average values of each physicochemical factor, at the four different sampling times and standard deviations of the four measurements.

Table S2. Total number of bacterial cells and fungal colony-forming units measured in transect 1, 2 and 3 measured per gram of dry soil of the nine sites in the four sampling dates (February 2010, July 2010, February 2011, July 2011).