DYNAMIC OF BACTERIAL COMMUNITY COLONIZATION IN HIGH-ALTITUDE MOUNTAIN ENVIRONMENTS

by

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ABSTRACT

Glacier forelands after glacier retreat are composed of harsh environmental niches characterized by severe climatic regimes and barren substrate with low total carbon and nitrogen content. Moraines represent ideal sites to study primary succession because glacier retreat releases an incoherent mineral substrate, where the primary succession and plant community establishment are key events in soil formation and fertilization. The underlying mechanisms driving this ecological succession remain still to be deepened. The physical and biogeochemical weathering processes provide soluble plant nutrient elements and when the plant colonization on parent materials occurs, the development of glacier foreland into fertile soils starts through rhizodeposition, exudation of nutritive substances, and decaying biomass. In these conditions, pioneer plants can select rhizosphere microbial communities able to promote plant growth thanks to the interactions with the system nutrient cycling. Moreover the rhizosphere community importantly contributes to the ecosystem functioning and carbon sequestration.

Main purposes of this study were: i) to characterize bacterial communities involved in soil neo-genesis processes under the environmental harsh condition and nutrient scarcity typical of high mountain moraines; ii) to assess the diversity, structure and role of bacterial communities associated to pioneer plant grown in a deglaciated alpine environment.

We studied first the Lobuche glacier moraines (Mount Everest area, Khumbu Valley, Nepal, about 5100 m a.s.l.), where we identified environmental niches characterized by different stages of biotic colonization, from bare mineral substrate to complex biological soil crusts (BSC). Seven sites of mineral proto-soil covered by BSC evidenced the ongoing soil development. The sites differed in several environmental parameters which could indicate different soil quality and developmental stages. Automated ribosomal intergenic spacer analysis (ARISA) showed distinctive bacterial community per each site and differences between the BSC and the below mineral substrate within the same site.

The second studied site was the Weisskugel glacier foreland in the upper Matsch valley within the upper Vinschgau Valley (South Tyrol). Upper Matsch valley showed a wide range of ecosystems: grasslands, bogs and poor fens, vertical rocky walls colonized by lichens, rocky glaciers, isolated pioneer plants, loosely organized floristic proto-communities, transition and mature grassland stages. The study area is below a glacier foreland at 2400 m a.s.l. The vegetation colonization was evolved since 1840 when the Matschter glacier began to retreat. The bacterial communities associated to different environmental matrices i.e. rock surfaces, proto-soils, riparian sediments, lichen thalli, and water springs biofilms were investigated by three molecular techniques with different taxonomic resolutions: denaturing gradient gel electrophoresis (DGGE), length heterogeneity-PCR (LH-PCR), and ARISA. Bacterial communities were mainly composed of Acidobacteria, Proteobacteria, and Cyanobacteria but variations occurred among the sites. Proteobacteria were more represented in sediments, biofilms, and lichens whereas Acidobacteria were mostly found in proto-soils and Cyanobacteria on rocks. Firmicutes and Bacteroidetes were mainly found in biofilms. UniFrac P values confirmed a significant difference among different matrices. Significant differences ($P < 0.001$) in beta diversity were observed at the genus–species level, except for lichens and rocks which showed a more similar community structure, while two distinct bacterial communities between lichens and rocks were found at deep taxonomic resolution.

In the same alpine ecosystem we investigated the effect of plant species on the rhizobacterial communities of 33 plant individuals belonging to 13 different pioneer species. We compared these bacterial communities to those from similar non vegetated patches as control. To obtain a culture-independent picture of the samples,

metagenomic DNA was extracted from both rhizosphere and bulk soil samples and analyzed by ARISA and DGGE. ARISA fingerprinting showed different genetic structure per each plant species, extremely different from bulk soil bacterial communities, and the DGGE analysis showed rhizosphere bacterial communities mainly composed by Acidobacteria and Proteobacteria. Unifrac P values of DGGE results confirmed the rhizosphere effect exerted by the different plant species ($P < 0.05$). We concluded that in early primary succession pioneer plant species can select distinct rhizobacterial communities. Moreover, the rhizosphere effect on the bacterial communities associated to 21 alpine plants belonging to 14 pioneer species within three floristic communities (RW, FI, M sites) was studied. When little sites surrounded by big stones (safe sites) are filled up of stone debris or mud, opportunistic pioneer plant species settle down and form new floristic consortia strongly influenced by the chemistry of the lytic materials. While RW site was a safe site of early developmental stage, FI site represented an intermediate stage and M site could be considered a later stage where floristic consortia are matured. ARISA fingerprints showed different bacterial genetic structure per each patchy floristic communities which differed also from the bulk soil (BS site) bacterial communities. ANOSIM and PERMANOVA analyses indicated as significant the differences among the ARISA profiles of the sites ($P < 0.0001$). When plants of the same species occurred within the same site, almost all their rhizosphere bacterial communities clustered together. The Unifrac significance value ($P < 0.05$) revealed significant differences between BS site and the vegetated sites with a weak similarity to the RW site. The intermediate plant colonization stage, FI site, did not differ significantly from the RW and the M vegetated sites. These results highlighted the peculiar effect of the different floristic communities rhizospheres on their soil bacterial communities. The percentage of N and C in the four soils confirmed a different developmental stage per each of the sites with a kind of gradient from BS through RW, FI up to the M site.

The biodiversity, structure and role of both the overall and the active rhizobacterial communities of the most common floristic associations in the valley (*Cetrario Loiseleurenion, Nardion strictae, Festucetum halleri*) were studied during the early and late growing season at two different time points of glacier retreat characterized by particular environmental parameters. ARISA and 16S rRNA gene pyrosequencing were applied to metagenomic DNA and RNA. The presence of nitrogen cycle key-players was detected with specific 16S rRNA gene PCR-DGGE and nifH gene pyrosequencing. The overall rhizobacterial community structure of the two transects in the early and late growing seasons were significantly different from the correspondent active rhizobacterial communities (PERMANOVA $P < 0.0001$). Within the overall bacterial communities, each plot differed significantly according first to the sampling season and then to the soil age, whereas within the active bacterial communities the plots clustered mainly according to the season. A marked shift in active Proteobacteria, Acidobacteria, Planctomycetes highlighted the different between the different vegetation plots, growing seasons and soil ages. Moreover, most of the rhizobacterial communities involved to N-cycle exhibited specific diversity according to the growing season. In high-altitude mountain environment, niches with different soil developmental stages coexist in the same area and different environmental constraints (growing season, site position, plant species) lead to the selection of specific pioneer bacterial communities characterized by peculiar taxonomic patterns and functional diversity.

Diversity and role of bacterial communities in soil formation and primary succession in alpine environments

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Abstract

Bacterial communities are key-players in proto-soil formation and pioneer plant colonization and growth. Especially in the high mountain environments, this process is fundamental to assess the pedogenetic processes in order to better understand the consequences of the rapid glacier melting and of the climate change. Here we review the most updated researches on this topic, particularly focusing on high mountain environments in temperate regions.

Introduction

The ecological research along foreland of a receding glacier represents a pivotal interface between the ecosystem development and primary successional stages. Along a glacier chronosequence, characterized by a set of sites with the same parent material and substrates (Walker et al., 2010), time is substituted by space and the distance from the retreating glacier is used as a proxy for soil age. The soil close to the glacier terminus is vegetation free and heterogeneously composed of distinct morphotypes i.e. recent sandy deposit, exposed rock materials, erosion channels, floodplains, and mudslides with low amount of carbon, nitrogen content and available nutrients.

Although the theory of primary succession was focused on plant establishment (Odum, 1969; Chapin et al., 1994; Connell and Slatyer, 1977), bacterial communities colonize freshly exposed substrates in the earliest stage of primary succession long time before vascular plants and lichens (Sigler et al., 2002; Sigler and Zeyer, 2002; 2004; Nemergut et al., 2007; Schmidt 2008; Nicol et al., 2005; Bardgett et al., 2007). Bacterial colonization is the first step of a cascade of processes which deal to the formation of a fertile soil where complex vegetation communities grow and development. Bacteria, archaea and fungi are crucial and fundamental actors capable of enriching proto-soil of nitrogen and carbon. On the other side, microbial respiration and denitrification act as opposite processes leading to a loss of nutrients and organic matter. In this complex equilibrium, microbes have to continuously answer to the habitat change and to the inter- and intra-species competition, through their ecological behavior and developmental strategies (r- or K-strategy).

Here we cover the dynamical processes of primary succession on high-mountain environments under the viewpoint of bacterial communities, enlightening their contribution in the pioneer plant establishment, growth, development and in the nitrogen cycle, one of the most important geochemical processes.

Colonization of high-mountain environments by microorganisms

On the glacier surface, microorganisms are carried by wind, dissolved ice (Segawa et al., 2005) or atmospheric precipitations (Sattler et al., 2001). Distinct sand storms carry microparticles of illite and smectite from Sahara desert to many alpine glaciers. Such kind of powder and fine particles can host several different bacterial cells, in viable or in quiescent form (Chuvochina et al., 2011, 2012). In an early primary succession bedrock, glacier sandy moraines, glacier stones, and glacier streams are colonized by bacteria come from atmosphere, glacier water or moraine watersprings. These bacterial taxa are able to adapt themselves to the alpine environment and to proliferate along the time, as it could be happened to strains affiliated to Actinobacteria, α-Proteobacteria, Deinococcus-Thermus, Cyanobacteria, and Bacteroidetes widely distributed in Sahara desert and found on Monte Bianco glacier (Chanal et al., 2006). Other bacterial cells may come from rainfall and snowfall. It has been recently shown the presence of an active bacterial community into clouds, opening fundamental questions about biogeochemical cycles in the sky (Sattler et al., 2001). In the last years, many researches were conducted to characterize bacterial communities in snow, glacier ice surface, cryoconite holes, glacier ice cores, glacier water (Steven et al., 2006; Lee et al., 2011; Margesin and Miteva, 2011; Edwards et al., 2013). Psycrophilic bacteria from different bacterial taxa were often found, even in active form, showing that glaciers are far to be only ice deposit and that glacier water, after ice melting, is already enriched of complex bacterial community (Margesin and Miteva, 2011). The psychrophilic bacterial species *Cryobacterium psychrophilum*, *Variovorax paradoxus* and *Janthinobacterium lividum* were specialized members of the snow biota, worldwide distributed in polar environment and high mountains (Segawa et al., 2005). Other important vectors of bacteria on glacier surface, especially in Alps and other strongly human-impacted glacier areas, are humans and livestock (tourism and sheep, cow or goat-farming) (Lee et al., 2011).

Bacteria taxa can be dispersed on glacier surface and selected by the harsh environmental conditions. Environmental characteristics deeply change during the day-time and along the seasons. Extremely cold temperatures chill bacterial cells, causing injuries in the cell wall and membranes and the final cell lysis. Under sunlight, stone surfaces can reach high temperature and let the ice melt. In Antarctica, summer temperatures normally range between -15°C and 0°C, but temperatures beneath rock particles can reach 17-20°C many hours per day (Mevs et al., 2000). Moreover, UV irradiation may damage DNA and enzymes causing the dead of the cell. Nutrient scarcity and the general lack of liquid water is another important limitation of growth. Some bacteria species seem to be able to answer to these environmental constrains growing and propagating in glacial ice or in soil permafrost. Their growth rate is strongly affected by temperature, oxygen and nutrient availability. For their rock/sand colonization and dispersion, microclimatic factors can impact the efficiency of their colonization success (Lipson, 2007). Temperature fluctuations on rock surfaces of about 40°C can exert a strong pressure on bacterial communities influencing enzymatic activities. These fluctuations can be higher depending on the rock mineral composition and color: i.e., blackish rocks such as oxidized iron rocks, black rock varnish or mica phyllosilicates minerals can absorb more light energy reaching quite high temperature under the sun (Mevs et al., 2000). UV radiation and freeze-thaw cycle can select microorganisms able to produce protective secondary metabolites, especially those selected on glacier surface. Soil moisture and texture which affect water and gas fluxes could be responsible for nutrient availability. pH and chemical composition of the bedrock (Mummey et al., 2005) are also important for the accessibility to mineral elements which favor microbial life. Siliceous rocks, broken as a result of freeze-thaw cycle, release mineral elements useful for bacterial growth such as apatite (phosphorous source), whereas calcareous rocks, dissolved by chemical weathering, usually do not release useful elements for microorganisms. Glacier veins, where water can move towards the valley, are enriched by oligominerals and carbon molecules from bedrock, dust, organic debris, pollution. This water provides nutrients and other elements necessary for bacterial growth (Price, 2000). Of course, spore-formers can be facilitated in the surveillance, but non-spore-forming can also be frequently found. While on clouds bacteria can be present at density of 1500 cells/cm^3 (Sattler et al., 2001), in snow they can be about 10,000 cells/cm^3 (Sattler et al., 2001). Ice cores from Swiss glacier showed a bacterial charge evaluated between 1×10^5 and 6×10^6 (Sharp et al., 1999), while in Guliya glacier (China) charges were between 1×10^4 and 5×10^5 (Christner et al., 2000). In cryoconite holes, on glacier surface, liquid water can be enriched by hydrocarbons and other nutrients, as well as, nitrogen compounds and rock mineral elements, helping the development of a relatively rich microbial community. This kind of complex community can sustain the growth and reproduction of animals, as demonstrated for Tardigrada, cold-resistant animals often found in these habitats (Kaczmarek et al., 2012). Moreover, the water can flux under the glacier surfaces, through cracks, enriching lower glacier layers of bacteria and nutrients. Recently, through 454 pyrosequencing, a great number of lithoautotrophic bacteria, autotrophic methane producing archaea and heterotrophic eukarya in the subglacial environment of Robertson glacier (Canada) was found, allowing the researchers to suggest their active contribution to the global carbon cycle over extended periods of time (Hamilton et al., 2013). Since it is well known that mountain glaciers are collectors of persistent pollutants such as pesticides, herbicides, halogenates and artificial radionuclides (Tieber et al., 2009), glacier environments are getting more attention from different points of view. The high natural mineral content (usually heavy metals or natural radioactive elements) led to the selection of bacteria resistant to metals and other toxic elements, thanks to cellular efflux pumps. Unfortunately for human health those resistant genes are strongly correlated to antibiotic resistance. Truly, antibiotic resistant bacteria can be easily found in glacier environments, associated to cryoconites (Brusetti et al., 2008) or ice cores (Segawa et al., 2013). In particular, while Brusetti et al., found uncharacterized ampicillin-resistant bacterial isolates in the Midtre Lowenbreen glacier cryoconites (Svalbard Islands), Segawa and coworkers showed a plethora of antibiotic resistance genes from both clinical and agricultural origin in at least 17 glaciers worldwide, except those in Antarctica.

Despite the harsh environmental conditions, autotrophs (Duc et al., 2009a; 2009b) and heterotrophs (Tscherko et al., 2003; Bardgett et al., 2007) may follow a succession playing crucial roles in ecosystem development. Besides the first step of soil development is characterized by geological and chemical processes, microorganisms can carry out mineral-bioweathering of bedrock surfaces (Gorbushina and Broughton, 2009) due to enzymatic reactions, pH reduction or release of oxalate, cianide, gluconic acid, siderophores (Mavris et al., 2010; Styriakova et al., 2012).

Bacterial strains belonging to *Arthrobacter, Janthinobacterium, Leifsonia* and *Polaromonas* or isolated from biofilms on rock substrates were able to dissolve granite, plagioclase and feldspar, thanks to oxalic acid excretion. Oxalic acid is highly corrosive against mineral rocks, dissolving the metal ligands by acidification of the mineral surface (Welch et al., 1999; Welch and Ullman, 1999). Oxalic acid can be accumulated in large amount when microorganisms are organized in biofilm, reducing the pH; free protons can wear oxygen away from minerals, weakening the chemical bond between the metal ion and the rock surface. The dissolved elements were incorporated in the biofilm matrix, which become a hotspot of nutrients on the bare bedrock. Moreover, cryptoendolithic *Cyanobacteria* could improve bioweathering during photosynthesis process, because of the substrate alkalization (Budel et al., 2004).The microbial activity facilitates the release of mineral cations essential for microbial growth.

Organic carbon may come from wind deposition of allochthonous organic matter i.e. plant and algal litter, organic deposition from living invertebrates and animals, soot, pollution or even dead animals and plants (Hodkinson et al., 2003). Carbon inputs are also originated by anoxygenic photosynthetic bacteria, oxyphototrophic *Cyanobacteria* and eukaryotic microalgae, which colonized cryoconite holes (Kastovska et al., 2007) and patch of snow (Sawstrom et al., 2002; Stibal et al., 2008a; 2008b). Moreover, ancient carbon pools may be used by heterotrophic microbial communities (Bardgett et al., 2007). Nitrogen fixation by microorganisms and nitrogen deposition by atmosphere (rain, pollutants, dust) are the only two processes to improve the amount of available nitrogen in the sandy moraines. Nitrogen fixation activity is low during the earliest pre-plant stages (Duc et al., 2009a; 2009b), as confirmed by the low abundance of the marker gene *nifH* found in those environments. Nevertheless, nitrogen deposition, and decomposition and mineralization of the organic matter could be the most important nitrogen transformation process (Brankatschk et al., 2011).

High bacterial diversity and new taxa strictly correlated to the carbon and nitrogen cycles were found in Antarctic soil (Ganzert et al., 2011). Acidobacteria seem to be more common in proto-soil with low carbon and nitrogen content, and neutral or alcaline pH, whereas Bacteroidetes seem to colonize acidic soil with organic matter supplied by mosses. Moreover, Acidobacteria were negatively related to the carbon mineralization process and they are thought to be essential actors in the balance of carbon cycle in high mountain environments.

Microbial development in primary vegetation successions

According to the vegetation succession theory, pioneer plants are the first colonizers of incoherent proto-soil, and, through a complex dynamical process, they lead to a plant community stable climax. This process is based on an orderly and continuing replace of one plant community by another one. This ecological development is affected by several environmental factors, such as local microclimate changes, mudslides, anthropogenic modifications. An ecological succession is mainly due to two contrasting physiological behavior: r-selection and K-selection. The r/K selection theory is based on simplified Verhulst model of population dynamics: $dN/dt = rN(1-n/K)$, where r is the maximum growth rate of the population (N), and K is the carrying capacity of its habitat. The fraction dN/dt is the dynamical growth of N with respect to time t (Odum, 1963). The two parameters r and K are important and have consequences on the rate of population growth. r-strategists are species which accentuate growth rate, producing a great amount of offspring and exploiting less competitive niches whereas K-strategists maximize K, with a more careful use of the natural resources and a stronger adaptability to live in high-competitive niches and investing more time and energy in fewer offspring.

While the r/K theory is well established in plant and animal ecology, in environmental microbiology is rarely deepened due to the extraordinary complexity of bacterial cell behavior. Surveillance, growth and reproduction are affected by different environmental factors and by the presence of competitors. Nevertheless, microbiologists consider bacterial species able to growth in laboratory conditions on carbon-rich media as a r-strategists, while K-strategists are bacterial species rarely isolated or intrinsically not culturable (Garland et al., 2001; Sigler and Zeyer, 2002). Genera such as *Bacillus* and *Pseudomonas* are believed to be r-strategists, while Acidobacteria and most of the Actinobacteria are considered K-strategists (Philippot et al., 2013).

Microbial ecologists tried to apply the r/K selection theory to the primary succession in high mountain environments. Ohtonen et al. (1999) firstly used molecular techniques to study microbial community composition from unvegetated sites, and in mycorrhizal and non-mycorrhizal pioneer plants from 20- to 80-year-old soils in a

primary succession along the forefield of Lyman glacier (Washington, United States).They measured microbial biomass with three independent methods. Microbial biomass increased over the successional time from the unvegetated soil to the vegetated soils due to a different community composition and to a community shift from bacterial-dominated to fungal-dominated communities. Hence, during the early stages of succession, the microbial community is not able to incorporate all the carbon substrate into its biomass, and thereby it increases respiration process. The later-stage microbial community is not able to reach high rate of respiration, being mostly linked to K-strategy, and remains in an "energy-saving state", accumulating carbon in the biomass. In this case, fungi are important carbon-sink of this habitat.

Many studies are conducted to assess the bacteria capability to colonize different successional stages. The first investigations of the initial colonizers of two receding glaciers (Sigler and Zeyer, 2002), Dammaglacier and Rotfirnglacier (Switzerland), showed the development of microbial populations from the youngest to oldest soil, due to the increase in microbial abundance and activity confirming the previous result of Ohtonen, who studied a different glacier forefield anyway. Following a different model of bacterial community succession, the composition of all the microbial communities from the Dammaglacier were significantly different, whereas the Rotfirnglacier microbial communities were quite similar. Moreover, the authors applied the ecological r/K selection to the microbial succession labeling microbial culturability as r-strategy (Garland et al., 2001). Opportunistic microorganisms able to grow on non-selective medium were dominant during the first 10 years of succession due to stress tolerance strategies against the harsher environmental conditions (Sigler and Zeyer, 2004). Microbial activity increased to a maximum in the 70 years soil, while decreased or reached a plateau in the 100 years soil. A shift from r- to K-strategists occurred in the late successional stages, and a metabolically more efficient population which invest few resources into reproduction was settled (Schipper et al., 2001; Ohtonen et al., 1999; Sigler and Zeyer, 2004).

Other studies about the same alpine environment were conducted and highlighted different aspects of the primary colonization. The bacterial community composition of proto-soils from siliceous or calcareous parental materials were not determined by bedrock type, being otherwise dominated by ubiquitous bacterial taxa, generally adapted to the oligotrophic conditions (Lazzaro et al., 2009). Consequently, the same authors designed a transplantation experiment of siliceous soil to calcareous one to assess possible shifts in bacterial community structure and diversity (Lazzaro et al., 2011). The natural growth dynamics of the bacterial populations did not change after the transplantation, and bacterial communities from bare soil, despite their simple species composition, could efficiently and dynamically answer to the changed environmental conditions. α-Proteobacteria were not affected by seasonal dynamics and the related physico-chemical variations, while Actinobacteria and Firmicutes showed markedly changes in abundance and composition.

Bacterial, archaeal and fungal communities on rock substrates from fine granite sand, near the glacier terminus, to well-developed soils, covered with vegetation, were studied to have a simultaneous picture of the microbial population. Shannon diversity index for Bacteria were high and constant along the forefield according to Schütte et al. (2009), but in contrast to Nemergut et al. (2007), who detected increasing diversity along the chronosequence. Different factors such as climatic conditions, soil texture, bedrock composition and soil age could influence the bacterial community structure. Proteobacteria, Actinobacteria, Firmicutes and Cyanobacteria were abundant in young soil, whereas Acidobacteria colonized especially old soils. To assess the dynamics of bacterial populations which could be limited by selection processes along the gradients of chemical and biological parameters, a two-dimensional sampling were performed in the same oligotrophic forefields (Hammerli et al., 2007). Sites of different age and vegetation showed significant differences in soil properties, such as pH, ammonium concentration, and total organic carbon although those variations were not particularly high as expected. Isolated strains of the genus *Pseudomonas* were tested for local adaptation by a reciprocal transfer experiment. Bacteria from young soil increased three-fold their fitness on King's B medium with sterile old soil. Bacteria from both young and old soils showed the same fitness performance on old soil. Bacteria from old soil showed same fitness both on young and old soil. The isolates did not show a significant interaction with their local environment and there was a limited local expansion of soil bacteria. This lack of local adaptation could be explained by environmental constraints such as exclusion of competition, different migration behavior of bacteria from young and old soil, or the ability of early colonizing bacteria to survive under the glacier ice.

Along an age gradient of 20-years of an early successional soil in Southeastern Peru, evenness, phylogenetic diversity and number of phylotypes increased up to the oldest soil (Nemergut et al., 2007). Moreover, during the first 4-5 years of the succession, photosynthetic and nitrogen-fixing bacteria increased dramatically the amount of nutrients and organic matter many years before the occurrence of mosses, lichens and vascular plants (Schmidt et al., 2008). At 0 m from the glacier tongue, samples were dominated by Comamonadaceae which probably come from glacier ice because they are widely distributed in cold environments such as glacier ice core (Sheridan et al., 2003) and Antarctic ice (Gordon et al., 2000). Some of those genera were N-fixing, but most of them were heterotrophs. In this case, microbial heterotrophs seemed to be the first colonizers and they were probably influenced by the same environmental factors which control other microbial communities.

Microbial growth dynamics after pioneer plant growth
When the vegetation appeared along a primary succession, it could be influenced by environmental factors, ecological competition and mineral nutrient stress (Grime, 2001) and plants could be distinguished in: competitors (low stress and low disturbance), stress tolerators (high stress, low disturbance), ruderals (low stress, high disturbance).
Pioneer plants could play a crucial role in soil development with alteration of parent materials, slope stabilization, accumulation of litter and rhizodeposition. Microorganisms can convert rhizodeposits in other organic substrates, which can improve soil water retention capability to hold water and nutrients. Moreover, plants supplies fixed sugars to microorganisms, which in turn supply to plants fixed nitrogen, bioavailable phosphate. Plant growth promoting rhizobacteria can also improve plant growth and health, protecting it against parasites (Singh et al., 2004).
Studies about the effect of pioneer plant on rhizobacterial community in alpine ecosystems were focused especially on single plants along a chronosequence, while studies about the effect of the overall plant cover regarded different ecological niches and pointed out the higher significance of environmental parameters on the bacterial communities. In Antarctic environments along a latitudinal gradient, bacterial diversity of dense vegetation from different location were comparable, whereas bacterial diversity of "fell-field" vegetation decreased with increasing of latitude (Yergeau et al., 2007a; 2007b). In permafrost vegetated soils characterized by meadow, steppe or desert steppe soil moisture, C/N ratio and pH were main driving factors of the microbial diversity (Zhang et al., 2012). In high elevated arid grassland, a strong plant effect was demonstrated for the perennial bunchgrasses *Stipa*, *Hilaria* and for the invading annual grass *Bromus* (Kuske et al., 2002).
Along the chronosequence of the Rotmoosferner glacier in Austria, a 4 years old soil did not exhibit plant colonization due to the short period of ecosystem development whereas the first pioneer plants appeared within 14-20 years old soils due to the specific adaptations to the environmental conditions. Plant in stable symbiosis with diazothrops occurred late up to the 48 years old soils, due to the scarcity of phosphorous, essential for the nitrogen cycle. Moreover, the energy required for the maintenance of the symbiosis is usually very high, and this is why stable symbiosis are rarer under these critical environmental condition (Merbach et al., 1999). Woody plants colonize occasionally very young soils, but, due to the low growth rates, they are often replaced by more competitive perennial herbs. During the succession, when the vegetation cover and the plant diversity increased, the heat stress which affect soil bacteria is reduced while soil moisture is maintained. Nutrient availability and root exudation increase microbial root colonization. The organic matter become accumulated and K-strategists are favored against r-strategists. Moreover, the abundance and the activity of bacteria increased during the succession due to the soil age and to the shift from annual vegetation to a perennial vegetation. Actually, perennial plants are known to be able to exude more carbon, increasing the nutrient availability in the late successional stages (Bardgett et al., 1999).
During the early succession, plant species could not select specific rhizobacterial communities, whereas pioneer plants on 15 years old soils in Glacier bay (Alaska) could select for specific rhizobacterial communities (Bardgett and Walker 2004; Knelman et al., 2012). In Glacier bay, the vegetation succession is faster due to a different developmental pattern based on different climates (Matthews, 1992). After 75 years, plant species effect on the rhizobacterial communities were observed because of the increasing range of plant exudation patterns, which favor the selection of specific microbial communities characterized by higher microbial diversity. The perennial pioneer

plant *Leucanthemopsis alpina*, characterized by extensive root development and longevity (Körner, 1999), was chosen to assess the influence of a pioneer plant on the rhizobacterial community along a chronosequence of soil development (5, 50, and 70 years) in the forefield of the Dammaglacier (Switzerland; Edwards et al., 2006). The pioneer plant colonization creates hotspots where microbial growth and activity resulted to be higher than in adjacent bare soil of the same age. The size of the overall bacterial community was not significantly affected by soil age or by the presence of *L. alpina*. But, interestingly, K-strategist bacteria colonized more efficiently the young soil where the plant was growing, while the r-strategists where commonly found in non-young soils, especially in the 50- and 70-year-old soils. This was maybe due to the increased concentrations of labile nitrogen and carbon. The rhizobacterial communities associated to *L. alpina* was clearly different from the interspace community in the young soils, but these differences tended to disappear in the older ones. The relative similarity of the profiles strongly reflected labile carbon and nitrogen availability. The influence of *L. alpina* depended on soil age and decreased from the youngest soils up to the minimal influence in the oldest.

On the other side, variable results were obtained assessing the influence of *Poa alpina* on the soil microbial community in primary succession along four different stages of grassland development on the Rotmoosglacier. In the pioneer stage, the rhizobacterial community was determined by the environmental conditions, whereas, in the later stages, plant selected for a specific microbial community related to soil properties and carbon supply (Tscherko et al., 2004).Variable results were also found when the same authors studied the impact of the pioneer plant *L. alpina* within isolated patches on biological and chemical–physical soil parameters. In this case, they found that *L. alpina* rhizosphere could influence bacterial communities even 20 cm far from plant. The contradictory results with respect those of Edwards et al., were explained because Edwards and coworkers sampled bare soil at only 15 cm from the plant, indeed under the effects of *L. alpina* rhizosphere.

Microbial role in pioneer plant growth promotion in high mountain environments
As shown above, mountain soil bacteria are key-players in biogeochemical cycles on rock surfaces, incoherent sands, proto-soil, and pioneer plant rhizosphere. Particularly in plant rhizosphere, the exchange taking place between plants and microorganisms at soil-root interface leads to stone micro-particles bioweathering and lisciviation, nitrogen and carbon enrichment, plant organic compound decomposition or mineralization, soil pH modification. In an alpine oligotrophic environment, bacteria drive the symbiotic or non-symbiotic nitrogen fixation due to nitrogenase enzyme able to reduce atmospheric N into soluble ammonium.

Ammonium is used by plant for the biosynthesis of enzymes, nucleic acids, chlorophyll and other macromolecules whereas plant provide bacteria with carbohydrates. The most studied microorganisms involved in nitrogen fixation are symbiotic nitrogen fixing bacteria associated to legume host plant. Rhizobia are a paraphyletic group mostly belonging to α-Proteobacteria (i.e. *Sinorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Azorhizobium*, *Allorhizobium* and *Mesorhizobium*). Other bacteria of the genus *Frankia* sp. has an actinorrhizal symbiosis with nodular rhizosphere of plants such as *Alnus*, *Myricae*, *Betula*, or *Coriaria* (Benson and Silvester, 1993; Kennedy et al., 1997; Simonet et al., 1999).

Nitrogen fixation in rhizobia is the result of a complex chemical and genetic dialogue between bacterial cells and plant roots. Among all the different rhizodeposits and exudates released by roots, flavonoids are the starter gun of the nitrogen fixation process. Flavonoids, released by the root cells, are diffused in the rhizosphere: concentrations equal to 10^{-9} M are enough to chemioactract mobile rhizobia, while 10^{-6} M are necessary to activate the expression of all the bacterial genes involved into root nodulation (*nod* genes).Three *nod* genes can produce nodA, nodB and nodC proteins, that can alter the morphologic development of roots, prompting the shape changes of root hairs. This shape change is promoted by the *nod* factor, a chitin with a fatty acid chain that promotes a rapid cell reproduction and the formation of the nodule. Then, bacteria may enter into the nodule and start the symbiosis (Dakora 1995; 2003). Several constrains can control the establishment of the symbiosis through many mechanisms including genetic compatibility check, environmental conditions, soil chemistry, rhizobial genetic range. If the symbiosis can move on, rhizobia are able to fix significant amount of gaseous N but, if the soil humidity increases drastically, N becomes more mobile, and leaching and denitrification processes significantly decrease N availability to the plant.

However, there are bacteria which can fix nitrogen in non symbiotic association with plant. A great number of non-symbiotic bacteria help plant growth by feeding plant individuals of a significant amount of nitrogen. β-Proteobacteria such as *Burkholderia* and *Herbaspirillum* are often associated to nitrogen-fixation, although several strains can act as plant or even-human pathogens (Hayat et al., 2010). The free-living diazotrophs *Azotobacter* species are more common in more mature soils, where a significant amount of reduced sugars are available. Other non-symbiotic nitrogen-fixers are some strains of *Clostridia* (Kennedy et al., 2004) more common in anaerobic soils, such as high-mountain marshlands, ponds, wet soils, which needs a higher C/N ratio. *Azospirillum* are usually found in close associations with the family of the *Graminaceae* (Kennedy et al., 2004), where it provides plants of nitrogen, but also promotes plant root-growth, increasing water, mineral and nutrient uptake from the surrounding soil.

Moreover, such asymbiotic nitrogen-fixers could be considered potential key-players in nitrogen accumulation after glacier retreat, when proto-soil without pioneer plants, has to be enriched of nutrients. *Cyanobacteria* are considered one of the most important bacterial taxon in deglaciated oligotrophic environment, since they can provide proto-soil of both fixed nitrogen (through nitrogen reduction) and fixed carbon (through oxygenic photosynthesis) (Chapin et al., 1991; Fritzsheridan, 1988; Davey et al., 1991; Kastovska et al., 2005). The relative importance of *Cyanobacteria,* however, is still debated, since in many cases, they represent only a minor part of the overall bacterial communities in proto-soils, even if the proto-soil is fully exposed to light (Esposito et al., 2013). Therefore, due to the low concentration of nitrogen, which is not part of the mineral composition of the initial soil, the foreland of a retreated glacier, represents also a unique habitat to study the asymbiotic nitrogen fixation to assess which are the main actors.

The first investigation about asymbiotic diazotrophic diversity in high altitude alpine soil was carried out by Duc et al. (2009). Acetylene reduction tests showed nitrogen fixation along the chronosequence with higher activity in rhizosphere according to previous studies of the influence of rhizosphere on the associated bacterial community, that showed how exudation and reduction of environmental stress (i.e. plant cover effect) help bacterial diversity (Tscherko et al., 2004; Miniaci et al., 2003). Nitrate reductase activity in early successional stage in Rotmoosferner glacier moraine was found to be 23-times higher in rhizosphere of *P. alpina* than in bare soil (Deiglmayr et al., 2006). With the increase of soil age, in rhizosphere of *P. alpina* the amount of nitrate decreased while ammonium increased. Authors explained that this observation was maybe due to nitrate immobilization by root or by microorganisms, rather than nitrification activities. Nitrifying microorganisms could be inhibited by heterotrophic bacteria in N-poor environments such as the glacier foreland (Bengtson and Bengtsson, 2005), while nitrogen fixation and ammonification could guarantee the ammonium amount in these soils.

Although nitrogen fixation is a complex process which requires several proteins, basis of the process is the nitrogenase reductase (*nif*) gene. The highly conserved amino acid sequence of nifH protein lets *nifH* gene an useful marker gene to assess the diazotrophs diversity in the environment. The diversity of *nifH* genes was generally found to be higher and with a broader environmental distribution in glacier moraines than in forest and agricultural soils (Poly et al., 2001; Rösch et al, 2005; Widmer et al., 1999). Actually in forest soils or in agricultural soils, *nifH* diversity was mainly due to Proteobacteria only. New *nifH* cluster or *nifH* genes referred to Firmicutes were widely distributed in 8 years old unvegetated soils in polar environments (Deslippe and Egger, 2006). The most common *nifH* sequences were related to *Geobacter* species, a taxon able to perform both oxic and anoxic metabolisms. Its physiologic plasticity is very useful to answer to environmental fluctuation between aerobic and anaerobic conditions, that happens during wet periods (snow melting, rainfall). *Cyanobacteria* are pivotal source of nitrogen in arctic and alpine environment where they colonize recently exposed bedrock. Cyanobacterial communities of the Damma glacier moraine were quite similar to the ones found in the biological soil crusts of the Chihuahuan desert, but in Damma region they were found to be not shaped in well-organized crusts. Maybe in such kind of high-mountain environments, *Cyanobacteria* are mainly symbiont of mosses, as frequently occurred in vegetation patches of mountain forelands (Solheim et al., 2004). Moreover, the nitrogen cycle in rhizosphere soil of *L. alpina* was characterized by quantifying functional genes involved in nitrogen fixation and mineralization, nitrification and denitrification at two time points along a chronosequence transect in Damma glacier valley (Töwe et al., 2010). The functional gene abundance did not change between time points, but they were more abundant in rhizosphere than in unvegetated soils. Since plants grown on 10 years old soils were

able to accumulated big amounts of nitrogen, plants on young soil were strongly more dependent on asymbiotic nitrogen fixation. The results were confirmed *in situ* by Brankatschk et al. (2010), who observed that early and transient successional stages were mostly dominated by N-fixation and organic matter mineralization, while nitrification and the following denitrification, which cause nitrogen loss, were increased in later successional stages, when plant cover and microbial associations were fully established.

Experimental design in high-mountain environmental microbiology
The experimental designs applied in high-mountain environmental microbiology have been changed since the last decade. While the earliest researches applied phenotypic analysis to understand microbial community behavior in such environments (Insam and Domsch, 1988; Ohtonen et al., 1999), the application of modern molecular tools have been become extensively used since last few years only. There is a variety of techniques to study the microbial diversity in high mountain environments. Traditional methods are based mainly on culturing methods, which use a variety of culture media designed to select for several different microbial taxa. Culture-based methods are important to isolate and study bacterial strains, but they are not the best tool to evaluate the overall microbial diversity, given that the conditions they offer are usually selective for a particular population of microorganisms. It is estimated that less than 1% of the known bacterial species can be isolated by using the traditional techniques, since the vast majority of microorganisms is not able to grow due to the lack of required environmental conditions that cannot be provided in the laboratory (Curtis et al., 2002). Other bacteria are intrinsically not cultivable, due to physiologic constrains such as quorum sensing growth limitations or the necessity to growth in co-culture with other species. Moreover K-strategists are less probable to be cultivated than r-strategists.

In the last decades several biomolecular methods have been developed to study uncultivable microorganisms, allowing a new perspective for the analysis of microbial communities diversity and structure. This approach is based on the Polymerase Chain Reaction (PCR). DNA extracted from the sample is analyzed to detect microorganisms. The most common approach is the PCR amplification of the 16S rRNA conserved gene. In particular this gene is broadly used because of its interesting features, namely its essential function, its evolutionary properties, and its characteristic of having highly conserved regions as well as species-conserved regions. Moreover, 16S rRNA gene sequences are commonly used as a housekeeping genetic marker to study bacterial phylogeny and taxonomy, mainly because it is present in all bacteria and the fragment is large enough (1,500 bp) for bioinformatics purposes (Woese, 1987; Neefs et al., 1993).

Once DNA has been amplified, a crucial point is the separation of the amplified fragment from the non-target sequences. Different techniques are available for sequences separation. Cloning libraries involve the ligation of the amplified genes into a plasmid vector and the transformation of *Escherichia coli*, followed by the screening of the obtained clones. Alternatively, fragments with the same size but different sequences can be separated thanks to the Denaturing Gradient Gel Electrophoresis (DGGE). This technique allows the analysis of different microbial communities simultaneously on the same gel. Sequences are separated because of the different dissociation behavior of the DNA fragments. After the run it is possible to cut the single bands and sequence them (Muyzer et al., 1996). The sequences identification is done by comparing them with those of known organisms in a large database such as Ribosomal Database Project (RDP) and GenBank.

Electrophoretic profiles can be used to represent the investigated microbial structure by displaying the community in a band or peak profile that can be used for statistic comparison. Basically, each band or peak represents a taxon. This approach is useful to assess microbial communities differences among samples. If applying automated methods, fragments are marked with a fluorescent chromophores, separated trough capillary electrophoresis and detected by a "CCD camera" after being excited by a laser-light. Among these methods, Terminal-Restriction Fragment Length Polymorphism (T-RFLP) implies that the 16S rRNA gene is amplified by using two specific primers with two different fluorophores, and then digested with a restriction enzyme. The laser will detect only the terminal fragments obtained after the restriction. The different sizes of the digested terminal fragments of the 16S rRNA gene represent the different taxa (in some cases at the genus level) (Liu et al, 1997). Microbial structure can be studied as well with another automated method called Automated Ribosomal Intergenic Spacer Analysis (ARISA), based on the investigation of the amplified intergenic region between the 16S and 23S rRNA genes (ITS). Being able to detect differences up to a single-nucleotide, the technique shows a high resolution analysis, up

to the subspecies level, and reproducibility is guaranteed by instrumental automatism (Fisher and Triplett, 1999; Cardinale et al., 2004).

Although automated techniques to analyze microbial communities profiles give an huge amount of information with respect to traditional techniques, they are not sufficient to describe microbial diversity in depth. Next-generation sequencing is becoming a routinely used technique, able to provide deeper insights into complex microbial communities. Pyrosequencing is a flexible, parallel-processing, and easily-automated method for DNA sequencing, with a higher throughput and coverage of phylotypes compared to other techniques. One of the primers used to amplify the fragment of interest is modified with biotin. The fragment is mixed with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase, the substrates adenosine 5' phosphosulfate (APS) and luciferin. Later, the four nucleotides are added one at a time, iteratively, in the nucleic acid polymerization reaction. Pyrophosphate (PPi) is released during the ATP-conversion operated by ATP sulfurylase, and light is emitted, while luciferin is converted into oxyluciferin. This latter reaction is catalyzed by luciferase. The light produced emits a signal, detected by a camera, proportional to the number of nucleotides incorporated during DNA synthesis. The process is repeated with each one of the four nucleotides (dAGP, dGTP, dCTP and dTTP) until the DNA sequence of the single stranded template is synthesized. The sequential collection of images taken by the camera is analyzed to measure the light intensity in order to work out the amount of a specific dNTP incorporated in a given attempt. The imagines analysis permits to calculate the number of sequences per each bead (Dewell et al., 2005; Sogin et al., 2006).

Additional techniques involve MySeq and HiSeq sequencers (Illumina), that can provide millions of DNA reads from a sample.

Since the most informative techniques are linked to the high-throughput DNA sequences, a technology available since last few years, most of the published papers related to the glacier moraines are based on old-dated techniques, or on T-RFLP and DGGE. The application of such new techniques will increase the robustness of these kind of analysis. Moreover, the extension of the experimental designs on metagenomic RNA will help to understand the role of the active bacterial communities, defining the key-players in these complex environments. Lazzaro et al., (2012) tried to identify the dominant physiologically viable microorganisms in the unvegetated forefields of two receding glaciers, Damma and Tsanfleuron (Switzerland) and to relate possible functional changes with different composition of the viable bacterial communities. Application of Reverse Transcription-T-RFLP on metagenomic RNA allowed to recognize that viable-bacterial abundance was not affected by seasonality in vegetated-free soils. Pyrosequencing or extensive metagenomic approach on functional genes linked to nitrogen cycles, as well as, other metabolic processes can improve our comprehension of the high mountain systems. With this respect, any kind of environmental microbiological experiments will benefit of a more holistic approach, taking account the multidisciplinarity such as soil chemistry, phytosociology, hydrology, glaciology, pedology, geochemistry and geology.

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Microdiversity in the earliest stages of ecosystem succession in a high elevation mountain moraine in Himalaya

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Abbreviations: BSCs: Biological Soil Crusts; DLs: Deep layers

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Abstract

The diversity and structure of bacterial communities inhabiting seven biological soil crusts (BSCs) and related deeper layers (DLs) were investigated in a high elevation mountain moraine. The influence of physical-chemical parameters and the bacterial taxa involved in the nitrogen cycle were also studied. Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprints showed a co-clustering of BSC and DL within each site and DistLM multivariate analysis confirmed significant relationship of microbiome structure with the peculiar biological, climatic, and chemical parameters of each site. The BSC bacterial communities differed from the ones of the DLs in terms of bacterial taxa composition. While *Acidobacteria*, α- and β-*Proteobacteria*, *Cyanobacteria*, *Clostridia* and *Sphingobacteria* were widely distributed in both BSCs and DLs, *Gemmatimonadetes* and *Anaerolinae* were retrieved only in BSCs, whereas *Actinobacteria*, γ-*Proteobacteria*, *Bacteriodetes*, *Flavobacteria* and *Firmicutes* were associated to DLs only. Moreover, the microbiological biomass of the BSCs was forty times higher. AOB population, probably composed of cold and desiccation tolerant, were also identified. BSCs may contribute to increase the natural heterogeneity of soil properties at a centimeter spatial scale.

Introduction

The biodiversity of microbes in mountain ecosystems has received increasing attention with regard to climate warming concerns, glacier retreats, soil reclamation and shift in the distribution of vascular plants. The moraines of receding glaciers is constituted by a barren mineralogical substrate released from ice that is extremely nutrient poor with little nitrogen, no organic carbon or available phosphorus (Schulz et al., 2013). As succession proceeds, this oligotrophic substrate, as a result of the interactions with the climatic and biotic components of the ecosystem, undergoes to physical, chemical and microbiological transformations that finally result in the generation of conditions suitable for plant biocenosis. Thus, the forefield of glaciers provides a unique opportunity to study the first stages of soil neogenesis. In young successional stages, pioneer microorganisms are among the first players in colonizing moraine barren debris, providing crucial ecosystem services for element biogeocycling, water sequestration, nutrient accumulation and soil development. This stage of succession can last for several years in high elevation environments (Schmidt et al., 2008). In association with lichens, mosses, pioneer microorganisms persist in complex structures named Biological Soil Crusts (BSCs). BSCs are considered as architects of the soil in arid environments, quickly establishing on barren substrate and affecting its composition through their microbial activities. On the other side, the physical and chemical composition of the soil matrix, like pH, organic matter and redox conditions, affects the dynamics of the structure and functionality of the associated microbial communities, in a complex interplay between the biotic and abiotic components of the ecosystem (Schulz et al., 2013). The role of Cyanobacteria in BSCs has been largely reviewed, with regard to their role in primary productivity, while little attention was devoted to the structure and functionality of bacteria in the first stages of succession, before plant establishment, on mountain environments. Bioweathering, exerted by bacteria and fungi, is a key mechanism for the improvement of water retention and nutrient availability in moraine barren substrate (Mapelli et al., 2011; Mapelli et al., 2012). A time-independent mechanism, driven by bacterial bioweathering activity, is responsible for the generation of oasis of fertility in the Midtre Lovenbreen moraine in Svalbard islands (Borin et al., 2010). Rusty strips were observed around several conglomerate rocks in the moraine, surrounded by a flourishing area, densely colonized by mosses. The chemiolithoauthotrophic activity exerted by an iron-oxidizing bacterial community, including *Acidiothiobacillus ferrivorans* as a dominant species, was able to bioweather and leach minerals from pyrite-rich stones, triggering key changes in the moraine matrix composition (Borin et al., 2010; Mapelli et al., 2011; Mapelli et al., 2012). The release of iron oxyhydroxides from the pyrite rocks determined a gradient of low pH that, in turn, was responsible for an increase of the cation exchange capacity and a higher water holding capacity. Such physical-chemical modifications in the composition of the moraine substrate were the drivers for enhancing nutrient availability and improve soil structure, in association with carbon and nitrogen fixed by Cyanobacteria (Borin et al., 2010). Thus, the increase of water activity (A_w), mediated by bacteria metabolism, represents a crucial factor to generate suitable physical-chemical condition for plant establishment, followed by the cascade of events that finely result in soil maturation. An intriguing question is if similar mechanisms of forefield primary colonization occurs worldwide, independently from the geographical locations. We investigated a site of primary colonization located at high altitude and low latitude in the Himalayan Mountains in the moraine of the

Lobuche glacier (5050 m a.s.l), focusing on the characterization of the bacterial counterparts. The Himalayan plateau is a threatened environment, characterized by extreme cold and dry conditions, resulting in a minimal liquid water input to soil. In the Lobuche glacier forefield, seven spotted sites of primary colonization, hosting BSCs, were studied, with the aim to unveil the possible contribution of bacteria in modifying the geochemical properties of the moraine barren soil.
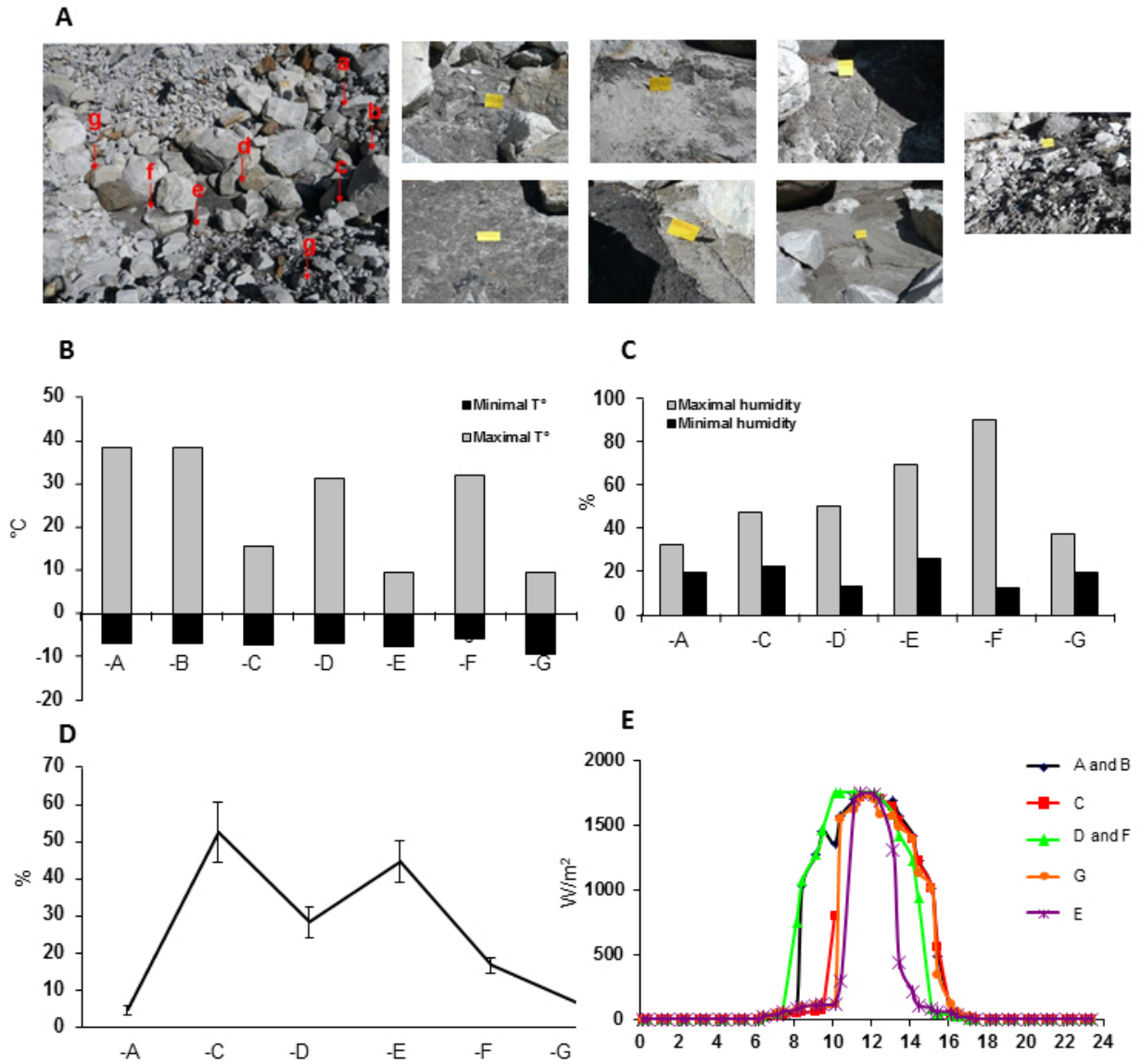

Figure 1 - Microclimate parameters in the NP09LOG1 site. (A) Topographical distribution of the investigated sites and crust morphological features; (B) minimal and maximal temperatures measured within the sites; (C) minimal and maximal air humidity recorded within each site; (D) crust moisture measured on the top surface of each site; (E) PYR and PAR photon flux profile within the investigated sites.

Materials and Methods

Site and sampling

In October 2009 a site, NP09LOG1, showing evidences of primary colonization was identified in the moraine of the Lobuche glacier at 5050 m a.s.l. (Fig.1A). The region is classified as a cold desert, experiencing long winter periods with temperatures below freezing, while summer periods are short and arid, although snowmelt and occasionally rainfall pulses locally increase moisture content. The site was located in a stone valley in the moraine. Samples were collected from 7 stations (labeled-A, -B,-C, -D, -E, -F and -G), presenting BSCs at apparent different stages of development. Site -B lacked of the crust layer and was composed of the same mineralogical substrate retrieved in the upper layer. All the sites, with the exception of -G, were located in a small valley in the moraine. Site -G was located along one of the slope of the valley. The total height of the site, considering the sampled stations, was 218cm, and the total length was 19.1m. Additional details are reported in Supplementary Table 1 at the end of the manuscript. For each station of primary colonization, both the crusty surface, containing the BSC, and the deep layer beyond, composed of a fine-sandy mineralogical substrate, were collected. Each sample, both the crust and the deep layer, was collected in triplicate for molecular ecology analysis and stored at -20°C until the analysis in the laboratory. Samples for physical-chemical characterization were air-dried for 24h and stored at room temperature until the analysis in the laboratory. A sample from each station was collected and immediately soaked in methanol for further intact lipid analysis. Sampling activities last 3 days, during which environmental and biochemical parameters were recorded in order to assess the climatic influence of the environment in each station. The recorded parameters encompass: minimal and maximal air temperature, air humidity, the percentage of crust moisture (hygrometer Sama Tolls, Italy), the redox potential, the solar radiation flux density.

Geochemical analysis and Intact polar lipid (IPL) composition analysis

About 200 g of samples were oven-dried at 105 °C until constant weight and then acid digested (HNO_3 concentrated 65% and H_2O_2 30%) in a Milestone high performance microwave oven (MLS Mega, Gemini BV Laboratory, Apeldoorn, The Netherlands). Metals and total phosphorous were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES, Spectro Ciros CCD, Spectro GmbH, Kleve, Germany). Total nitrogen concentration was measured using the Dumas combustion method with a TruSpec N analyzer (LECO Corporation, St. Joseph, MI). The pH_{H2O} of the samples was measured using an Accumet AP85 pH (Fisher Scientific Ltd., Pittsburgh, PA).

IPL analysis was performed by High Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (HPLC/ESI-MS2), as described by Jaeschke et al. (2009) and by Brandsma et al. (2012). Main IPL classes were identified through diagnostic fragmentation patterns in MS2 mass spectra as previously shown (Kato et al., 1996; Brügger et al., 1997; Keusgen et al., 1997; Fang and Barcelona, 1998). Then, pointed mass spectrometric analysis was applied to better characterize and quantify each of the identified IPL classes. IPL with a diacylglyceryl-trimethylhomoserine (DGTS) or a phosphatidylcholine (PC) head group was measured in cation mode by parent ion scanning (m/z 300–900). IPL with a phosphatidylethanolamine (PE), or a phosphatidylglycerol (PG), or a sulfoquinovosyldiacylglycerol (SQDG) head group was measured by neutral loss scanning (m/z 300-900) for losses of 141 Da, 189 Da and 261 Da respectively. The C number and unsaturation degree of the fatty acid molecules were calculated using the m/z ratio of the molecular species. Information on single fatty acid compositions of the major IPL species was based on fragment ions or neutral losses diagnostic for fatty acids, obtained in the data dependent MS2 experiments (Brügger et al., 1997). PGs, PCs, PEs, SQDGs and DGTSs quantification was done by comparing the peak height of each IPL class and their constituent IPL species to the respective peak heights of measured quantities of commercial standards (C16:0/C16:0 PC, C16:0/C16:0 PG; C16:0/C16:0 PE (Sigma Aldrich, Italy); SQDGs usually contain C16:1/C18:2 SQDG (> 60 %), plus C16:0−16:1, C18:0−18:1 and C20:5 fatty acid combinations, and a standard of C14:0/C18:1 DGTS, which was purified from IPL extracts of *Isochrysis galbana* (CCMP 1323) as described by Brandsma et al. (2012). Detection limits were 50–100 pg for the glycerophospholipids, 100 pg for the DGTSs and 1 ng for the SQDGs. Reproducibility of all IPL quantifications was within 10% error between duplicated runs.

DNA extraction and ARISA fingerprinting
Total DNA was extracted from the BSCs and DLs using the Power Soil kit (MoBio), according to the manufacturer's procedure. The DNA was quantified and stored at -20°C until use ARISA-PCR was conducted on a standard amount of DNA on each sample by using the primer set ITSF, 5'-GTC GTA ACA AGG TAG GCC GTA-3' and ITSReub, 5'-GCC AAG GCA TCC ACC 3', as previously described (Cardinale et al., 2004). Denatured ARISA fragments were run by STAB-Vida Inc. The data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems), and a threshold of 40 fluorescent units was used, corresponding to two times the highest peak detected during the negative control run.

DGGE of 16S rRNA gene fragments for the study of bacterial microbiome, 16S rRNA of β-Proteobacteria for ammonia-oxidizing bacteria (AOB) and of NifH gene for the study of N₂-fixer bacteria
PCR amplification of the 16S rRNA was performed using the 907R and 357F primer, adding a GC-clamp to the forward primer (Muyzer et al., 1993). PCR reaction was performed in 0.2 ml tubes in a final volume of 50 μl containing the diluted buffer 1 X, 1.5 mM MgCl$_2$, 5% DMSO, 0.12 mM of a mixture of dNTPs, 0.3 μM of each primer, 1 U Taq polymerase, and 10 ng of template. If necessary, DNA was properly diluted. The amplification program consisted of an initial denaturing step at 94° C for 4 min, followed by 10 cycles of 94°C for 0.5 min, 61°C for 1 min, and 72°C for 1 min; followed by further 20 cycles of 94°C for 0.5 min, 56°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 7 min. 2 μl of the PCR products were checked by electrophoresis in 1% agarose gels.
Despite two different protocols were applied to study by DGGE the diversity of *NifH* gene for nitrogen fixing bacteria, we obtained amplification only for samples from sites -F and -G. These PCR products were processed as previously described (Demba Diallo et al., 2004).
PCR amplification of 16S rRNA of β-Proteobacteria for the study of ammonia-oxidizing bacteria (AOB) was performed by adopting a nested-protocol, essentially as previously described (Offre et al., 2009). This nested PCR approach was demonstrated to capture a wide range of diversity of ammonia oxidizers bacteria (Kowalchuk et al., 1997) yielding AOB phylogenies that are similar to those based on the *AmoA* gene (Purkhold et al., 2003). DGGE separation of the PCR products was performed as described previously in this paragraph. The DNA eluted from DGGE bands was amplified using 907R and 357F primers (without the GC-clamp) for bacterial 16S rRNA (Muyzer et al., 1993) and using primers 357F and 518R for the amplification of β-Proteobacteria 16S rRNA(Offre et al., 2009). The PCR was performed in a final volume of 50 μl with the same condition as above and using the following protocol: 95° C for 5 min, 30 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products obtained were sequenced by Macrogen Inc. (Korea). AOB sequences were analyzed by a maximum likelihood tree generated by MEGA (Tamura et al., 2007), rooted with *Pseudomonas syringae* 16S rRNA gene sequence.

Statistical analysis
Non-metric multidimensional scaling (NMDS) was carried out to explore similarities between sites, based on the resemblance matrix generated using Bray–Curtis similarity on the matrix for IPLs and ARISA within each sample. The same set of environmental data was used in the distance-based multivariate analysis for a linear model (DistLM) to determine which significant environmental variables explained the observed similarity among the samples. The Akaike Information Criterion (AIC) was used to select the predictor variables. The contribution of each environmental variable was assessed, firstly using "marginal tests" to assess the statistical significance and percentage contribution of each variable taken alone, and secondly a "sequential test" was employed to evaluate the cumulative effect of the environmental variables explaining biotic similarity. A distance-based redundancy analysis (dbRDA) was used for graphical visualization of the DistLM results. Significant differences in microbial community composition were investigated by permutational analysis of variance (PERMANOVA; Anderson, 2001), considering the sampling area as a fixed and orthogonal factor. Ecological diversity indices were calculated from the matrix of ARISA OTUs. All the statistical tests were performed by PRIMER v. 6.1 (Clarke et al., 2006), PERMANOVA+ for PRIMER routines and PAST software.
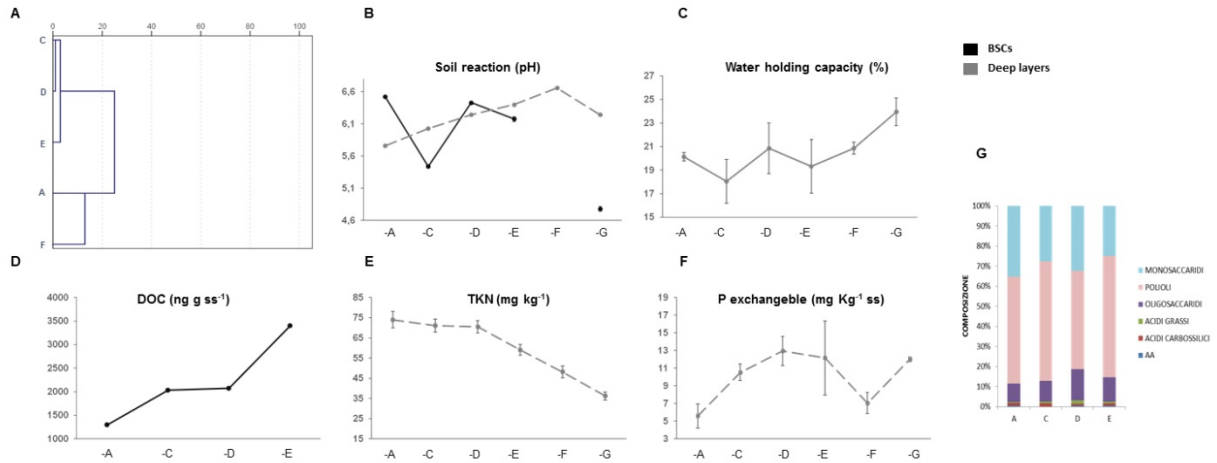
Figure 2- Comparison of selected physico-chemical parameters between crusts and DLs. (A) Cluster analysis for the similarity of DLs on the basis of the investigated physical-chemical properties; (B) pH values recorded within each site; (C) water holding capacity; (D) DOC values; (E) TKN, considering both organic and inorganic nitrogen; (F) exchangeable phosphorous; (G) Dissolved Organic Matter (DOM).

RESULTS

Microclimate within the NP09LOG1 site

The seven sites presented peculiar microclimatic settings according to their topographic location within the site (Fig.1A). While the minimal temperature dropped below zero at night for all the sites, the maximal temperature, recorded during daytime, varied from above 30°C (station -A, -B, -D) to 15°C (-C) and 9°C for the coldest sites -E and -G (Fig. 1B). Similarly, we observed a large range of variability among minimal and maximal air humidity among the sites, although there was not a linear correlation among maximal temperature, air humidity and the moisture recorded on the crust surface (Fig.1 C-D). Differential solar radiation fluxes, both in terms of time of sun exposition and cumulative solar energy in 24h, affected the seven sites. While sites -A, and -F were exposed to the solar irradiation for about 8h and received a cumulative PYR of about 37000 (KU m^{-2} 24^{-h}), site -E was largely in the shade, experiencing only 4 h of sun radiation with a cumulative PYR of about 19000 (KU m^{-2} 24^{-h}). A similar trend was observed for PAR, photosynthetically active radiation, in the sites (Fig. 1E).

Geochemical properties of the NP09LOG1 site

Both glacier transects are characterized by a thin grainsize with a high contingent of sand, with the main mineralogical phases represented by quartz, feldspar and mica. Soil texture was composed of over 90% sand and<6% loam and traces of clay in almost all samples.

Noteworthy, SEM-EDS analysis performed on BSCs identified a tight connection between mineral and organic constituents in the superficial crusts. Organo-mineral association were detected at different scales and showed a differentiated morphology (Supplementary Figure 1).

Physical-chemical features of BSCs and deep layers

The collected samples, BSCs and DLs, were variable in their physical-chemical properties (Fig. 2). Nevertheless, DLs shared a high degree of similarity in their physical-chemical properties, indicating that the bare substrate under the BSCs was featured by extremely similar nutrient conditions (Fig. 2A). In general, pH values close to neutrality were observed for both BSCs and DLs, ranging between 5.4 and 6.6 with the exception of the crust in site -G that was more acidic (pH= 4.7) than other sites (Fig. 2B). The water content in DLs, measured as water holding capacity, is dramatically impaired, recording values of ≈ 20% (Fig. 2C). A slow but persistent increase in DOC was recorded in BSCs, that could be correlated to the biological processes acting within each site. TKN (Total Kjeldhal Nitrogen), including both organic nitrogen and ammonia, decreases in concentration along the site,

with -G recording values that were almost half of those observed in -A (Fig. 2D). Exchangeable phosphorous, often a nutrient-limiting nutrient in bare soils, showed the lowest values for sites -A and -F in DLs (Fig. 2E). Dissolved organic matter (DOM) analysis detected about 100 different molecules, ranging among mono- and oligosaccharides, polyols, fat acids and carboxylic acids (Fig. 2G). Polyols represented the vast majority of DOM molecules, glycerol was the most abundant molecule, accounting for 80% of all detected compounds in the examined BSCs. A multivariate analysis was performed in order to correlate microclimate parameters with the recorded physical-chemical features of the sites. The percentage of carbon (C) and nitrogen (N) retrieved in the sites were strictly correlated to the moisture recorded on the surface of the BSCs (r=0.941; $p<0.05$ and 0.05 respectively) and with the maximal air temperature (for C, r=-0.908, $p<0.1$; for N, r=-0.976, $p<0.05$). While, the thickness of the crusts was positively linked to both air maximal and minimal temperature (r=-0.957; $p<0.05$; r=-0.99; $p<0.05$) and solar radiation (for PYR r=-0.984; $p<0.05$; for PAR r=-0.957; $p<0.05$). Similarly, pH values recorded within the BSCs are positively affected by the photon flux (for PYR r=0.974; $p<0.05$; for PAR r= 0.974, $p<0.05$).
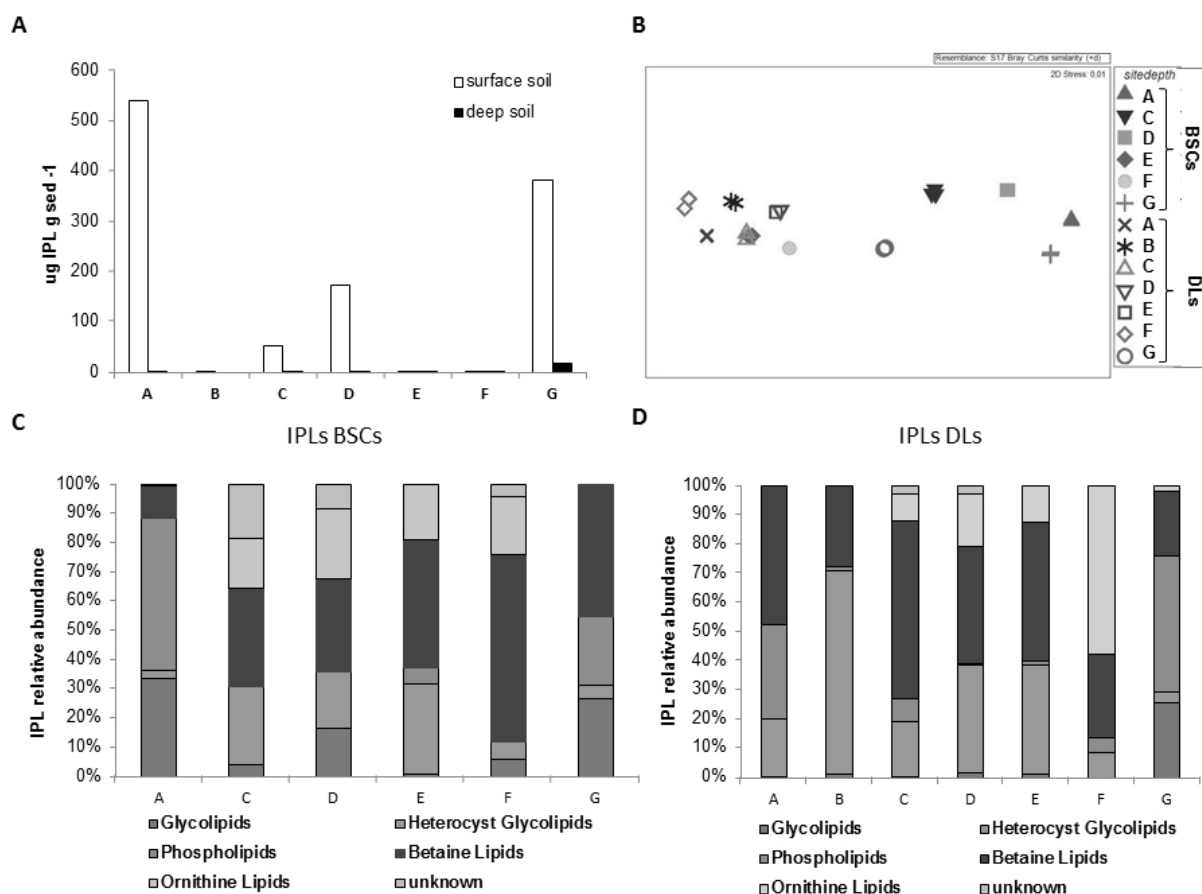

Figure 3 - Intact polar lipid analysis of NP09LOG1 site in the Lobuche moraine. (A) sum of all intact polar lipids within crusts and deep layers; (B) Nonmetric multidimensional scaling (NMDS) results based on quantitative IPL matrix showing a sharp clustering of crust samples and deep layers; (C) Relative distribution of intact polar lipids in BSCs; (D) Relative distribution of intact polar lipids in DLs.

Overview of the biological communities associated to BSCs as assessed by Intact Polar Lipid (IPL) analysis
Most of the microbiological biomass was found in the BSCs, reaching forty fold higher levels than in deep layers (Fig. 3A). Sites -A, -C,-D and -G were featured by higher amount of intact polar lipids, suggesting that they hosted a larger microbial communities compared to the other sites (Fig. 3A). The combined effect of site and depth on the lipid composition of the biological assemblages in the study site clearly showed a separation among BSCs of -A, -

C, -D and -G sites respect to deep layers and BSCs in -E and -F sites (NMDS analysis, stress value =0.01) (Fig. 3B). Heterocyst glycolipids represented about one third of the total intact lipids, suggesting that Cyanobacteria were the dominant component of the microbial communities (Fig. 3C-D). Betaine lipids, typical of eukaryotic organisms, were most abundant in sites -A, -C,-D and -G, representing respectively 19.4, 5.9, 18 and 55.8% of total intact polar lipids, and showing a similar abundance profile of heterocyst glycolipids, typical of N_2 fixing Cyanobacteria (19.3, 17.4, 40.4, 22% respectively). This similarity in abundance reinforces the link between prototroph and higher eukaryotic communities, like lichens and mosses. High relative levels of ornithine were observed in sites -C and -D (9. 2 and 41.5% respectively), suggesting a prevalence of bacteria over eukaryotes within these sites. Sites -A and -G were featured by higher levels of phospholipids (75.7 and 24.25% respectively) which origin may be related to eukaryotic cell, mainly fungi. In these sites, together with phospholipids also betaine lipids are abundant, while heterocyst glycolipids are relatively low represented respect to the other two classes of lipids (19.3 and 22% respectively), suggesting that in these two sites phosphorous is immobilized in phospholipids, thus no P-limiting conditions exist, and that a microbial community, typical of more mature soil, may be present. A DistLM was applied with the aim to identify the environmental variables shaping the microbial community composition, indicating that both the maximal air humidity (p=0.0001) and crust moisture (p=0.0088) affected the structure of the microbial composition, as assessed through the quantification and identification of intact polar lipids. Among physical-chemical parameters, both nitrogen (%N) and DOC are inputs for the observed diversity (p= 0.0024 and 0.0001 respectively), while the combined effect of pH and both carbon and nitrogen percentages explained 89.65% of the total data variation and was highly significant (F=13.16, p=0.0003).

Variability of the Bacterial Community Structure as Revealed by Community Fingerprinting
ARISA (Automated Ribosomal Internal Spacer Analysis) fingerprinting was adopted to evaluate spatial patterns in the structure of the bacterial communities associated to the sites, aiming to identify the correlation between the structure and the environmental conditions that characterize each site. A sharp difference could be envisaged among sites according to cluster analysis, with a percentage of similarity that dramatically dropped to less than 10% (Fig.4A), indicating that each site hosted a peculiar bacterial assemblage (PERMANOVA test for samples separation according to the site, p=0.0001). The effect of depth in shaping the structure of the site-associated microbiome was strengthened by PERMANOVA test, showing a sharp separation between the bacterial communities inhabiting the BSCs and DLs (p= 0.003). Noteworthy, the cluster analysis emphasized a co-clustering of BSCs and DLs within each site, indicating a link between the structure of the bacterial communities in the upper and lower layer within each site (Fig. 4A). Since DLs share similar physical-chemical features (Fig. 2A), such clustering of BSCs and DLs within each site suggested a BSC-mediated effect in influencing the structure of the microbial communities in the underlying deep layers. The combined effect of site and depth accounted for a statistical relevant difference for the bacterial communities associated to the sites (PERMANOVA test, p=0.0001). The Shannon diversity index significantly decreased in BSCs respect to DLs, suggesting the presence of both biotic and abiotic constrains that contribute to shape the biodiversity in the upper layers (Fig.4B). A DistLM multivariate analysis was performed in order to correlate the differences in the structure of microbiomes in the different sites with abiotic environmental parameters (Fig.4C). Only BSCs were analyzed since the majority of microbial biomass occurs in these superficial layers. We observed that among abiotic factors, the recorded temperatures (for maximal temperature p=0.0002, for minimal temperature p=0.0003), the air humidity (p=0.0001) and the crust moisture (p=0.001) exerted a statistical significant influence in determining the structure of the bacterial communities associated to each site, according to PERMANOVA test (Fig. 4C).Thus, an overlap exists about the environmental factors that shaped the structure of both the overall microbial communities hosted within each site. Noteworthy, these environmental parameters may affect the water content within each site. All the investigated physical-chemical parameters showed a key role in modulating the structure of the bacterial communities associated to BSCs, including pH (p=0.001), N, C and H percentage content (p= 0.0239, .0001 and 0.0001 respectively) and DOC (p=0.0048).
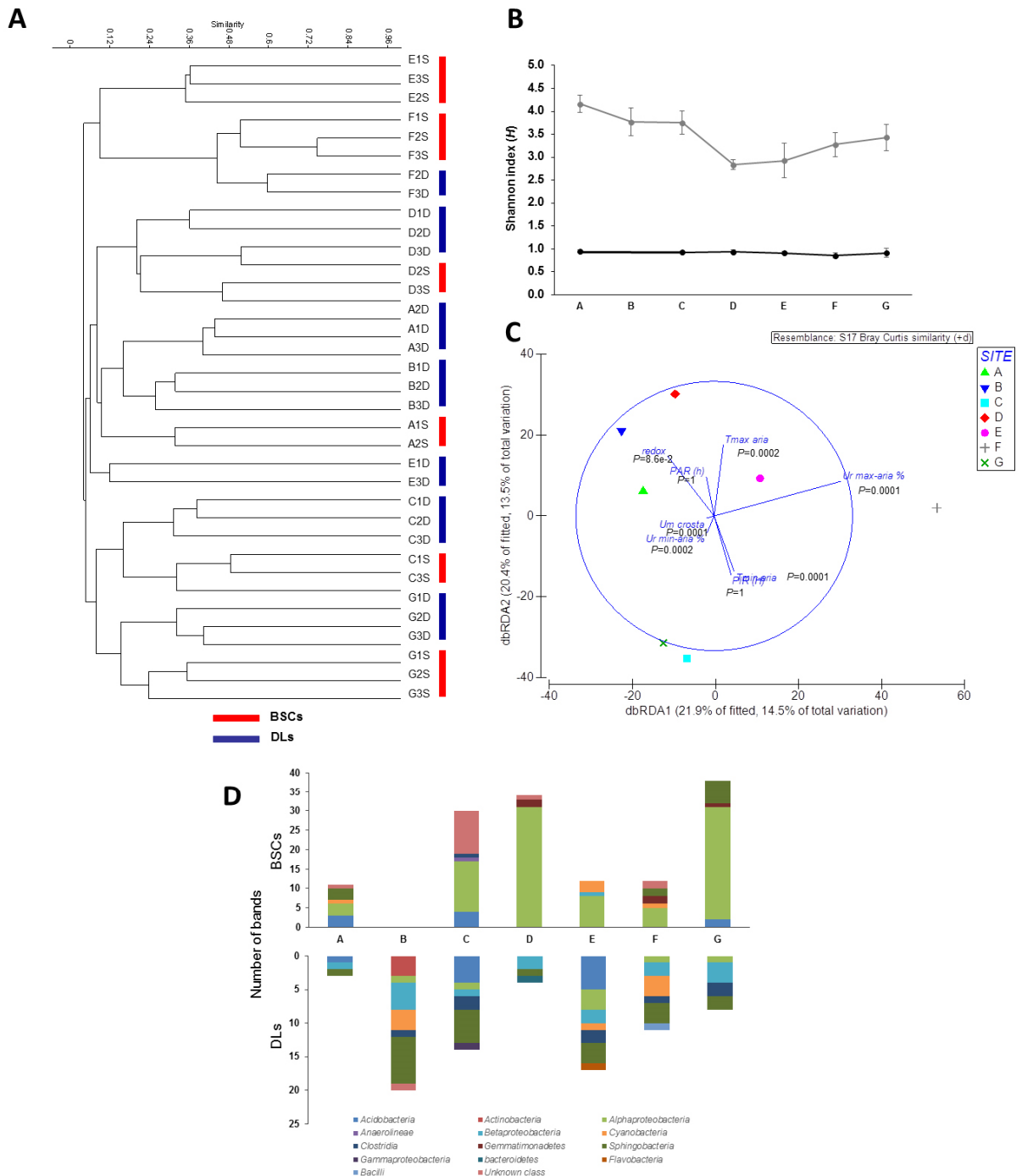
Figure 4- ARISA fingerprinting analysis of the bacterial microbiome and dominant component of the bacterial communities as assessed by DGGE analysis. (A) Cluster analysis of quantitative ARISA profiles of surface and mineral layers within each site; (B) Comparison of diversity, through the Shannon index, of BSCs and DLs on the basis of ARISA profiles; (C) dbRDA ordinations of the quantitative ARISA dataset overlaid with the partial correlations of the tested environmental variables explaining the clustering of crust and deep layers; (D) Phylogenetic identification of DNA fragments that were excised from the DGGE gel and successfully amplified and sequenced.

Cultivation-independent analysis of site associated bacterial communities

A 16S rRNA gene PCR-DGGE analysis was performed to explore the dominant components of the bacterial communities associated to each site, investigating both crust and deep soils. A total of 214 bands were successfully re-amplified, encompassing 137 bands for BSCs and 77 for DLs. Between 10.9% and 1.3% bands for BSCs and DLs respectively were classified as unknown bacteria since they did not exhibit significant homology with any of the sequences deposited in the Ribosomal Database Project (RDP). The analyzed sequences were affiliated to 13 classes, among which*Acidobacteria, α- and β-Proteobacteria, Cyanobacteria, Clostridia* and *Sphingobacteria* were common to both BSCs and DLs. *Gemmatimonadetes* and *Anaerolinae* were specifically associated to BSCs, while sequences affiliated to *Actinobacteria, γ-Proteobacteria, Bacteriodetes, Flavobacteria* and *Firmicutes* were retrieved only in DLs (Fig. 4D). The distribution of the retrieved classes within each site and in crust and deep soils gave rise to a variegated distribution, indicating thata peculiar bacterial community is hosted in each fraction. Sequences related to *Cyanobacteria* were relatively low represented, presumably due to a bias of the primers adopted for the analysis. In BSCs, the great majority of sequences (89/137) were affiliated to *α-Proteobacteria*, represented by different orders like *Rhizobiales, Sphingomonadales, Rhodospirillales* and *Caulobacterales*. In DLs, the predominant class was *Sphingobacteria* (22/77) with the order *Sphingobacteriales* and the *β-Proteobacteria* class (15/77) with the order *Burkholderiales*.

Diversity of Ammonia Oxidizing Bacteria (AOB) in BSCs

Bacterial metabolism alters the physical-chemical features of the environment in a way that can generate valuable conditions for the colonization and growth of other bacterial populations. We focused on bacteria involved in nitrogen cycling, considering the key role of nitrogen in ecosystem development. Despite several attempts, we failed to study *NifH* diversity through a DGGE approach. We retrieved few sequences affiliated to *Azospirillum* and *Bradyrhizobium* sp. in site -F and of *Azospirillum* and *Sinorhizobium* sp. in site -G. However, the diversity of AOB communities associated to BSCs was extensively studied. Out of the 177 sequences, 117 could not be assigned to any known class within the *Proteobacteria* phylum according the Ribosomal Database Project, 7 were classified as *Burkholderiales*, 11 as *Methylophilales*, 4 as *Pseudomonadales* and 38 as *Nitrosomonadales*. Thus only 21.4% of the retrieved sequences were close matches to the *Nitrosospira*-like group of AOB in sites -A, -B, -D and -G (Supplementary Fig. 2A), with the majority of sequences clustering within the *Nitrosospira multiformis* lineage previously described (Kowalchuk and Stephen, 2001) and few sequences clustering within the four clusters of *Nitrosospira*-like sequences as previously reported by Purkhold et al., 2003 (Purkhold et al., 2003) (Supplementary Fig. 2B).

Discussion

Biological Soil Crusts are structural and functional components of unvegetated drylands and mainly consist of Cyanobacteria, microfungi, lichen, mosses and an heterogeneous group of bacteria. Despite an increasing interest in the functionality of BSCs as pioneer colonizers in arid and cold mountain ecosystems, attention was mainly devoted to prototrophs, the primary producers, while the bacterial component of BSCs was relatively poorly investigated. The role of these bacteria in soil development has been recently documented in the Midtre Lovenbreen moraine in Svalbard islands. Here, in synergy with carbon and nitrogen fixation exerted by Cyanobacteria, the pyrite bioweathering activity of *Acidiothiobacillus ferrivorans* promoted dramatic changes in the chemical-physical features of the barren moraine, improving water holding capacity. These changes, driven by the iron-oxidizing bacterial community, supported valuable conditions for plant biocenosis (Borin et al., 2010; Mapelli et al., 2011; Mapelli et al., 2012). In the present study, we investigated the microbiological and bacterial counterparts of BSCs and DLs in a site presenting evidences of primary colonization in the Lobuche moraine in the Himalayan plateau at 5000 m a.s.l. Despite the relative little size of the site (~50 square meters), the seven investigated examples of primary colonization appeared as distinct microsites. Each microsite was characterized by a set of specific soil physico-chemical properties including organic matter, pH and soil nutrient concentration, by a set of microclimate parameters and by a different microbiological and bacterial composition. Furthermore, each BSC differed from the other ones and obviously from the respective deep layer. Therefore, as shown by

multivariate statistical analysis, at this initial step of pioneering colonization, a series of biological, climatic and chemical factors within each microsite may differentially determine a heterogeneity of topsoil properties at a centimeter scale.

According to IPL analysis, most of the microbiological biomass was found in the BSC at levels 40 times higher respect to the deep layers. A similar profile of reduction concerned also the measured nutrients which occurred at higher concentration in the superficial layers. The accumulation of biomass in the BSCs may implicate a faster turnover of nutrients in the crust, with intermediate metabolites bound to the microbial loop and not released in the underlying mineral layer. A similar trend of biomass and nutrients, mainly concentrated in the crusts surface rather than in bare soils, was observed along an elevation gradient from 5300 to 5900 m a.s.l in the Tibetan plateau (Janatkova et al., 2013). Considering both microbiological and nutritional abundance, it seems that the microbial life is restricted to the few millimeters of the superficial crust layer and that the microbial structure and nutritional status of the deep layers are only indirectly affected, presumably by leaching of organic matter. Despite this, it is hard to speculate about the stages of soil formation that may occur in the investigated primary colonization sites. Multivariate analysis highlighted that some microclimate parameters, including the gradient of temperature, pH and solar irradiation affected both morphological features of the crust, like the thickness, and the moisture content. Variations in water activity, due to environmental factors, primarily affected the microbiological activity in the crust, rather than in the mineral layer, since water retention is more efficient in BSCs (Cable and Huxman, 2004; Aguilar et al., 2009). The value of 20% of WHC, that were recorded for the deep layers in NP09LOG1 site, were similar to those found in Svalbard islands in unvegetated soil (Borin et al., 2010). Even under low water activity values, microorganisms can be metabolically active, given their short duplication time, quick metabolism and the adoption of mechanisms to escape from desiccation. The most abundant molecule among DOM was glycerol, acting both as cryoprotectant and in improving resistance to dryness, together with trehalose, well known as compatible solute able to withstand with osmotic stress(Bartels and Sunkar, 2005). Indeed, the Himalayan plateau is characterized by unfavorable extreme cold and dry conditions, resulting in a minimal liquid water input to soil. Our findings are in agreement with previous studies indicating that microorganisms from environments with a high-osmotic potential exhibit cross-tolerance to freeze stress through high intracellular levels of osmolytes and/or the ability to form biofilms (Wilson et al., 2012). The IPLs analysis, offering an overview of the biological components within each site, highlighted that every BSCs represented a microsite, with a peculiar composition in terms of plant, bacterial and fungal lipids. These sharp differences are mainly attributed to both water activity, affected by air humidity and crust moisture, and to physical-chemical properties like pH and nitrogen and carbon-based nutrients present in the surface layers. The influence of water availability at the microbial scale in BSCs has been documented with certain *Cyanobacteria* that quickly respond to water inputs moving toward the wet soil surface or refuge in deeper layers during dryness (Garcia-Pichel and Pringault, 2001). Similarly, microbial activity in BSCs in hot deserts quickly resumes to a metabolic active state under hydration conditions, induced by dew or precipitation (Rajeev et al., 2013). On the other hand, the metabolic activities performed by the microbial community within each site increased the small-scale heterogeneity by modifying soil chemistry, mostly related to carbon and nitrogen cycling and micro- and macro-nutrients availability, thereby creating distinct soil microsite. A high degree of soil heterogeneity, with the generation of clearly different microsites, was retrieved in semiarid grassland in Central Mexico and was mainly related to the dominant prototroph constituting the BSCs, being cyanobacteria, lichens or mosses (Concostrina-Zubiri et al., 2013). The majority of biomass in the BSCs was represented by N_2-fixing Cyanobacteria, primary producers within soil crusts in arid and semi-arid cold environments worldwide (Pointing et al., 2009; Janatkova et al., 2013). Cyanobacteria diversity dramatically increasing in the first 4 years after ice retreat in deglaciating soils, passing from ice-dwelling Cyanobacteria to those typically associated to biological soil crust-form (Schmidt et al., 2008). To some extent, the observed sharp differences in the microbiological community structure are not surprising, considering soil heterogeneity and the patchy distribution of microorganisms, as already described also in Arctic moraines (Green and Bohannan, 2006; Schütte et al., 2009). Thus, the within-site variation in deglaciating moraine is expected to be large, as confirmed by the bacterial pattern distribution in the seven investigated sites. We determined that the bacterial communities associated to the crusts differed significantly and that such differences were reflected in depth, with mineral soils underlying the BSCs presenting distinct bacterial assemblages. This can be explained by the dynamic changes of

the physical environment in glacier foreland that can affect primarily the surface layers and then are reflected to the underlying mineral soils through leaching processes (Schutte et al., 2009). Since the physical-chemical features and the nutritional status of DLs are statistically similar, any observed differences in the bacterial microbiome structure in DLs are mainly influenced by the top crusts. The presence of sequences affiliated to *Actinobacteria*, *Proteobacteria* and *Acidobacteria* were reported also in other cold habitats, including the Pindari glacier in the Indian Himalayan region and in soil samples from western Himalaya (Gangwar et al., 2009; Shivaji et al., 2011; Srinivas et al., 2011). The occurrence of identical phyla in geographically distinct areas is indicative of the ability of these bacteria to adapt similar metabolic strategy to survive to freezing and remain active at low temperatures (Shivaji et al., 2011). The microbiota inhabiting the BSCs was mainly represented by taxa that are generally retrieved in mature soil like *Rhizobiales*, *Sphingomonadales* and *Caulobacterales* (Haichar et al., 2008). This finding suggests that the superficial crusts may represented microsites of fertility, developing over the mineralogical substrate (Borin et al., 2010). *Rhizobiales* are of particular interest since this order encompasses many bacteria with the ability to fix atmospheric nitrogen, playing a key role in nitrogen cycling in combination with *Cyanobacteria*.

In DLs, *Sphingobacteriales*, which belong to the phylum *Bacteroidetes*, was the dominant order, whose abundance was positively correlated to increasing rates of carbon mineralization in soil (Fierer et al., 2007). In addition, most of the *Sphingobacteriales* sequences were affiliated to *Chitinophagaceae* at the family level. These bacteria are able to degrade chitin, mainly of fungal origin, and other complex polymeric organic compounds, to obtain C and N to supply their growth (Del Rio et al., 2010) and represent a dominant component of microbial communities of Antarctic soil, glacier forefields and alpine debris-covered glaciers (Ganzert et al., 2011; Franzetti et al., 2013). Although only few sequences were retrieved in DLs, *Burkholderiales* deserve attention since bacteria affiliated to this order are considered as typical inhabitants of ice environments, commonly found in deglaciating soil at initial and medium stages of development (Nemergut et al., 2007; Mapelli et al., 2011). During ice melting, these bacteria are seeded in the mineral substrate and their relative abundance decreases during soil development (Nemergut et al., 2007).

The metabolic activities of bacterial populations drive geochemical changes in mineral soil properties, facilitating the colonization by other bacterial phyla. For instance, Cyanobacteria and other N_2-fixing bacteria increase the concentration of ammonia in soils, generating suitable nutritional conditions for the establishment of ammonia oxidizer and nitrifying bacteria. In our study we focused on AOB community diversity, although only 20% of the AOB retrieved sequences were affiliated to the *Nitrosospira*-like group, as already experienced for AOB communities from 23 different soil ecosystems in North America (Fierer et al., 2009). Among altitude dependent factors, temperature was clearly demonstrated to have a huge influence in the structure of AOB communities, with a decrease in AOB activity under warming conditions (Avrahami et al., 2003; Avrahami and Bohannan, 2007). Similarly, soil moisture affected the activity of AOB community, as shown by the increase in the relative content of *amoA* copies in the Atacama desert experiencing a desert blooming event due to sporadic rainfall (Orlando et al., 2010). Thus, the retrieved sequences of *Nitrosospira*-like AOB may be representatives of cold and desiccation tolerant AOB population colonizing the Lobuche moraine.

In conclusion, the study of primary colonization events in the forefield of the Lobuche glacier in the Himalayan plateau suggest that BSCs may contribute to the fundamental inherent natural heterogeneity of dry land cold ecosystems at a smaller yet unexplored spatial scale, boosting those geochemical changes that over time allow the establishment of plant biocenosis.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. NP09LOG1 site features related to the size of the site and BSC morphological properties.

Station	Crust color and morphology	Crust thickness (cm)	Deep layer thickness (cm)	Relative height (m)*	Distance (m)[#]
NP09LOG1-A	Grey	1	60	1.14	-
NP09LOG1-B	No crust	-	60	1.35	1,30
NP09LOG1-C	Brown-gray	0.2	>2	2.20	3
NP09LOG1-D	Brown-gray	0.2	30	2.65	4,9
NP09LOG1-E	Black	0.2	0.8	3.08	3,5
NP09LOG1-F	Light gray	>0.1	2	3.30	2
NP09LOG1-G	Black, globular	0.5	15	1.70	2,90

* Relative height was calculated by tracing a rope along the site and measuring the relative height of each site respect to this prefixed height.
[#] The distance among the sites was measured starting from site –A.

Supplementary Table 2. Mineral and texture composition of the investigated sites.

| Site	Sand (% ss)	Loam (% ss)	Clay (% ss)	USDA classification	Mineralogical phases
NP09LOG1-A	93.10	6.05	0.85	Sandy	Quartz, Feldspar, Mica
NP09LOG1-B	n.d[#]	n.d	n.d	n.d	n.d
NP09LOG1-C	94.81	5.18	0.01	Sandy	Quartz, Feldspar, Mica
NP09LOG1-D	94.11	5.49	0.40	Sandy	Quartz, Feldspar, Mica
NP09LOG1-E	94.81	5.18	0.01	Sandy	Quartz, Feldspar, Mica
NP09LOG1-F	89.10	5.25	5.65	Sandy	Quartz, Feldspar, Mica
NP09LOG1-G	n.d	n.d	n.d	n.d	n.d

n.d: non determined
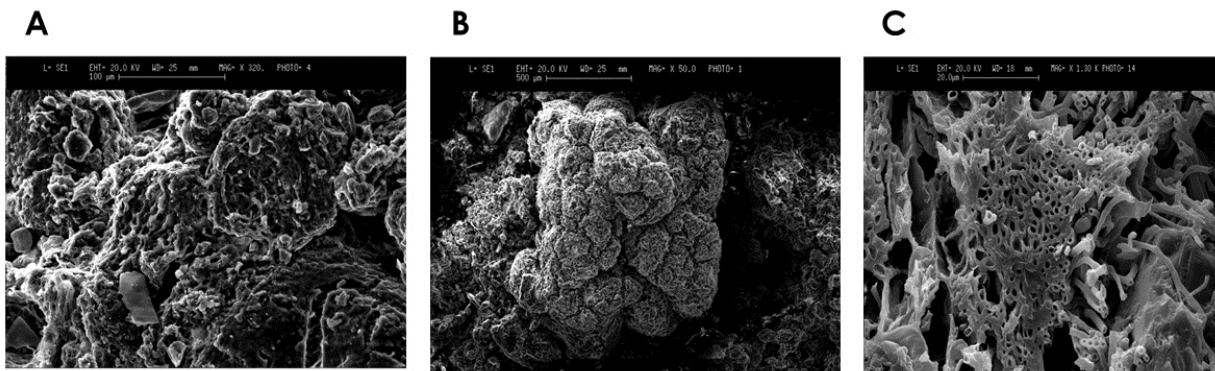
Figure SM 1 - SEM photomicrographs illustrating the tight association within mineral and organic constituents in the BSCs. (A) dark organic crusty patina developed on quartz grain; (B) occurrence of calcium carbonate nodule at the top of the crust; (C) nodules constituted by coalescent carbonate microtubules. Photomicrographs are relative to the crust layer in site –A; the scale bar is reported in each capture.
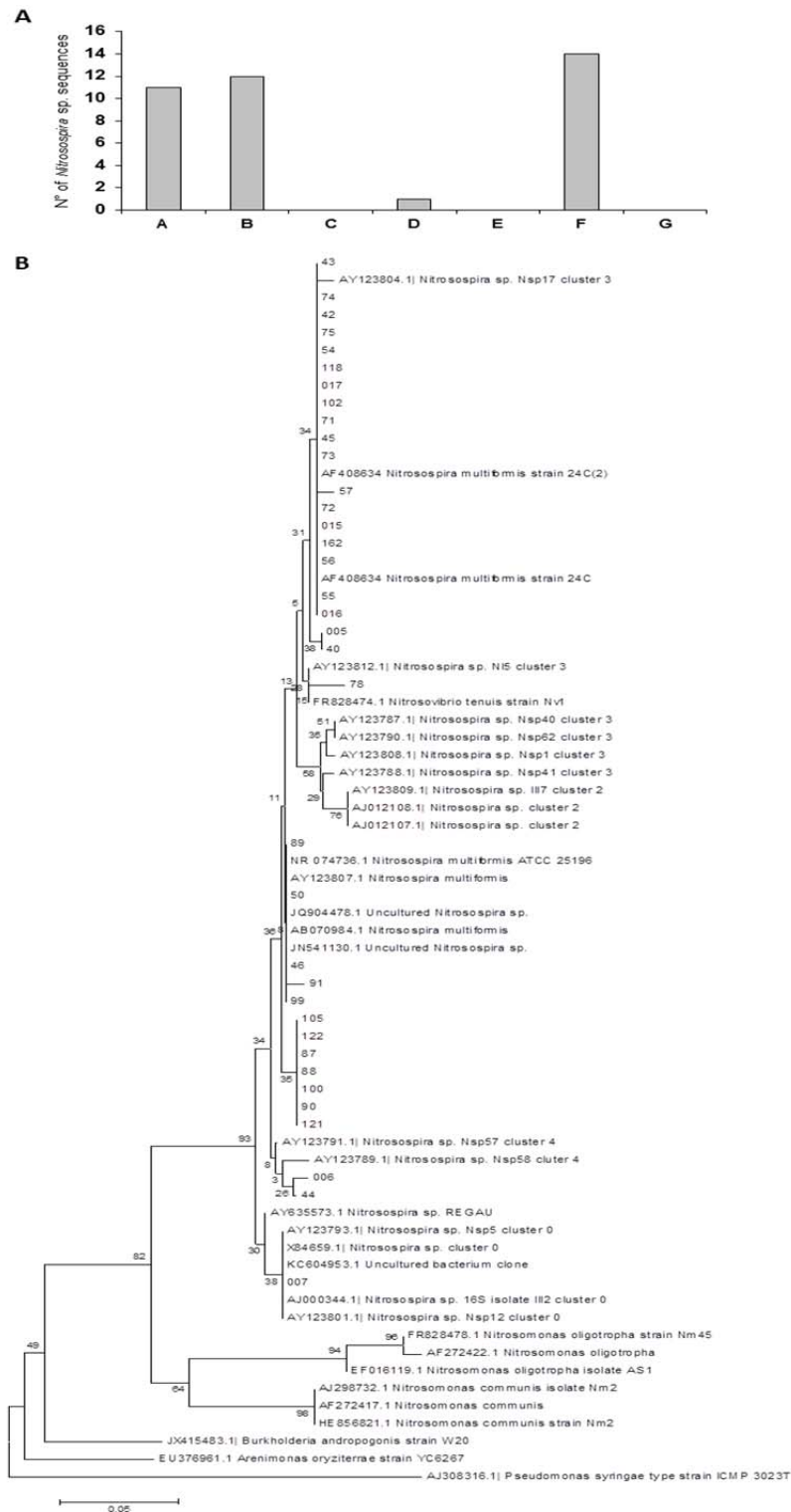
Figure SM 2 - Diversity of AO bacteria in BSCs. (A) Distribution of Nitrososospira-like sequences within the studied sites; (B) Phylogenetic tree of the Nitrososospira-like sequences. The taxonomic identification was performed according to neighbour-joining method and is based on the 180 bp fragment of 16S rRNA gene partial sequencing amplified with primers specific for β- Proteobacteria.

A three scale analysis of bacterial communities involved in rocks colonization and soil formation in high mountain environments

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Concise title: Bacterial communities colonizing glacier foreland.

Abstract Alpha and beta diversities of the bacterial communities growing on rock surfaces, proto-soils, riparian sediments, lichen thalli and water springs biofilms in a glacier foreland were studied. We used three molecular based techniques to allow a deeper investigation at different taxonomic resolutions: Denaturing Gradient Gel Electrophoresis (DGGE), Length Heterogeneity-PCR (LH-PCR) and Automated Ribosomal Intergenic Spacer Analysis (ARISA). Bacterial communities were mainly composed of *Acidobacteria*, *Proteobacteria* and *Cyanobacteria* with distinct variations among sites. While *Proteobacteria* were more represented in sediments, biofilms and lichens, *Acidobacteria* were mostly found in proto-soils and *Cyanobacteria* on rocks. *Firmicutes* and *Bacteroidetes* were mainly found in biofilms. UniFrac p-values confirmed a significant difference among different matrices. Significant differences ($p < 0.001$) in beta diversity were observed among the different matrices at the genus-species level, except for lichens and rocks which shared a more similar community structure, while at deep taxonomic resolution two distinct bacterial communities between lichens and rocks were found.

Introduction

Glacier forelands are affected by several biotic and abiotic factors triggering primary succession and ecosystem development [39]. Soil formation processes in recently deglaciated areas are a function of chemical composition of the mother rock, temperature, moisture regimes, time since exposure and topography [23]. Microbial communities, as free living cells or in biofilms, are the first colonizers of newly exposed rocks. They influence the rate of mineral weathering and the reactions to synthetize secondary minerals [20], the amount and the quality of organic content in the soil and in last instance the quality of the ecosystem services. They can achieve a very wide array of biochemical reactions and the investigation of the dynamics of pioneer bacterial communities is useful for drawing guidelines about land management.

Most bacterial community fingerprinting techniques are PCR-based amplifications using environmental DNA as template [46]. Downstream analyses are focused on the separation of single products using gel or capillary electrophoresis [49]. However, the relative richness of a few abundant species could hide the presence of rare species giving an incomplete picture of the overall composition. Furthermore, a high number of amplicons could give misleading results due to different organisms with the same fragment length [42, 17]. Some limits of PCR-based techniques are connected to the intrinsic pitfalls of PCR itself, including an incomplete detection of less abundant amplicons, the formation of chimeras and a selective amplification of best fitting template [28, 41, 45]. Nonetheless, the limits of each technique alone might be overcome with a polyphasic approach [6, 12, 40].

This article reports the use of Denaturing Gradient Gel Electrophoresis (DGGE) [35], Length-Heterogeneity PCR (LH-PCR) [44] and Automated Ribosomal Intergenic Spacer Analysis (ARISA) [13] for the description of bacterial communities in 27 samples from five different high mountain sites, representing the five most common ecological matrices, namely rocks, proto-soils, glacier stream sediments, rock-colonizing lichens and water springs biofilms. The aim was a polyphasic description of alpha and beta diversity at different taxonomic resolution of bacterial communities belonging to these environments.

Materials and methods

Study area and samples description

The study area is located in the high Matsch valley, a S-E oriented valley in South Tyrol (Italy). The valley had an average rainfall of about 550 mm per year in the period of 1970-2000. The main rock types are schist and gneiss [24], while the most diffused soil types are acidic leptosols, regosols and umbrisols (mean pH = 4.3) derived from carbonate-free mother rocks. The whole study area is located above the tree line (2000 m a.s.l.) where a quick retreating glacier has left 3.3 Km^2 of foreland in the last 150 years [31]. A total of 27 samples were collected in the summer of 2011: 8 samples from rock surfaces (R), 7 from proto-soils (SL), 4 from glacier stream sediments (SD), 5 from rock colonizing lichens (*Rhizocarpon* spp.) (RL) and 3 from biofilms in water springs (B) (table 1). Rock samples were taken by scratching the rock surface with a sterile scalpel and collecting 0.05 - 0.1 g of grinded material. Sediments and proto-soil samples were collected in sterile 25 mL tubes, while lichens and biofilm were collected in sterile bags. Samples were kept at -80° until DNA extraction. DNA from rock, lichen thalli and biofilm samples were extracted with the CTAB method [2], while proto-soil and sediment sample DNA were extracted using the PowerSoil DNA isolation Kit (MO BIO, Arcore, Italy).

Sample number	Latitude	Longitude	Altitude
1.1R, 2.1R, 4.1SL, 5.1SL, 8.5R, 117RL, 118R, 120RL, 121SL, 134SL, 138RL	N46 46.587	E10 41.913	2453
52.1SD	N46 46.032	E10 42.017	2344
50.1SL	N46 46.342	E10 41.849	2381
45.1SD	N46 46.497	E10 41.817	2416
46.1SD	N46 46.498	E10 41.818	2417
48.1SD	N46 46.608	E10 41.816	2428
70.1R, 71.1R, 73.1SL, 76.1R, 78.1B, 80.1B, 81.1B	N46 46.669	E10 41.848	2472
77.2RL	N46 46.684	E10 41.793	2482
90.1R	N46 47.108	E10 42.277	2848
88.1RL	N46 47.154	E10 42.161	2842
93.1SL	N46 47.157	E10 41.924	2812

Table 1 - GPS coordinates in UTM system for each sampling point, each suffix describe the nature of the sample: R-Rock surfaces, SL-Proto-soils, SD-Stream sediments, RL-Rock colonizing lichens, B-Watersprings biofilms

Fingerprinting analysis
For DGGE, the 16S rRNA gene from environmental DNA samples was amplified using the primers 357F-GC [34] and 907R [33] in a volume of 30 μL [21] in an Eppendorf Mastercycler thermocycler. DGGE was run in a BioRad DCode Universal Mutation Detection System. The gel was made with 7.5% w/v acrylammide and a denaturing gradient of 40%-60% w/v of urea/deionized formammide (where 100% denaturant is 7 M urea and 40% deionized formammide) was used. Runtime was 16 h at 90 V. Discrete bands were excised from the gel using a sterile blade, diluted in sterile MilliQ water and used as template for PCR reamplification with the primers 357F and 907R. PCR products were sequenced by STAB Vida Lda. (Caparica, Portugal). For LH-PCR and ARISA an amount of 10 - 300 ng of template DNA was used, depending on the matrix analyzed. For LH-PCR, the primers 27F and 338R [44] were used, the former labeled with 6-FAM dye. The PCR conditions followed Brusetti et al. [10]. For ARISA, the primers ITSF and ITSReub labeled with 6-FAM were used as in Cardinale et al. [13]. PCR products were sent to STAB Vida Lda. for capillary electrophoresis. Electropherograms were analyzed using the Peak Scanner Software 1.0 as previously described [10]. Data matrices were exported and checked separately, according to the environmental matrix, in order to avoid biases due to fragments with nearly similar length but deriving from different organisms.

Statistical and bioinformatics analysis
A set of bioinformatics tools written in Biopython [14] (downloadable from http://pro2.unibz.it/projects/blogs/ emerge/?page_id=140) were implemented for the screening of electropherograms. Those tools allow multiple contemporary screening of sequences using the BLAST [1] algorithm, gathering the most useful data for identification of the closest relative in a .xls workbook, increasing significantly the check accuracy in comparison with eye screening. All sequences were submitted to the RDP-Ribosomal Database Project [15] web server to assign taxonomy. Sequences, submitted to the Genbank/EMBL/DDBJ databanks under the accession numbers HF546411 to HF546516, were used to construct a phylogenetic tree with the Maximum Likelihood using the Kimura 2-parameter model [30]. Phylogeny was tested with 1000 bootstrap replicates. The analysis was done with the MEGA5 software. The resulting tree was submitted in Nexus format to the UniFrac [25] web server to test for differences among the environments based on the UniFrac metric with 1000 permutations.

Matrices obtained from LH-PCR and ARISA were normalized with the formula $(x/\sum x)*1000$ where "x" is the fragment height in units of fluorescence, then transformed on a logarithmic scale. The Shannon index (H'), the Evenness (J') index and the Kruskal-Wallis non parametric test coupled with a post-hoc Mann-Whitney pairwise comparison test were calculated by using PAST software [26] on the normalized non-transformed matrices. The

transformed matrix was imported in "R" environment, and a Non-Metric Multidimensional Scaling (NMDS) was done with Bray-Curtis distance measure and K=3.
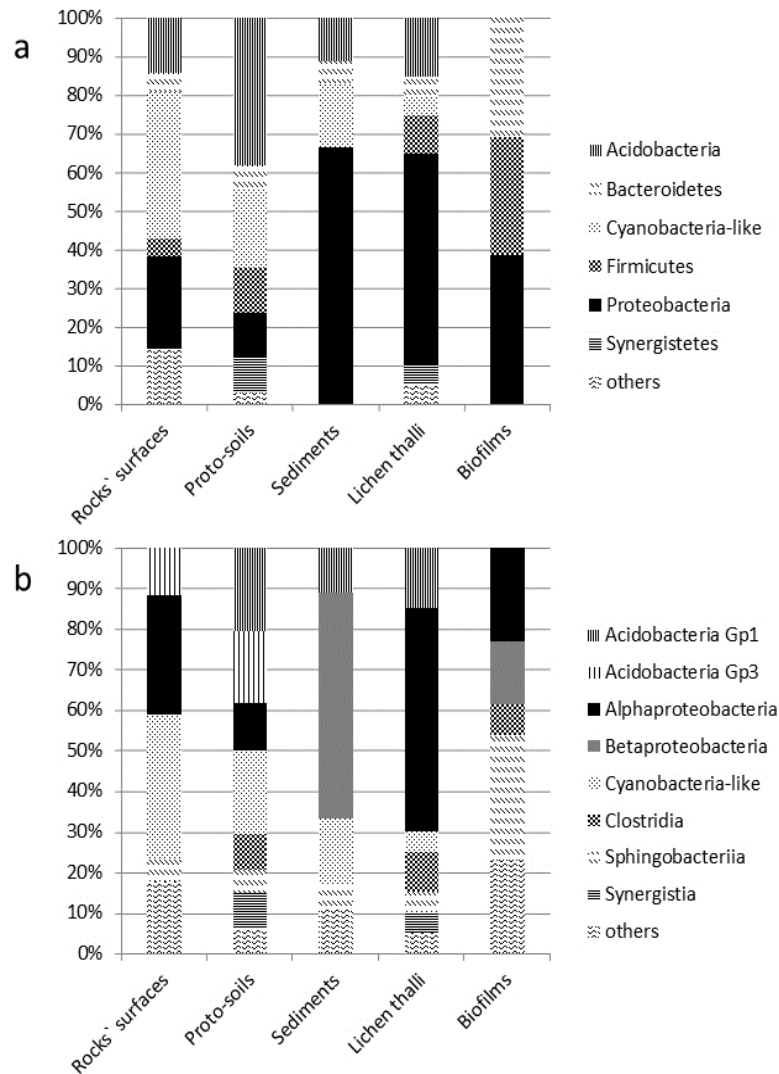

Figure 1 - Stacked barcharts representing the relative abundance of each bacterial phylum (a) or class (b) in each environmental matrix

Results

Bacterial diversity

We obtained 106 sequences from the DGGE profiles. There were specific differences in community composition in each environmental matrix (fig. 1): *Cyanobacteria* were mostly represented in rocks, *Acidobacteria* in proto-soil samples, β- and γ-*Proteobacteria* in sediments, α-*Proteobacteria*, *Acidobacteria* and *Firmicutes* in lichens, while biofilms had equally abundant sequences belonging to *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The UniFrac p-value of the phylogenetic tree, considering all the environments (Supplementary Material 1), was highly significant (<=0.001). A high significant difference was found between rock surface and biofilm, and between sediments and all the other environments but biofilms (table 2). DGGE showed the presence of bacteria with high identity with uncultured organisms isolated in environments such as soils at a high elevation (14.8 % of DGGE

sequences) [18, 38, 47], volcanic rocks (15.9 %) [16, 29], or uncultured microorganisms involved in the cycle of metals (8.6 %) [11] or in the oxidation of methane (5.3 %) [32].

	R	RL	SD	SL
B	<0.001**	0.02*	0.12	<=0.001**
R		0.05*	<0.001**	1.00
RL			<0.001**	0.95
SD				<0.001**

Table 2 - UniFrac p-values for all pairwise comparison, *marginally significant (0.01-0.05), **highly significant (<0.001). Suffixes' acronyms are reported in Table 1 caption

Alpha-diversity analysis from LH-PCR and ARISA data

In table 3 the number of OTU, the H' index and the J' index for both LH-PCR and ARISA of each sample are reported. LH-PCR gave an average of 25 peaks per sample, ranging from 299 bp to 457 bp. The highest number of Operative Taxonomic Units (OTU) was found in the sample 4.1SL (35 OTU), while the lowest was in sample 80.1B (15 OTU). Some OTU were found in certain environmental matrices only: rocks had 4 unique OTU, proto-soils 4, stream sediments 2, while lichens and biofilms did not show any unique OTU. The matrix obtained from ARISA had an average of 45 peaks per sample, ranging from 202 bp to 1387 bp. The highest number of OTU was found in the sample 71.1R (97 OTU), while the lowest was in sample 90.1R (15 OTU). Rocks had 44 unique OTU, proto-soils 20, lichens 15, sediments 9 and biofilms 8. The average values of H' and J' indices were respectively comprised between 2.25 and 2.74 and between 0.443 and 0.540 for LH-PCR and respectively between 3.19 and 3.61 and between 0.694 and 0.783 for ARISA. The Kruskal-Wallis test found significant differences ($p < 0.05$) among the proto-soil and the other environmental matrices in LH-PCR fragment richness and H' diversity, while for the evenness no significant differences were found. Slight significant differences ($p = 0.05$) were found between rock's lichens and rock's surfaces for the ARISA OTU richness and H' index, while proto-soils showed significant difference with biofilm bacterial communities ($p = 0.023$).
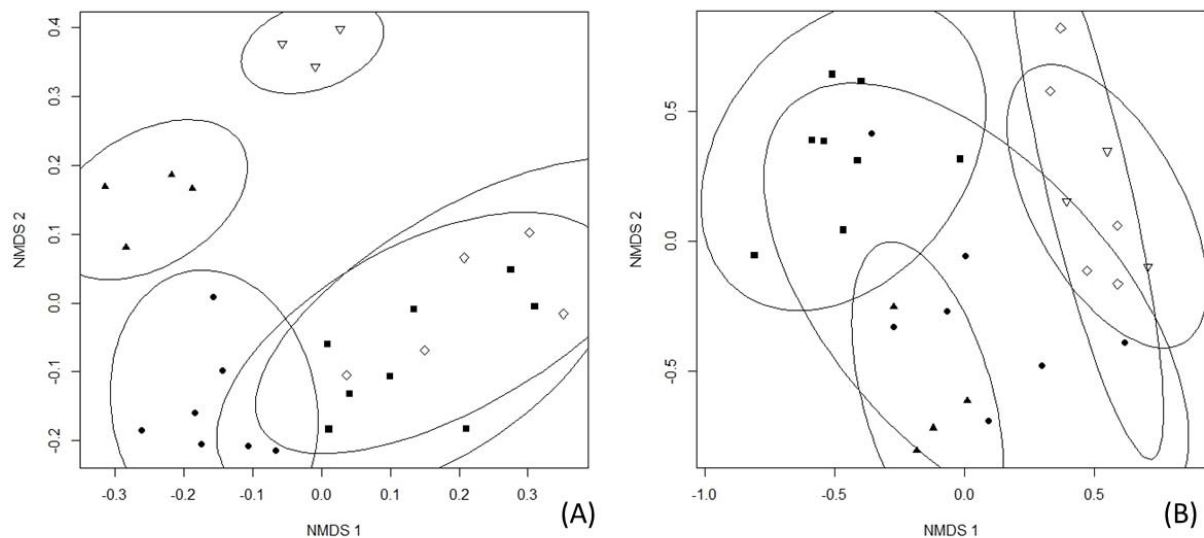

Figure 2 - NDMS ordination plots from LH-PCR (a) and ARISA (b) including rock surfaces (squares), proto-soils (circles), stream sediments (triangles up), lichen thalli (diamonds) and waterspring biofilms (triangles down). Full shapes are abiotic, empty shapes are biotic samples

Sample number	LH-PCR			ARISA		
	N. OTU	Shannon H'	Evenness J'	N. OTU	Shannon H'	Evenness J'
1.1R	30	2.534	0.4202	67	3.814	0.6767
2.1R	29	2.396	0.3785	38	3.36	0.7578
8.5R	30	2.555	0.4291	80	4.042	0.7116
70.1R	29	2.789	0.5611	40	3.561	0.8795
71.1R	20	2.502	0.6104	97	4.183	0.6762
76.1R	20	2.542	0.6353	59	3.864	0.8081
90.1R	19	2.282	0.5154	15	2.361	0.7069
118R	17	1.92	0.401	59	3.661	0.6596
4.1SL	35	2.715	0.4315	52	3.63	0.7254
5.1SL	33	2.894	0.5476	28	2.94	0.6759
50.1SL	30	2.761	0.5272	29	2.869	0.6073
73.1SL	27	2.304	0.3709	27	3.019	0.7584
93.1SL	30	2.772	0.5329	27	2.962	0.7163
121SL	26	2.883	0.6873	67	3.93	0.7599
134SL	32	2.818	0.523	58	3.74	0.7256
45.1SD	16	2.356	0.6591	27	2.918	0.6852
46.1SD	25	1.89	0.2647	35	3.359	0.8217
48.1SD	22	2.282	0.4451	49	3.516	0.6865
52.1SD	29	2.457	0.4025	59	3.686	0.6762
77.2RL	26	2.243	0.3622	34	2.882	0.525
88.1RL	28	3.043	0.749	38	3.483	0.8572
117RL	19	2.166	0.4592	43	3.311	0.6372
120RL	16	2.053	0.4869	36	3.067	0.5966
138RL	22	2.649	0.6428	29	3.21	0.8546
78.1B	24	2.413	0.4653	53	3.716	0.7755
80.1B	15	2.192	0.5966	20	2.725	0.763
81.1B	18	2.275	0.5404	49	3.68	0.8091

Table 3 - Number of fragments and diversity indices for each sampling point for each fingerprinting method. Suffixes' acronyms are reported in Table 1 caption

Beta-diversity analysis from LH-PCR and ARISA data
Two distinct NMDS ordination plots were obtained from LH-PCR and ARISA data (Fig. 2). The stress values were 0.13 for LH-PCR and 0.18 for ARISA. The LH-PCR ordination plot (Fig. 2a) showed that biofilm, sediments and proto-soils formed well-defined clusters. Lichens and rocks grouped together on the right side of the first axis. ARISA plots showed biotic matrices grouped in a mixed cluster, while sediments and rocks formed distinctive groups. Proto-soils were sparsely scattered in the plot (Fig. 2b).

Discussion
The techniques used in this study have different phylogenetic resolutions due to the different gene targets (16S rRNA gene for DGGE and LH-PCR vs the 16S-23S rRNA genes Internal Transcribed Spacer for ARISA) and to the fragment separation procedures by electrophoresis (length polymorphisms for LH-PCR and ARISA vs sequence polymorphisms for DGGE). DGGE is known to be helpful in naming the genus/species of environmental entities through partial 16S rRNA gene sequencing, although it has a lower sensitivity relative to capillary

electrophoresis. LH-PCR can be used to assess bacterial community structures at the genus/species (broad taxonomic resolution) [7, 10] while ARISA can determine the subspecies level (deep taxonomic resolution) through the detection of each internal spacer length polymorphism within the different copies of ribosomal operons in a bacterial cell [13, 19]. As found by DGGE, the prevalence of *Cyanobacteria* and the detection of sequences affiliated to the UV-resistant *Deinococcus* sp. [5] on rock surfaces supports the importance of autotrophy and ionizing-ray resistance in highly oligotrophic environments exposed to sun irradiation. The prevalence of *β-Proteobacteria* in riparian sediments agrees with previous studies carried out on a nearby valley with a similar geochemical background [4], where the authors surveyed bacterial communities from a glacial stream. Moreover, the prevalence of *α-Proteobacteria* in *Rhizocarpon* spp. lichens is in agreement with Bjelland et al [8] and Grube et al [22], who demonstrated the predominance of *α-Proteobacteria* in *Rhizocarpon geographicum*, *Cladonia arbuscula*, *Lecanora polytropa*, *Umbilicaria cylindrical* and other lichens. A high level of bacterial diversity was also found by Hodkinson et al [27], who detected even *Acidobacteria* in different lichens' species, especially in genus *Ophioparma*. *Acidobacteria* seems to be ubiquitous in terrestrial environments of Matsch Valley, since all our samples, except biofilms, were characterized by their presence. The prevalence of *Acidobacteria* in proto-soils is consistent with other data on pioneer plant rhizosphere, carried out in vegetation plots at 2400 m in the same valley. The three oligotrophic water springs hosted a complex biofilm mainly dominated by aerobic, anaerobic and chemotrophic microorganisms, such as *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (*Clostridia*). While other authors found *Cyanobacteria* in oligotrophic mountain freshwater basins [3], we did not detect any presence of photoautotrophic bacteria, probably because of the intertaxa competition and a more torrential water regime of the springs.

UniFrac P-values showed a wide ecological distance among bacterial communities belonging to different environmental matrices and justifies the use of a NMDS ordination plot to screen the beta-diversity [9]. By considering the overall results, we noticed that proto-soils were the richest environment in bacterial diversity, as confirmed by a Kruskal-Wallis test, DGGE sequencing and ARISA NMDS. With the increase in carbon content along moraine chronological sequences [48], bacterial species richness tends to increase as well [37]. On the other hand, as shown by the LH-PCR NMDS plots, highly oligotrophic environments, such as stream sediments, led to the development of a genetically specialized bacterial community with a low level of genus/species variability. The discrepancies between the clustering pattern of lichens in the NMDS ordination plots of LH-PCR and ARISA could be explained by the different resolution of the two techniques. We hypothesize that, while on a broad taxonomical scale, lichens harvest diversity from the surrounding environment (the rock on which they lay), at a subspecies level their communities are affected by other kinds of factors, similarly to biofilms. The finding of *Acidobacteria* as an important fraction of bacterial diversity on rocks' surfaces and lichens seems to support this conclusion. *Acidobacteria* were also found in pioneer plant-associated bacterial communities from Matsch Valley and seems to be one of the principal contributors to Matsch Valley overall biodiversity, probably due to the particular lithological substrate of the valley. Matsch Valley is characterized by peralluminous acidic rocks, mainly schist and gneiss [24], that contributes to the overall soil acidity. However, there is not a clear linkage between the presence of *Acidobacteria* and the soil/rock acidity, and the ecological importance of *Acidobacteria* is still debated, although it seems they are particularly adapted to unstable temperatures and freeze–thaw cycles [43]. A deeper analysis of the bacterial diversity made by high throughput tagged-pyrosequencing techniques may help to address this issue.

With this study we described the taxonomic distribution of bacterial communities involved in rock, sediment and proto-soil colonization, as well as diversity parameters through the use of three fingerprinting techniques. The polyphasic approach not only overcomes the limits of each technique alone but may also reveal patterns of diversity that help us formulate complex hypotheses about the distribution of organisms at different taxonomical levels.

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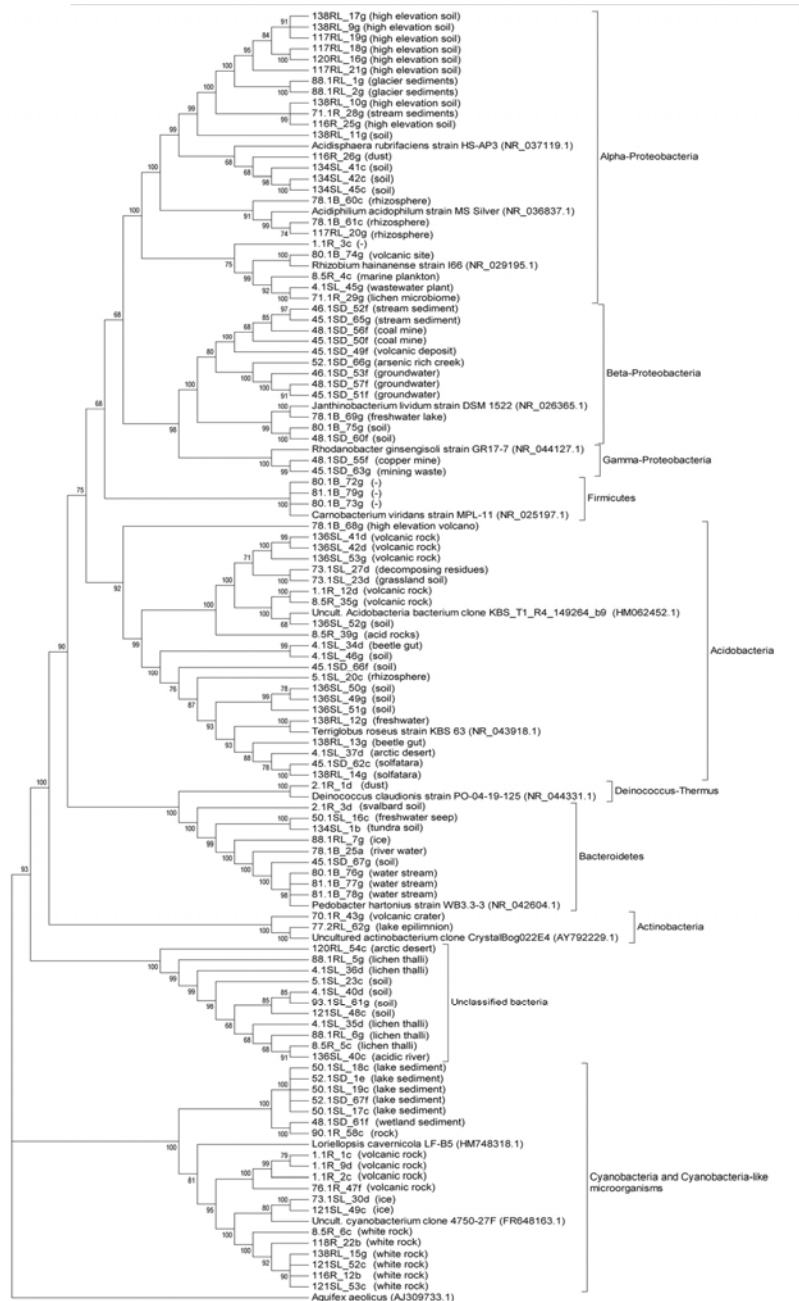
SUPPLEMENTARY MATERIALS

Figure SM 1 - Phylogenetic tree of the partial 16S rRNA genes DGGE fragment. Samples included rock surfaces (R), proto-soils (SL), stream sediments (SD), lichen thalli (RL) and waterspring biofilms (B) The tree was obtained through the Maximum Likelihood method (log likelihood = -8221.70) by MEGA software. Bootstrap percentages are given on branches

Pioneer plants species select peculiar rhizosphere bacterial communities in a high mountain environment

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Abstract
The rhizobacterial communities of 29 pioneer plants belonging to 12 species were investigated in an alpine ecosystem to assess if plant of different species could select for specific rhizobacterial communities. And, we compared the rhizobacterial communities with bacterial communities of three bare sites. To allow a culture-independent perspective, metagenomic DNA was extracted from both rhizosphere and bulk soil samples and analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Denaturing Gradient Gel Electrophoresis (DGGE). ARISA fingerprinting and ANOSIM analyses showed rhizobacterial genetic structure extremely different from bare soils bacterial communities and almost all the rhizabacterial communities clustered strictly according to the plant species. DGGE analysis showed rhizobacterial communities mainly composed by Acidobacteria and Proteobacteria whereas bacterial communities associated to the bare soil were composed by Acidobacteria and Clostridia. UNIFRAC significance calculated on DGGE results confirmed the rhizosphere effect exerted by the 12 species and showed different bacterial communities ($P < 0.05$) associated to almost all the plant species. These results pointed out that peculiar rhizobacterial communities were select by pioneer plants of different plant species in a natural high mountain ecosystem during an early primary succession.
Keywords: bacterial communities, pioneer plants, Alps, rhizosphere, ARISA

Introduction
In natural, agricultural and forest ecosystems, the rhizosphere is a biologically active zone of extreme importance because of the crucial role of plant–microorganism interactions in nutrient cycling, carbon sequestration, and ecosystem functioning (40). The increasing interest in plant growth promotion, nutrition, and biological control of soil-borne plant pathogens requires a proper understanding of the structural and functional diversity of the bacterial communities in the rhizosphere. Several studies have been carried out to reveal the impact of biotic and abiotic factors on the below-ground microbial diversity. It has been demonstrated that the plant species and plant species composition select for taxonomic and functional groups in the rhizosphere due to different root exudation and rhizodeposition. For instance, crops grown in monoculture or on agricultural soils have revealed evidence for plant species effect on specific bacterial communities (8, 13, 24, 42, 44). Using a culture-independent analysis, peculiar bacterial communities have been observed in the rhizosphere of field-grown strawberry (*Fragaria ananassa* Duch.), oil-seed rape (*Brassica napus* L.), and potato (*Solanum tuberosum* L.). Moreover, the plant species effect increased after planting the same crops in consecutive years (42). However, studies of non-agricultural plant communities have indicated variable results. A high degree of plant effects was demonstrated for the native perennial bunchgrasses *Stipa*, *Hilaria* and for the invading annual grass *Bromus* (27), for eight native herbaceous plants in Germany (7) or for *Nardo-Galion-* and *Lolio-Plantaginion* grasslands in Ireland (2). Other experiments indicated the influence of both plant species and soil type (29, 30). Different studies showed that soil characteristics (18, 25, 26, 48), soil texture (39), soil mineral composition (4), pH (28), season, and land management (21) can exert a greater effect on rhizosphere bacterial ecology than plant species and plant species composition.

In a natural ecosystem it is difficult to assess the effect of vegetation on the rhizosphere bacterial communities, especially in high mountain environments characterized by variable environmental parameters (successional stage, pH, rainfall, moisture, mineral composition, sampling season, slope) within a size-limited area typical of early and transitional successional stages.

In a successional chronosequence, resulting of a continuous glacier retreat, patchy vegetation can colonize harsh environmental niches with high fraction of coarse-grained mineral skeleton, low total carbon and nitrogen content, and severe climatic regimes (31). We can suppose that pioneer plants, which colonize early and transitional successional stages, could select different rhizosphere microbial communities able to promote plant growth in these oligotrophic conditions. But only few studies have examined how rhizosphere directly impact microbial communities in the young alpine ecosystems (9, 32, 45, 46). The major focus of these studies was to highlight the relationship between the chronosequences in alpine ecosystems and different microbial communities in the rhizosphere of pioneer plants and related bare soil. In an early successional stage, the rhizosphere microbial community of *Poa alpina* L. was strongly influenced by harsh abiotic constrains, but under more favorable environmental conditions, the plant could select for a specific microbial community (45). Interestingly, along a

similar chronosequence, the pioneer plant *Leucanthemopsis alpina* (L.) Heywood exerted a contradictory rhizosphere effect showing a specific microbial community in the early succession stage only (9). However, the study of the spatial extent of *Lc. alpina* on the microbial community and physical-chemical parameters in an early successional stage (5, 10 years) did not exhibit significant trends, supporting the conclusion of Tscherko *et al.* (45). Moreover, Tscherko *et al.* (46) did not clearly show a selective effect of different plant species on the bacterial communities in the rhizosphere, because this effect seemed to be related with the soil age.

To avoid the clear correlation between rhizobacterial community and soil age along a chronosequence, we decided to investigate the impact of plant belonging to different species on rhizobacterial communities sampling plants grown on a deglaciated terrain of the same age. Aim of the work was to assess if different plant species selected specific rhizobacterial communities.

Materials and methods

Study site and soil samples

The study area is located in the upstream sub-catchment of the Saldur river (46° 46' 30" N; 10° 41' 46" E; 2,400 a.s.l.) in the high Matsch valley (inner Central Alps, South Tyrol, N Italy). The sub-catchment has a drainage area of 11 km^2. The dominant geological processes are periglacial and the streamflow is dominated by the glacier dynamics. The overall valley had an average rainfall of about 550 mm per year in the period of 1970-2000. In 2011, the mean temperature during the plant growing season was 7.3°C in July, 10.3°C in August, and 8°C in September and the mean precipitation was 2.7, 2.5, 3.6 mm per day, respectively. The main rock types are schist and gneiss (15), while the most diffused soil types are acidic leptosols, regosols and umbrisols (mean pH = 4.3), derived from carbonate-free bedrocks. The study site is entirely located above the tree line (2,100 m a.s.l.), where a quick retreating glacier has left 3.3 km^2 of foreland in the last 160 years (23). The different stages of glacier retreat were reconstructed by analyzing the historical maps of the Third Austro-Hungarian topographic survey (the so called "Franzisco-Josephinische Landesaufnahme") dated 1850, an aerial photograph of 1945, and ortophotos from 2006. On the basis of those photos, our sampling site was ice-free since 1850.

Rhizosphere and soil sampling were carried out in September 2011, at the end of the plant growing season. Thirteen of the most representative plant species of the 160 years old successional stage were identified in a plot of 5 m × 5 m, *i.e. Festuca halleri* All., *Gnaphalium supinum* L., *Leucanthemopsis alpina* (L.) Heywood, *Linaria alpina* (L.) Mill, *Minuartia sedoides* (L.) Hiern, *Potentilla aurea* L., *Saxifraga bryoides* L., *Sedum alpestre* Vill., *Senecio carniolicus* (Willd.) Braun-Blanq, *Sibbaldia procumbens* L., *Silene acaulis* (L.) Jacq, *Veronica bellidioides* L. [Varolo *et al*. submitted]. The sampling site of each pioneer plant individuals was chosen to be as much as homogeneous possible in terms of altitude, aspect, slope, physical and chemical composition. Soil was a sandy silt soil with humus, having an average texture of 72.3 ± 5.0 % of sand, 21.0 ± 4.1 % of silt, 6.6 ± 1.3 % of clay, and 4.6 ± 1.3 % of humus. Total organic carbon was 2.6 ± 0.8 %, while pH was 4.5 ± 0.3 %. The average chemical composition of the sampled soils was ammonia 3.4 ± 1.0 mg kg^{-1} d.m., total P 0.7 ± 0.1 mg kg^{-1} d.m., total K 7.4 ± 1.0 mg kg^{-1} d.m., total Ca 3.4 ± 0.6 mg kg^{-1} d.m., total K 7.4 ± 1.0 mg kg^{-1} d.m., total Mg 13.4 ± 1.7 mg kg^{-1} d.m., total Fe 45.4 ± 6.9 mg kg^{-1} d.m., and total Al 29.4 ± 5.6 mg kg^{-1} d.m. No nitrate and calcium carbonate were detected.

One or more rhizosphere samples were collected per each plant species. After pulling out each plant, we collected the soil adhering to the roots. An amount of about 4 g of rhizosphere soil for each plant was extracted. Three replicates of bulk soil were collected from the same area as a control. Soil samples were put in sterile bags and transported in refrigerated boxes to the laboratory as soon as the logistic constraints permitted and stored at -80°C until the analyses.

Molecular analysis of the bacterial communities

Total DNA of the rhizosphere and soil samples was extracted using Ultraclean Soil DNA Extraction kit (MO-BIO, Arcore, Italy) following the manufacturer's instruction. Microbial analyses were carried out using culturing-independent methods, i.e. DGGE (33) to describe the bacterial diversity associated to each plant species and ARISA (3) to describe the structure of the overall rhizobacterial communities.

For the DGGE analysis, primers GC357f and 907r were used as described by Sass *et al*. (38). DGGE was run in a BioRad DCode Universal Mutation Detection System. Polyacrylamide gels were done according to Muyzer *et al*. (33). The gels were stained for 30 min in 1 × TAE buffer containing SYBR® Safe - DNA Gel Stain (Invitrogen, Milan, Italy). Visualization and digital image recording was performed with UVTec (Cambridge, UK). DGGE bands were excised and reamplified as described (33). Sequencing was performed by STAB-Vida Inc. (Caparica, Portugal). Identification of 16S rRNA genes was done by a comparison with the EMBL/Genebank/DDBJ database and RDP database using BLASTN and Classifier respectively. All sequences were submitted to the RDP-Ribosomal Database Project (5) web server to assign taxonomy.

ARISA fingerprint was performed as described elsewhere (3) with the ITSF/ITSREub primer set. Denatured ARISA fragments were run by STAB-Vida Inc. The data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems), and a threshold of 40 fluorescent units was used, corresponding to two times the highest peak detected during the negative control run. If the baseline varied inconsistently, the sample was made run again. Output matrix was obtained as by Rees *et al*. (37).

Statistical analysis

Multivariate analysis was performed on ARISA profiles that were normalized with the formula $(x/\sum x)*1000$ where "x" is the fragment height in units of fluorescence and then log transformed to balance the advantage of analyzing non transformed data, which keeps relative abundance information, and binary data, which down-weigh abundant groups. In order to assess changes in rhizobacterial community structure between different plant species, nonmetric multidimensional scaling (NMDS) was applied because it avoids the assumption of linear relationships among variables, and it is reported to be the most effective ordination method for ecological community data (Clarke, 1993). PERMANOVA (based on Bray-Curtis similarity) was performed to test significant differences in the profile composition between the plant rhizospheres and bare soils. PERMANOVA is a nonparametric permutation-based statistical test of significant differences between two or more groups, based on ant distance measure (Anderson et al 2001). It is an analogue of the univariate ANOVA and it calculates the F value. The null hypothesis was rejected when significance value was < 0.05.
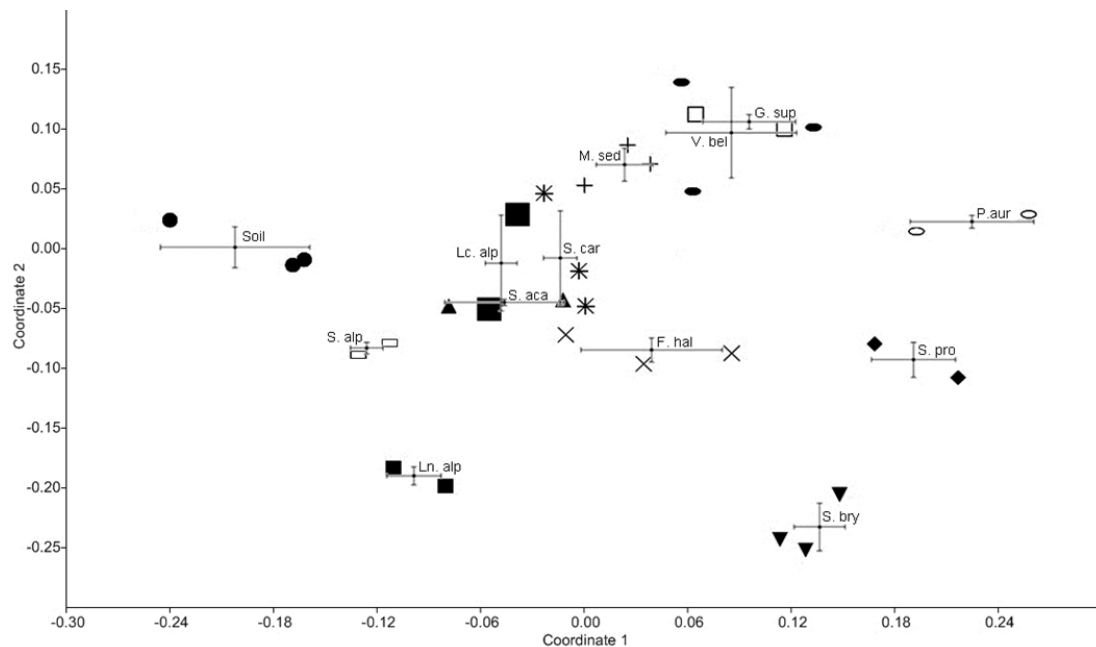

Figure 1 - Non-Metric Multidimensional Scaling analysis of the ARISA patterns of the bacterial communities associated to the 12 pioneer plant species rhizosphere and of the unvegetated soil. Symbols are as follows: black circle - bare soil; little black square - Linaria alpina; big black square - Leucanthemopsis sp.; black triangle - Silene acaulis; cross - Festuca halleri; asterisk - Senecio carniolicus; plus - Minuartia sedoides; white square - Gnaphalium supinum; black oval - Veronica bellidioides; white oval - Potentilla aurea; black diamond - Sibbaldia procumbens; black wedge - Saxifraga bryoides. Values are means ± SD.

The Nexus format of the phylogenetic tree of the DGGE identified bands performed by MEGA5, was submitted to the UniFrac web server to test differences among samples based on the UniFrac metric with 100 permutations (Hamady et al., 2010). All the statistics analyses were performed using PAST program (Hammer et al., 2001).

Sequence data
Sequences were submitted to the Genbank/EMBL/DDBJ databanks under the accession numbers from HF930771 to HF931020.

Results
ARISA provided the fingerprints of both rhizosphere and bare soil bacterial communities. Due to the high sensitivity of the automated sequencer, complex profiles with peaks ranging from 155 bp to 1477 bp were obtained and the internal transcribed spacer region (ITS) richness varied from 29 to 166 peaks. The electropherograms, characterized by distinct peaks number and intensity, revealed a large shift in bacterial community structure across the different plant species (data not showed). *S. carniolicus* and *G. supinum* showed the major peaks between 489-541 bp (13 % and 15 %, respectively) and also between 592-751 bp (33 % and 35 %, respectively), like *S. alpestre* (35 %). Between 489-751 bp were the 59%, 57%, and 54% of the peaks of *Lc. alpina*, *V. bellidioides,* and *S. acaulis*, respectively. *S. procumbens* had 14% of the bands between 279-331 bp and the major peaks between 489-695 bp. The major peaks of *M. sedoides* (85%) were widely distributed between 332-751 bp. The 90% of the peaks of *P. aurea* were between 279-695 bp. *S. procumbens*, *M. sedoides*, and *P. aurea* had no significant peaks from 752 to 1477 bp. *S. bryoides* showed 10% of peaks between 279-331 bp and the major bands (40%) between 542-751 bp. *F. halleri* had 7% of peaks between 155-280 bp and the most bands until 807 bp. *Ln. alpina* exhibited a completely different profile characterized by 12% of peaks between 155-280 bp and widely distributed bands along the entire profile. The unvegetated soils revealed a high degree of similarity among replicates, and 92% of their peaks were evenly present between 279-981 bp. Comparing soil samples with all the rhizosphere samples, PERMANOVA confirmed significantly different microbial community structures (F=1.58; P < 0.0069).
We studied the pioneer plants using a Rho NMDS analysis (Figure 1). On the NMDS plot with the deviation standard, the replicates samples of bare soils, *F. halleri*, *S. alpestre*, *S. procumbens*, *M. sedoides, P. aurea, Ln. alpina,* and *S. bryoides* showed separate clusters based on microbial community structure whereas the replicates of *G. supinum* and *V. bellidioides* clustered more strictly together like the ones of *Lc. Alpina, S. carniolicus, S. acaulis.*

DGGE was performed to investigate the different microenvironments in terms of their dominant bacterial population. 250 sequences of more than 300 bp were obtained from all sample profiles. RDP facilitated the determination of putative taxonomic affiliation of the recovered sequences. Major bacterial taxa included Acidobacteria Gp3 and Gp1, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Sphingobacteria, Actinobacteria and Firmicutes. Although many bands were detected in almost all the samples, shifts in bacterial communities were visible. Members of the Acidobacteria order were present in all the rhizosphere and bulk soil samples except in *S. bryoides* and *S. procumbens* rhizospheres. They were the most abundant taxa with percentage from 35 % associated with *M. recurva* up to 74 % with *G. supinum*. We found also Proteobacteria in almost all the samples, but less abundant than Acidobacteria. However, Proteobacteria represented 74 % of the rhizosphere bacterial communities in *S. procumbens*. Sphingobacteria were 75 % of the bacterial communities associated to *S. bryoides* but they were recovered in few samples and with low percentages. Members of Firmicutes and Actinobacteria taxa were even less abundant, being present in four and three plant species respectively. We did not found Proteobacteria, Sphingobacteria, Actinobacteria taxa associated to bare soil samples. Despite bias associated with sampling, DNA extraction, PCR amplification and DGGE run, the pattern of differences in bacterial communities between bare soils and plant rhizosphere was supported by the pairwise UniFrac distance ordinations. Comparing each pair of environments, the UniFrac significance (*P* values < 0.05) showed that the bare soils samples were significantly different from almost all the rhizosphere (except *Lc. alpina*) and almost all the plant species exhibited peculiar bacterial communities associated to the rhizosphere. The first axis of PCA analysis, using the weighted UniFrac distance matrix, explained 29.21% of the total variation along the first axis, 14.38% along the second axis

and 9.68% along the third axis and showed the significant shift of the bacterial communities (Figure 2). The wide distribution of the samples in all the quadrant suggesting a different bacterial community composition.
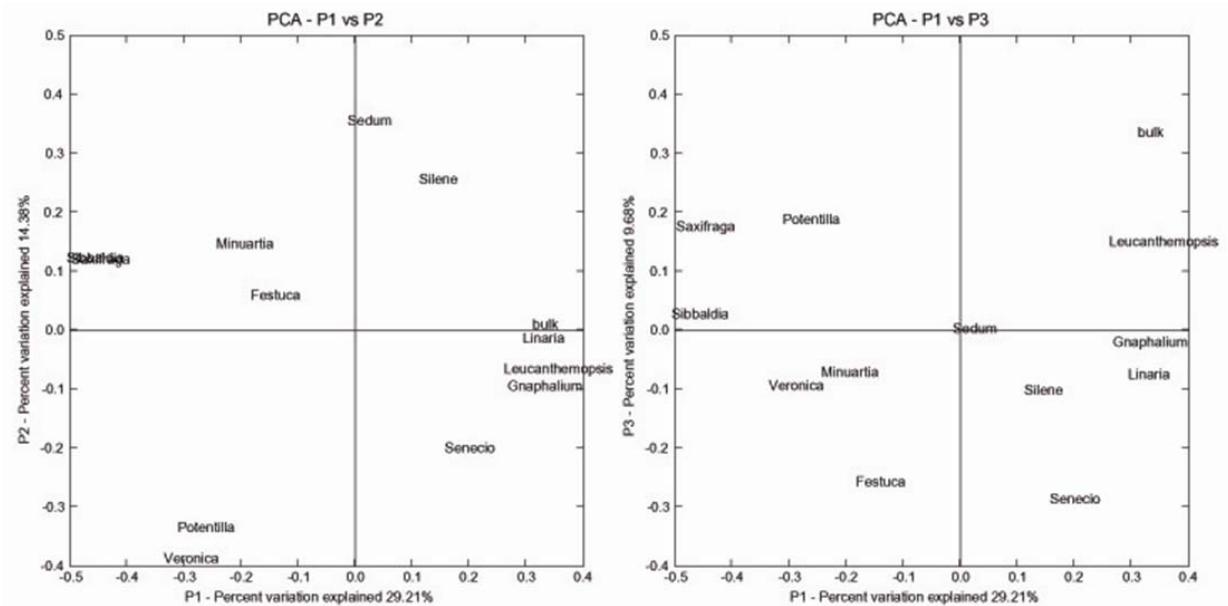

Figure 2 - Principal Component Analysis plot of the 12 pioneer plants and the bare soil according to Unifrac distance matrix.

Discussion

The study supports the ability of pioneer plant of different species to select peculiar rhizobacterial community in a natural alpine ecosystem. Our study of rhizobacterial communities detected a distinct clustering of bacterial communities according to 7 of 12 plant species and to bare soil. The UniFrac analysis showed shifts in bacterial diversity which suggest a specialized physiologies adapted to the peculiar rhizosphere conditions.

Basically soil type, soil characteristics, growth stage, management practices, and growing season may shape the composition of the microbial communities in the rhizosphere (11, 18, 21, 24, 28, 29, 39, 47, 48). A number of studies have assessed the structural and functional diversity of rhizosphere bacterial communities associated with different plant species according to specific root exudation and rhizodeposition (1, 10, 20). Most of these previous investigations were conducted either in artificial microcosms such as pots or on agricultural soils such as orchards or crop monocultures. It is more difficult to assess the association between plant species and rhizosphere bacterial communities in a natural ecosystem, like our alpine ecosystem.

To show that rhizobacterial communities of pioneer plant were strictly related to the plant species in the natural high mountain ecosystem, we designed the experimental sampling plan to minimize as much as we could all the environmental variables (rainfall, moisture, sampling season) within a size-limited area characterized by the same soil parameters (successional stage, pH, moisture, mineral composition). Moreover, to exclude the correlation between rhizobacterial community and soil age along a chronosequence, we decided to investigate plant of different species grown on a deglaciated terrain of the same age. We chose plants growing on a 160 years soil because, due to the quick glacier melting in the last 80 years, it represents the only transitional step of our glacier morain between earliest stages (< 10 years) and mature soil (> 500 years). As shown by aerial photos, ortophotos, and a topographic survey, the Matscher glacier, one of the glacier tongue of the Weisskugel glacier, has been retreating accordingly to a discontinuous movement. Consequently, we had not a constant gradient of soil age, but rather distinct block stages in which soil age is invariable. Moreover, the 160 years old stage is characterized by harsh environmental niches with poor mineral skeleton, low total carbon, low nitrogen content and suffered severe climatic regimes. In this environment, it is still possible to find single plant individuals not interrelated in proto-floristic complex communities. And being more stable than an earlier successional soil, the 160 years old soil hosts a larger number of plant species. Hence, it was possible for us sampling several single

individuals of the same species, avoiding the influence of the rhizosphere of other plant species. We sampled at least two field replications, in order to identify consistent plant species effect on the rhizobacterial communities.

Some previous studies investigated a single plant species along alpine chronosequences to assess the effect of pioneer plants on rhizobacterial communities and their results were variable. The rhizosphere of the pioneer plant *Lc. alpina* was different from the interspace community in an early successional stage and also different from the rhizosphere and interspace communities of a late successional stage. However, rhizosphere and interspace communities in the late successional stage were quite similar. It seems that the influence of *Lc. alpina* depends on soil age linked to the different nutrient availability (9). Some *Lc. alpina* individuals occurring in the early successional stage (5, 10 years) of a glacier forefield (Dammaglacier, Switzerland) were studied to assess the effect of this pioneer plant on microbial community structure and functions (32). Fingerprinting analyses of 16S rRNA gene (T-RFLP, DGGE) showed similar bacterial communities to 40 cm of distance to the plant and, therefore, they could not demonstrate the selective effect of the rhizosphere on the microbial community. Another single plant studied along a chronosequence was *P. alpina* (45) which in the pioneer stage did not exhibited a selective role on its rhizosphere bacterial community due to the harsh environment. However, under more mature stages, the plant could select for a specific microbial community but related to soil properties and carbon supply. Although Tscherko et al. (2005) found that microbial biomass, rhizosphere enzyme activity, fungal/bacterial PFLA, Gram positives, Gram negatives and Gram positives/Gram negatives ratio were higher for late successional plant species than for early pioneer plants, this did not conclusively prove a more pronounced plant species effect in the late successional stage (75, 135 years) than in the first 43 years of succession because the result could be a consequence of the different soil age on which the studied plants grown.

These studies performed several cultural-independent techniques: PFLA used by Tscherko *et al.* (45, 46) could point out only the different concentration of bacterial/fungal fatty acids and compare the Gram positives/Gram negatives ratio. This biochemical approach was less discriminatory than the molecular methods to provide the microbial community structure in soils (41). Furthermore, RFLP and DGGE analyses, used by Edwards *et al.* (9) and Miniaci *et al.* (32) to assess the bacterial community structures, give fewer information than ARISA analysis, although theoretically, each DGGE band could represent a distinct bacterial phylotype and RFLP, being automated, is more useful than DGGE for data reproducibility. DGGE and RFLP based on the analysis of 16S rRNA gene cannot distinguish among closely related groups whereas ARISA, targeting the intergenic 16S-23S highly variable ITS region, shows more sensitivity and enable identification on the species or even sub-species level. Comparing T-RFLP and ARISA analyses, Danovaro *et al.* (6) suggested that ARISA is more accurate for evaluating the biodiversity of different aquatic environments. Moreover, ARISA analysis appeared the more suitable method for diversity analysis of soil microbial community structure (34). Thanks to its high phylogenetic resolution and the estimated OTU richness, ARISA can describe different bacterioplankton communities along an estuarine gradient in Moreton bay, Australia (19).

In our study, the NMDS and ANOSIM analyses of ARISA profiles and the Unifrac significance values of DGGE analysis were useful culture-independent techniques to compare rhizosphere bacterial communities and to point out the ordination of the samples structure among the different site. Within the stable block stage of 160 years old in an alpine environment, the measured differences in rhizobacterial composition, supported hypothesis that the plant species could exhibited an effect on the rhizobacterial communities.

The plant rhizosphere samples did not cluster together with the bare soil samples showing a pronounced rhizosphere effect exerted by the 12 alpine plant species. The significant difference between soil samples and the rhizosphere of 12 different plant species showed a clear selection carried out by the rhizosphere not showed in previous studies on pioneer plants *Lc. alpina* or *P. alpina*. Moreover, the NMDS analysis showed a selection of seven different plant species on the rhizobacterial community. These results confirmed some studies about plant species selection of rhizobacterial community. Despite the different glacial forefield dynamics and early primary succession events, Knelman *et al.* (22) demonstrated plant species effect on bacterial communities of early colonizer plants (*Alnus sinuata, Picea sitchensis*) in the Mendenhall glacier forefield (AK, USA). Kuske et al., 2002 demonstrated an influence of plant species comparing the rhizosphere bacterial communities of three plant species of an arid high elevation grassland.

On the other hand, the two cluster made up of *S. acaulis*, *Lc. alpina* , *S. carniolicus* and *G. supinum*, *V. bellidioides* could confirm the variable previous studies because a successional chronosequence characterized by alluvial fan, scree, are not stable and take back the environment to the harsh pioneer stage where the plant was not able to exhibit a selective role on its rhizosphere bacterial community. The Unifrac distance values, which were almost all significant, confirmed the plant species effect.

According to Fisher *et al*. (12), Ranjard *et al*. (36) within an intergenic region ranged from 155 bp to 1477 bp, Gram positive organisms have spacer lengths of about 400 bp, due to the absence of tRNAs in the spacer region of many Gram positive bacteria (14). ITS size of more than 500 bp belong to Gram negative bacteria. Our ARISA profiles suggested that some species like *S. alpestre, M. sedoides,* and *P. aurea* had an even distribution of Gram positive and Gram negative bacteria within the rhizosphere bacterial communities. Moreover, *Lc. alpina, V. bellidioides, S. acaulis, G. supinum,* and *S. carniolicus* hosted bacterial communities dominated by Gram negative bacteria whereas *M. recurva, S. procumbens, S. bryoides,* and *F. halleri*, exhibiting significant percentages of ITS size below 331bp, showed an important presence of Gram positive bacteria. *Ln. alpina* showed a majority of ITS size below 200 bp supporting Tscherko *et al*. (46) where Gram positive organisms are predominantly associated to *Ln. alpina* rhizosphere. Members of Acidobacteria and Proteobacteria phyla (Gram negative bacteria) were widely distributed in our samples, confirming the strict ecological and physiologic relationship within them to support ecological niches like rhizosphere soils (35, 43). It was confirmed the prevalence of both phyla in mountain oligotrophic soils whereas plant growing on agricultural soil showed predominance of Actinobacteria and Firmicutes (42).

In conclusion, by using culture-independent methods, our research reveals the plant species effect exerted on the rhizosphere bacterial communities, despite the harsh environmental condition of the natural alpine ecosystem. This study, focusing on different pioneer plant species, highlights the species effect as shown previously in artificial microcosms only. It will be interesting to investigate if and how different pioneer plant species associated in a proto-floristic community continue selecting the associated bacterial communities in the rhizosphere.

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Safe-site effects on rhizosphere bacterial communities in a high-altitude alpine environment

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Abstract

The rhizosphere effect on bacterial communities associated to three floristic communities (RW, FI, M sites) which differed for the developmental stages, was studied in a high-altitude alpine ecosystem. While RW site represented an early developmental stage and FI site an intermediate stage, M site could be considered a later stage where floristic consortia were more matured. The percentage of N and C in the four soils confirmed a different developmental stage per each of the sites with a kind of gradient from the unvegetated BS site through RW, FI up to the most complex M site. The floristic communities were composed of 21 alpine pioneer plants belonging to 14 species. Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprints showed different bacterial genetic structure per each floristic consortium which differed also from the bulk soil (BS site) bacterial communities. When plants of the same species occurred within the same site, almost all their rhizosphere bacterial communities clustered together exhibiting a plant species effect. Unifrac significance value ($P < 0.05$) on 16S rRNA gene sequencing from Denaturing Gradient Gel Electrophoresis (DGGE) bands revealed significant differences ($P < 0.05$) between BS site and the vegetated sites with a weak similarity to the RW site. The intermediate plant colonization stage FI did not differ significantly from the RW and the M vegetated sites. These results pointed out the peculiar effect of the different floristic communities rhizospheres on their soil bacterial communities.

Introduction

A glacier foreland after glacier retreat is composed of habitats characterized by severe climatic regimes and barren substrate with low total carbon and nitrogen content (Matthews, 1992). Rock cracks, concave surfaces and little depressions could ensure protection from wind, cold and other harsh environmental conditions (Jumpponen et al., 1999; Mizuno, 1998) helping the accumulation of nutrients and the growth of pioneer plants. Safe-sites are defined as little areas, often surrounded by big stones, filled up of stone debris or mineral mud (Harper et al., 1961). Here, opportunistic pioneer plants could settle down and form first floristic consortia, significantly affected by the geochemistry of the lytic material. Indeed, physical and biogeochemical weathering processes provide soils of soluble nutrients and when the plant colonization on parent materials occurs, the development of glacier foreland into fertile soils is enhanced by rhizodeposition, root exudation, decaying biomass and root mass development. Safe-sites can be severely affected by geological dynamics, such as sudden mudslides, alluvial fan sliding, scree movement, that take back the habitat to an early pioneer condition. Consequently, safe-sites cannot reach the climax but only a stable stage of middle maturity (Pignatti, 1994).

Furthermore, pioneer plants could select rhizosphere microbial communities able to promote plant growth thanks to the interactions in nutrient cycling and carbon sequestration (Hayat et al., 2010). Nevertheless, in a natural ecosystem it is difficult to assess the effect of vegetation on the rhizosphere bacterial communities, especially in high mountain environments characterized by variable environmental parameters (successional stage, pH, rainfall, moisture, mineral composition, sampling season, and slope) within a size-limited area typical of early and transitional successional stages. The impact of single plants on microbial communities in an alpine glacier forefield has previously been studied to highlight the relationship between the rhizosphere bacterial communities of pioneer plants or of the related bare soil and the chronosequence (Edwards *et al*. 2006; Miniaci *et al*. 2007; Tscherko *et al*. 2004; 2005). In an early chronosequential stage, the rhizosphere microbial community of *Poa alpina* L. was strongly influenced by the environmental conditions, but in transition and mature stage, plants could select for a specific microbial community (Tscherko *et al*. 2004). Along a similar chronosequence, the pioneer plant *Leucanthemopsis alpina* (L.) Heywood showed an opposing rhizosphere effect with a specific microbial community in the early successional stage only (Edwards *et al*. 2006). The study of the spatial extent of *Lc. alpina* on the microbial community and on the physical-chemical parameters in an early successional stage (5, 10 years) did not exhibit significant trends, supporting the conclusion of Tscherko *et al*. (2004).

However, in a safe-site, the pioneer vegetation interrelated in floristic consortia often exhibited ground stems and root tangle with large nets. In this case, a safe-site could be equaled to a transitional or even mature grassland for root tangle and plant community structure. The floristic community effect in such a habitat were observed in natural as well as in artificial experimental sites (Haichar et al., 2008). Osanai et al. (2013) demonstrated that co-occurring plant species from native grassland selected their microbial communities. The effect was generally

smaller than for species that generally do not co-occur naturally, such as those from agricultural crop systems (Costa et al. 2006), improved grassland systems or fertilized grassland fields (Patra et al. 2006; Benizri et al., 2005). Nunan et al. (2005) found a weak influence of plant community or no effect of plant species on the structure and diversity of the root-colonizing bacterial community when comparing five co-occurring grass species from an upland grazed grassland in Scotland. Moreover, topography and other uncharacterized environmental factors seemed to be main drivers of the bacterial community composition.

On the other hand, studies about the effect of plant cover on microbial community in cold environments regarded different ecological niches and pointed out the higher significance of environmental parameters than the influence of the floristic consortia. In Antarctic environments along a latitudinal gradient, bacterial diversity of dense vegetation from different locations were comparable whereas bacterial diversity of "fell-field" vegetation decreased with increasing latitude (Yergeau et al., 2007a; 2007b). In permafrost meadow, steppe or desert steppe soil characteristics were driving factors of the microbial diversity (Zhang et al., 2012). In high elevation arid grassland, a strong plant effect was demonstrated for the perennial bunchgrasses *Stipa*, *Hilaria* and for the invading annual grass *Bromus* (Kuske *et al*. 2002).

Consequently, aim of this work was to investigate if in different safe-sites on a deglaciated terrain of the same age floristic consortia could select for specific rhizobacterial communities.

Materials and methods

Study site and soil samples

The study site is located into the upstream sub-catchment of Saldur river (46° 46' 30"N; 10°41'46"E; 2,400 a.s.l.) in the high Matsch valley (South Tyrol, Italy) with a drainage area of 11 km^2. The main geological processes are periglacial and the streamflow is characterized by the glacier dynamics. During the 1970-2000, the valley had an average rainfall of about 550 mm per year. In 2011, the mean temperature of the plant growing season were 7.3°C in July, 10.3°C in August, 8°C in September and the mean precipitations were 2.7, 2.5, 3.6 mm per day, respectively. The dominant rock types are schist and gneiss (Habler et al., 2009) and the most common soil types are acidic leptosols, regosols and umbrisols (mean pH = 4.3), derived from carbonate-free bedrocks. The study site, a foreland of about 3.3 Km^2 left after a quick glacier retreat in the last 160 years (Knoll et al., 2010), was located above the tree line (2,100 m a.s.l). The analysis of the historical maps of the Third Austro-Hungarian topographic survey (the so called "Franzisco-Josephinische Landesaufnahme") dated 1850 and the aerial photograph of 1945 and of 2006 ortophotos, were helpful to reconstruct the different stages of glacier retreat. Thus, comparing these photos, our sampling site was ice-free since 1850.

Rhizosphere and soil sampling was carried out in May 2011, at the beginning of the plant growing season. Three safe-sites (RW, FI, and M sites) characterized by loosely organized assemblages of different plant species and a bulk soil (BS site) were sampled. The sites were less than 20 × 20 cm. RW site, below an iron rich rockface, was colonized by *Diphasiastrum alpinum* and *Gnaphalium supinum* L.; FI site, a floristic island between big rocks, was colonized by *Cladonia* sp., *Festuca halleri* All., *Polytrichum* sp., *Racomitrium* sp., *Sedum alpestre* Vill. and *Senecio carniolicus* (Willd.) Braun-Blanq,; M site, a safe-site surrounded by big rocks and characterized by a flatter area, was colonized by *Cetraria islandica* (L.) Ach*., Leucanthemopsis alpina* (L.) Heywood*, Potentilla aurea* L., *Rhododendron ferrugineum, Sibbaldia procumbens* L. and *Silene acaulis* (L.) Jacq. These sampling sites were chosen in order to share similar conditions in terms of altitude, features and geology.

The rhizosphere samples of each plant within a floristic community were collected. Each individual plant was carefully pulled out the soil, without damaging its single root system. After pulling out each plant and avoiding roots, 4 g of rhizosphere soil strictly adhering to the roots was collected with a pair of tweezers. Three replicates of bulk soil were collected as a control. Moreover, from each safe-site, 50 g of root-free soil was collected and put into plastic bags for soil chemical analysis. All the samples were immediately transported in refrigerated boxes to the laboratory as soon as the logistic constraints permitted and stored at -80°C until analysis.

Soil chemical analysis

Soil samples for chemical analysis were oven-dried at 105°C until constant weight and then acid digested (HNO$_3$ concentrated 65% and H$_2$O$_2$ 30%) in a Milestone high performance microwave oven (MLS Mega, Gemini BV

Laboratory, Apeldoorn, The Netherlands). To determine the total organic carbon content, soil samples were also acidified with HCl (6M) to eliminate all carbonates. Metals and total phosphorous were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES, Spectro Ciros CCD, Spectro GmbH, Kleve, Germany). Nitrogen and C were quantified with an Elemental Analyzer (Flash 2000, Thermo Scientific). The pH_{H2O} was measured using an Accumet AP85 pH (Fisher Scientific Ltd., Pittsburgh, PA).

Molecular analysis of the bacterial communities
Metagenomic DNA of the rhizosphere and soil samples was extracted using Ultraclean Soil DNA Extraction kit (MO-BIO, Arcore, Italy). Microbial analyses were carried out using Denaturing Gradient Gel Electrophoresis (DGGE; Muyzer et al., 1993) to describe the rhizobacterial diversity, and Automated Ribosomal Intergenic Spacer Analysis (ARISA; Cardinale et al., 2004) to describe the structure of the rhizobacterial communities.

For DGGE analysis, primers GC357f and 907r were used as described (Sass et al., 2001). DGGE was run in a BioRad DCode Universal Mutation Detection System (Biorad, Milan, Italy). Polyacrylamide gels were done according to Muyzer et al. (1993). Gels were stained for 30 min in 1× TAE buffer containing SYBR® Safe - DNA Gel Stain (Invitrogen, Milan, Italy). Visualization and digital image recording was performed with UVTec (Cambridge, UK). DGGE bands were excised and reamplified (Muyzer et al., 1993). Sequencing was performed by STAB-Vida Inc. (Caparica, Portugal). Identification of 16S rRNA genes was done by comparison with EMBL/Genebank/DDBJ database and RDP database using BLASTN and Classifier respectively. All sequences were submitted to the RDP-Ribosomal Database Project (Cole et al., 2009) web server to assign taxonomy. Sequences were submitted to the Genbank/EMBL/DDBJ databanks under the accession numbers HG763876-HG764130.

ARISA fingerprint was performed as described by Cardinale et al. (2004) with the ITSF/ITSREub primer set. Denatured ARISA fragments were run by STAB-Vida Inc. The data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems, Monza, Italy), and a threshold of 40 fluorescent units was used, corresponding to two times the highest peak detected during the negative control run. Output matrix was obtained as in Rees et al., 2004.
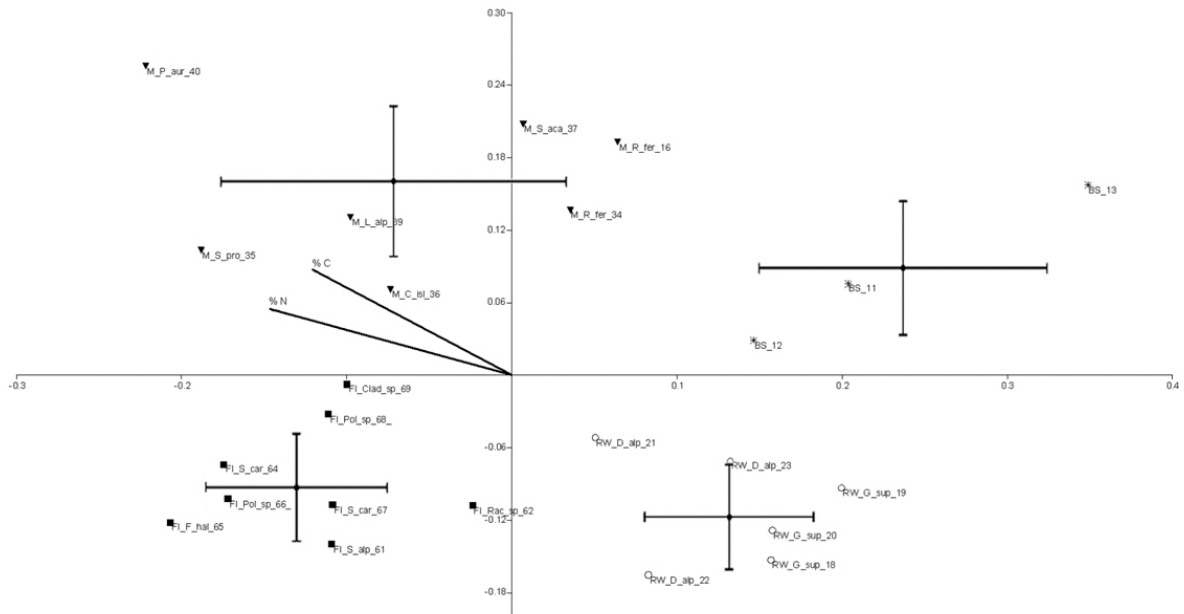

Figure 1 - PCA plot of the three safe-sites and the bare soil site according to Unifrac distance matrix

Statistical analysis

ARISA matrix was normalized with the formula (x/∑x)*1000 where "x" is the fragment height in units of fluorescence, then transformed on a logarithmic scale for multivariate analysis. Log-transformation was used to stabilize the sample variance, to reduce the interaction effect and to normalize the distribution of data. Moreover, log-transformation can combine the information of a binary matrix with the those of a non-transformed data matrix, hence preserving the relative abundance information and down-weighing dominant groups.

In order to assess changes in rhizobacterial community structure between floristic consortia, Non-Metric Multidimensional Scaling (NMDS) was applied with Bray-Curtis algorithm. NMDS does not necess of the assumption of linear associations among variables, being described as the most efficient ordination method for microbial ecology (Clarke, 1993). Bray-Curtis is not influenced by recurrent absent values into the matrix, a characteristic very common in ARISA (Clark and Warwick, 2001). ANOSIM (based on Bray-Curtis similarity) was performed to test significant differences in the profile composition of the different sites. ANOSIM is a nonparametric statistical test, based on permutation, which uses rank similarity matrix of an ordination plot to calculate an R test statistic on the null hypothesis H^0 that there are no differences among groups. When R is near to 0, H^0 is true, while when R is reaching 1, H^0 can be rejected and there is a discrimination between groups. When ANOSIM statistics approaches 1, the similarities within groups are larger than similarity between groups. We rejected H^0 when significance *P* value was < 0.05.

The Nexus format of the phylogenetic tree of the DGGE identified bands performed by MEGA5 was submitted to the UniFrac web server to test differences among sites based in the UniFrac metric with 100 permutations and the Bonferroni correction factor (Hamady et al., 2010). A Principal Component Analysis (PCA) on the DGGE sequence distance matrix for each pair of safe-sites was calculated through UniFrac metric. On the basis of the DGGE sequences, similar safe-sites tended to cluster together. In order to allow a broader view of those similarities, the first three principal components were considered.

The other statistical analyses such as Kruskal-Wallis test on soil C and N percentages or on NMDS sample position were performed using PAST program (Hammer et al., 2001).

Results

Soil chemical analysis

Soil resulted to be a sandy silt soil with an average texture of 72.3 ± 5.0 % of sand, 21.0 ± 4.1 % of silt, 6.6 ± 1.3 % of clay, and 4.6 ± 1.3 % of humus; pH was 4.5 ± 0.3 %. Average chemical composition of sampled soils was: total P 0.7 ± 0.1 mg/kg d.m., total K 7.4 ± 1.0 mg/kg d.m., total Ca 3.4 ± 0.6 mg/kg d.m., total Mg 13.4 ± 1.7 mg/kg d.m., total Fe 45.4 ± 6.9 mg/kg d.m., total Al 29.4 ± 5.6 mg/kg d.m. No calcium carbonate was detected. Since those safe-sites were located in proximity each other, their soil chemical composition did not differ substantially among sites (*P* < 0.05).

Safe-site	% Nitrogen		% Carbon		C/N	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
BS	0.05	0.01	0.62	0.16	11.5	0.61
RW	0.27	0.11	3.48	1.47	12.7	0.94
FI	0.72	0.35	10.4	6.03	14.2	1.46
M	0.98	0.85	19.3	18.3	17.5	3.79

Table 1 - Percentage of total nitrogen and carbon content and C/N ratio in the four safe-sites.

No nitrate was detected, while all the nitrogen found was represented by ammonia. Nitrogen increased along an ideal gradient from bare soil (0.05 % dry weight) to the most vegetated M site (0.98 % dry weight), and also total organic carbon grew up from BS site (0.62 % dry weight) to M site (19.3 % dry weight). The trend was confirmed by the C/N ratio which tended to increase constantly among site of more complex vegetative patterns (Table 1). Kruskal-Wallis non parametric analysis of variance showed significant differences among sites for both total nitrogen, organic carbon content and C/N ratio, except for M vs RW and M vs FI (*P* values shown in (Table 2).

p/R value	BS	RW	FI	M
BS		0.9630	0.9758	0.7937
RW	0.0124		0.9390	0.7434
FI	0.0077	0.0005		0.7055
M	0.0092	0.0009	0.0004	

Table 2 - *P* and R values of ANOSIM based on Bray-Curtis similarity of the four safe-sites as grouped after ARISA-NMDS plot analysis.

Genetic structure of bacterial communities in alpine bulk soils and plant colonized safe-sites

Due to the high sensitivity of the automated capillary electrophoresis, ARISA fingerprints of both rhizosphere and bare soil bacterial communities provided complex profiles with peaks ranging from 151 bp to 1437 bp and the 16S-23S rRNA internal transcribed spacer region (ITS) richness varied from 43 to 168 peaks. The electropherograms, characterized by distinct peaks number and intensity, revealed a shift in bacterial community structure across the different safe-sites plant communities (Figure 1). On the NMDS plot, samples from root-free soil (BS), safe-site of early developmental stage (RW), intermediate stage (FI), and from the most mature one (M) showed four separate clusters based on microbial community structure (Figure 1). According to axis 1, RW site and BS site are separated from M and FI sites. According to axis 2, BS and M sites are separated by RW and FI sites. The unvegetated BS site clustered in a specific group, differentiated by the plant rhizospheres, clustering closer to the rhizosphere bacterial communities of RW site than to those of FI and M sites. The NMDS separation is partially explained by N and C content, as shown by those variable vectors, which influenced more M site than the other safe-sites. ANOSIM analysis confirmed a highly significant difference among the four microbial community structures (R=0.81; *P*=0.0001) and the performed test showed significant differences in the pairwise comparisons of the sites with R values approaching 1 in most of the cases: BS vs RW (R = 0.96), BS vs FI (R = 0.97), BS vs M (R = 0.79), RW vs FI (R = 0.94), RW vs M (R = 0.74), FI vs M (R = 0.71). Where replicated individuals of the same plant species within each safe-site were found, it was possible to denote a plant species effect. This is recognizable within RW safe-site, where individuals from *D. alpinum* and *G. supinum* formed two clusters significant different along the first axis of NMDS (*P* = 0.032 at the Kruskal-Wallis test). In FI and M sites the tendency of individuals of the same species to cluster together seems to disappear, except for *R. ferrugineum*, maybe due to the higher number of species interconnected in the safe-site.

Diversity of the bacterial communities associated with alpine bulk soils and pioneer plants in safe-sites

DGGE was performed to investigate the different microenvironments of the three safe-sites and bulk soil in terms of their dominant bacterial population. 255 sequences of more than 300 bp were obtained from all sample profiles. RDP facilitated the determination of putative taxonomic affiliation of the recovered sequences. Major bacterial taxa included Acidobacteria Gp3 and Gp1, Sphingobacteria, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria (Figure 2). A noteworthy amount of uncultured bacteria were found. Shifts in bacterial communities were visible. Members of the Acidobacteria order were present in all the sites samples. They were the most abundant taxa with percentage from 33% associated with M site rhizospheres up to 57% in BS site samples; a decrease of their relative abundance is visible in FI and M safe-sites. Proteobacteria were not found in BS site, while they were scarcely present in RW and FI site rhizospheres (4%, 8% respectively). In M site Proteobacteria became more abundant than Acidobacteria (35%). In particular, the increasing abundance of Proteobacteria was

due to Alphaproteobacteria, more represented than Gammaproteobacteria and Betaproteobacteria. A significant amount of unclassified Proteobacteria was also evident in M site. Sphingobacteria were recovered with low percentages in RW, FI, and M sites rhizospheres whereas members of Actinobacteria taxa were even less abundant being present in FI and M sites rhizospheres only. We did not found Sphingobacteria or Actinobacteria taxa associated to BS samples. According to RDP classification, unclassified Acidobacteria or Proteobacteria, as well as other uncharacterized bacteria were quite common within all sites. For example, RW site was almost completely colonized by unclassified Acidobacteria and unknown Bacteria, except few sequences affiliated to uncultured *Burkhorderia* or to a Chitinophagaceae bacterium. Similarly, FI safe-site was mostly colonized by unclassified Acidobacteria, although more frequent sequences belonging to Bradyrhizobiaceae, Chitinophagaceae and other rarer taxa such as *Flavisolibacter* sp. or *Granulicella* sp. were found. Finally, M site, the most differentiated safe-site, counted the presence of unknown Bradyrhizobiaceae, *Bradyrhizobium* sp., uncultured Rhizobiales, as well as Chitinophagaceae, *Streptacidiphilus* sp., Thermomonosporaceae and Xanthomonadaceae.
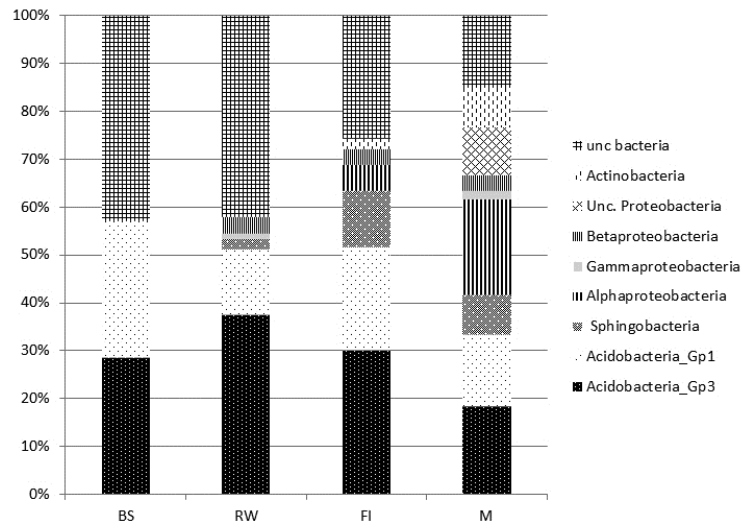

Figure 2 - DGGE identification of rhizobacterial communities of the three safe-sites and the bare soil site

Despite bias associated with sampling, DNA extraction, PCR amplification and DGGE run, the pattern of differences in bacterial communities between the unvegetated soils of the BS site and the rhizospheres of the three safe-sites was supported by the pairwise UniFrac distance ordinations. Comparing each pair of environments using the Bonferroni correction, the UniFrac significance (*P* values < 0.05) showed that the BS site samples were significantly different from FI and M sites rhizospheres, but not from RW site rhizospheres. Moreover, the FI site rhizosphere did not differ significantly from M and RW site rhizospheres, while the M site rhizospheres exhibited significant differences with the RW site. A PCA analysis of the UniFrac distance matrix was calculated to assess the overall sequence population similarity among safe-sites (Figure 3). The first axis of PCA analysis, explaining 45.58% of the total variance, showed a shift of the BS site from RW, FI, and M sites. The FI and M communities were located very close together in the same quadrant suggesting a similar bacterial community composition influenced by variables related to PC1. On the other hand, PC2 (32.58% of the variation) explained the differences between RW site and the other three sites. Finally, the third component (21.84% of the variance) differentiated FI from M and from BS and RW sites.

Discussion

Safe-sites are defined as environments immediately nearby a pool of seeds, where their germination, growth and establishment is favorable (Harper et al., 1961). In this respect, their availability, accessibility and geomorphological diversity in high mountain represent an important characteristics of this environment, since they

represent a micro-site where a list of ecological hazards (snow, wind, frost, irradiation) are less severe than in open terrains, and where plant propagules can resist, growth and reproduce. In Matschertal, belonging to south Tyrolean Alps, additional ecological hazards are represented by hot and dry summers, instability of the soil substrate and excessive animal grazing (Urbanska, K.M. 1997). Within each safe-site, more than one plant species can growth from seeds, specialized vegetative propagules or plant fragments (Chou et al., 1992). In such kind of environments, pioneer plants tends to growth in very complex coenosis, where roots are strictly intermixed and interrelated. A great diversity of root exudates from all these plants is released in rhizosphere, increasing the carbon amount of the safe-site. Due to the characteristics of safe-sites, usually well isolated among each other by rocks, sand or mud, an analysis to understand the occurrence of a vegetation effect on rhizobacterial communities cannot be done with traditional squared-plots, where more safe-sites are sampled smoothing possible differences between them. Hence, we decided to study three kind of safe-sites at different stages of morphological development, by sampling each single rhizosphere from all the growing plant individuals.

The vegetation complexity of the three safe-sites (RW, FI and M) raise from a simple colonization of two species (RW site), to the colonization of lichens, mosses and few herbaceous plant species (FI site), till M site, where five herbaceous species and one woody species (*R. ferrugineum*) were found. We discovered a distinct clustering of bacterial communities according to RW, FI, M vegetation types, significantly diverse from the unvegetated soil (BS site). We also found that a gradient in terms of C and N enrichment from BS site to the most developed M site was an important determinant of microbial community profiles. UniFrac analysis showed site-shifts in bacterial diversity which suggest a specialized physiologies adapted to the peculiar site environmental conditions. Moreover, the differences among safe-sites, according to C and N gradients, support the occurrence of a plant cover effect on the rhizosphere bacterial community within those safe-sites.
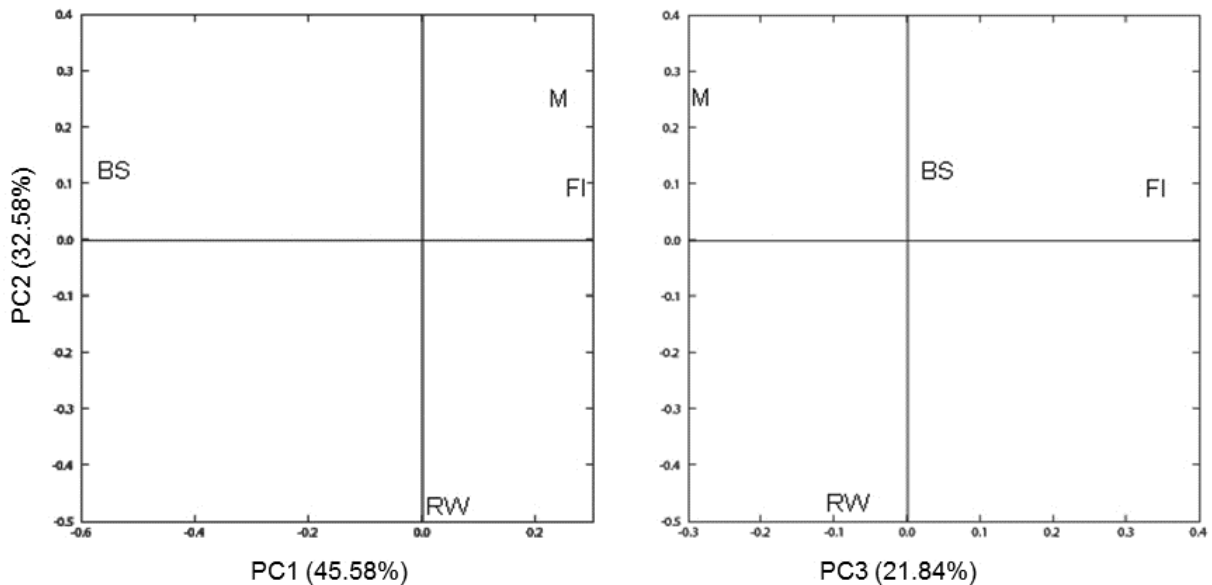

Figure 3 - PCA analysis of the UniFrac distance matrix calculated to assess the overall sequence population similarity among safe-sites.

Previous investigations of the rhizosphere effect were conducted on few single pioneer plants or in grassland plot. Almost all the researches on the rhizosphere effect associated to a single plant species were achieved on crop or other plants either in artificial microcosms such as pots or on agricultural soils such as orchards and crop monocultures. Most of these researches demonstrated that peculiar root exudation and rhizodeposition of different plant species could select the structural and functional diversity of the associated rhizosphere bacterial communities (Hirsch *et al.* 2003; Farrar *et al.* 2003; Bais *et al.* 2004). On the other hand, a consistent number of studies have showed that several environmental parameters i.e. soil type, soil characteristics, growth stage,

management practices, and growing season may influence the composition of the microbial communities in the rhizosphere (Marschner *et al*. 2001; Kowalchuk *et al*. 2002; Weinert *et al*. 2011; Hansel *et al*. 2008, Wu *et al*. 2008; Schutter *et al*. 2001; Kennedy *et al*. 2005; Lauber *et al*. 2008). Past studies about a natural alpine ecosystem, investigated single plant species along successional chronosequences and found inconsistent effects of pioneer plants on rhizosphere microbial communities. For example, while the rhizosphere bacterial community of *Lc. alpina* was different from the interspace community in an early successional stage, its rhizosphere bacterial community in the late successional stage was similar to that of the interspace. In this case, it seemed that the influence of *Lc. alpina* depended on soil age and that nutrient availability could influence the bacterial communities (Edwards *et al*. 2006). In another case, *Lc. alpina* individuals in the early successional stage (5, 10 years) of a glacier forefield showed no selective effect on the microbial community, since a similar bacterial community structure was apparent up to 40 cm of distance to the plant (Miniaci *et al*. 2007). Another single pioneer plant, *P. alpina*, did not exhibited a selective role on its rhizosphere bacterial community in the pioneer stage of a chronosequence, maybe due to the harsh environmental conditions of the plot where it was growing (Tscherko *et al*. 2004). However, by investigating a more mature soil, the same plant species could select for a specific microbial community, but related to soil properties and carbon supply.

On the other hand, safe-sites are more complex than single pioneer plant individuals in a cold environment, but show less complexity than a homogeneous plot carefully designed in mountain grasslands. Real safe-sites are much less homogeneous, being shaped by the history of the micro-area where they are, such as dynamical differences in climate, in geophysical features, or in biota colonization which determine complicated patterns and often unique rate of soil development (Matthews, 1992). In our case, due to the quick glacier melting in the last 80 years, the 160 years soil represents the only transitional step of the Matscher glacier moraine between earliest stages (< 10 years) and mature soil (> 500 years). As shown by aerial photos, ortophotos, and a topographic survey, one of the glacier tongue of the Weisskugel glacier has been retreating with a discontinuous movement. Consequently, there was not a constant gradient of soil age but distinct block stages where soil age is invariable. In this sense, the 160 years old stage is more stable than an earlier successional soil and it can host a larger number of plant species. Nevertheless it was possible to distinguish hundreds of safe-sites of which the three chosen were the most represented. Within the stable block stage of 160 years old, the measured differences in rhizobacterial composition and soil parameters, supported hypothesis that the plant community composition of each floristic consortium exhibited an effect on the rhizobacterial communities widely documented in studies done in quite different ecosystems. For example, Nunan et al. (2005) demonstrated a more important influence of the plant community composition than of the individual plant species on the root colonizing bacterial community in a upland grazed grassland, whereas Osanai et al. (2013) showed a significant impact of the plant species on the soil bacterial community composition. Similar results were obtained comparing the rhizosphere bacterial communities of three plant species of an arid grassland (Kuske et al., 2002).

The rhizosphere bacterial communities of RW site, characterized by only two different plant species, clustered more closely with the BS site than with the vegetated ones showing a simpler bacterial communities, as confirmed by the UniFrac analysis which not detected significant difference between the two sites. Although FI and M sites had a similarity of about 56 %, inside the FI site were found rhizobacterial communities of mosses and lichens which did not cluster strictly with the plant ones. The presence of lichens and mosses in the same site could explain why the bacterial community of the FI site represented an intermediate stage between the RW site and the M site. The M site, colonized by individuals of six plant species, could be considered a later stage where floristic consortia selected a more complex bacterial community which significantly differs from the one of BS and RW sites. The UniFrac analysis showed that the BS communities were distinct from ones of the FI and M sites and were weakly similar to the ones of RW site. Moreover, the intermediate plant colonization stage, FI site, did not differ significantly from the RW and the M vegetated sites. Previous studies (Lipson et al., 1999; Ohtonen et al., 1999; Tscherko et al., 2004) showed that the development of the soil microbial community in alpine glaciers was determined by the accumulation of soil TOC and total nitrogen. The increasing content of C and N in the floristic consortia corresponded with increased floristic developmental stage. Soil nutrients and C influenced the bacterial community composition along a chronosequences (Edwards et al., 2006), while in the Mendenhall glacier chronosequence (Knelman et al., 2012) they were not correlated with the rhizobacterial communities. These

different conclusions seem to strongly depend to the adopted experimental design. Cultural-independent techniques based on Phospholipid Fatty Acid (PLFA) determination (Tscherko et al. 2004; 2005), to point out the different concentration of bacterial/fungal fatty acids and to compare the Gram positives/Gram negatives ratio, or molecular methods like Restriction Fragment Length Polymorphism (RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) analyses (Edwards et al. 2006; Miniaci et al. 2007) could not have enough resolution to detect little changes in the bacterial community genetic structure due to faint environmental variables (Danovaro et al., 2006). The ARISA analysis we used, targeting the intergenic 16S-23S rRNA gene highly variable ITS region, showed more sensitivity and enable the detection up to sub-species level, increasing the chance of the analysis to detect very little effects on complex bacterial communities. Thanks to its high phylogenetic resolution and the estimated OTU richness, ARISA analysis appeared a more suitable method for diversity analysis of soil microbial community structure (Okubo et al. 2009).

Our results might support the capability of the different pioneer plant consortia to control the rhizobacterial species structure with an increase of bacterial diversity from BS to RW, FI, and M sites. Moreover, when plants of the same species within the same site clustered more strictly together, the high similarity in the rhizosphere genetic structures within individuals of each pioneer plant species was confirmed. Therefore, a plant community effect on the rhizosphere bacterial communities was found and the maintenance of single plant species effect within a floristic community, despite the harsh environmental condition of the natural alpine ecosystem and the tight complex root system of the safe-site.

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Dynamics of active and total rhizobacterial communities involved in nitrogen cycle in a deglaciated alpine environment

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Abstract

Glacier forefields are landscape characterized by primary successions where the interaction between microbial communities, pioneer plants and soil neo-genesis leads to the development from bare substrate to complex plant communities after the ice cover retreats. In a young soil, nitrogen is a key element that can help plant biocoenosis and the generation of a complex and stable bacterial community. In our research area in the Matsch Valley (Italy), plant colonization was evolved since 1840, when the glacier began to retreat. Three distinct floristic communities, at two different time points of glacier retreat and through an entire growing season were studied. DGGE on the 16S rRNA of ammonia oxidizing bacteria, ARISA and 16S rRNA gene pyrosequencing (83,000 OTUs) were applied to both metagenomic DNA and/or RNA, in order to assess the diversity of the overall bacterial taxa and of the active bacterial fraction. The free-living nitrogen-fixing bacterial community was monitored by *nifH* gene pyrosequencing of metagenomic DNA. The community structures of both the overall and active bacterial communities appeared to be affected mainly by the soil age. α-Proteobacteria and Acidobacteria were the most representative taxa. Active *Bradyrhizobia* were thoroughly found in all the samples. The characterization of the *nifH* gene showed an unexpected genetic diversity associated to free-living rhizobia that represent a "bacterial seed bank" of a great ecological importance in these severe environments.

Introduction

In alpine environments microbial communities are closely related to the availability and composition of soil nutrients which are affected by pronounced climatic fluctuations, characterized by extreme temperature and humidity variations (Brunetti et al., 2006). Low temperatures and heavy snowfall in winter time are followed by wet and dry periods after snowmelt. Summer and autumn are unpredictable with strong day–night variations in temperature (Körner, 1999) and significant physical and chemical changes of the soil with consequences on the local microbial communities could occur (Ley et al., 2001). A variety of studies (Nemergut et al., 2005; Freeman et al., 2009) have investigated specific microbial communities related to high water retention, UV exposure, and strong temperature fluctuations (Ensign et al., 2006; King et al., 2008) in oligothrophic unvegetated alpine soils (Kastovská et al., 2007; Schütte et al., 2009) characterized also by limited amount of aeolian and alluvial deposition of allochtonous C and N (Brankatschk et al., 2010) and release of ancient compounds from the mineral soil (Bardgett et al., 2007).

While microbial succession in recently exposed unvegetated soils of deglaciated terrains is a pivotal factor in early ecosystem development, the subsequent plant colonization may modify soil microbial community composition and function. In a young alpine soil characterized by C and N limitation due to the high fraction of coarse-grained mineral skeleton, one of the key plant roles is the accumulation of carbon (C) in the root exudates and litter (Grayston et al.,1998; Bardgett and Walker, 2004; Bardgett et al., 2005) with consequent changes in nutrient availability and soil pH. Due to translocation of photosynthetically fixed carbon into the rhizosphere, plant could stimulate microbial community activities (Marschner, 1995; Walker et al., 2003). Moreover, vegetation increases the moisture and thermal retention of soil microhabitats (Harris and Tibbles, 1997; Yergeau et al., 2007). Soil moisture, pH, bioavailable C and soil C/N were correlated with shifts in bacterial communities (Mannisto et al.,

2007; Wallenstein et al., 2007; Yergeau et al., 2007; Chu et al., 2011) which suggested a close interrelationship between the microbial community and soil characteristics in response to changes in seasonal conditions.

In the alpine, Arctic and Antarctic environments, the vegetation type together with the effects of nutrient availability and soil physical condition, has strong consequences on soil microbial community (Neufeld and Mohn, 2005; Yergeau et al., 2007; Chong et al., 2010; Chu et al., 2011; Zepp et al., 2003; Hughes et al., 2006). Arctic (Jonasson et al., 1995) and alpine soils (Kuske et al., 2002) are generally poor in nitrogen, whereas in the forefields of the Lyman Glacier (Washington, USA) (Othonen et al., 1999) and the Rotmoosferner and Ödenwinkelkess glaciers (Austria) (Tscherko et al., 2003, 2004) positive relationship between microbial biomass and soil organic carbon content occurred. Microbial community of Alaskan tundra has also shown significant seasonal changes at the species level (Wallenstein et al., 2007).

In the last years, different studies about glacier forefields were focused on different steps of the N cycle (Deiglmayr et al., 2006; Kandeler et al., 2006; Duc et al., 2009; Towe et al., 2010) because N is fundamental for the ecosystem development, but most of the deglaciated terrains do not contain any N.

In early primary successional ecosystems, N fixation strongly control early successional microbial and plant community dynamics (Matthews, 1992; Chapin et al., 1994; Edwards et al., 2006; Brankatschk et al., 2010). Recent investigation on the early successional soils of the Damma Glacier showed the free-living *nifH* gene abundance in the pioneer plant patches (Brankatschk et al., 2010), highlighting the relation between initial plant colonizers, bacterial community structure, and asymbiotic N fixation in the borderline of the unvegetated and vegetated landscapes.

Along a chronosequence, vegetation exhibits a gradient in plant-cover and the interaction between rhizobacterial community and vegetation might differ at different successional stages. Previous studies have investigated shifts in microbial community structure and activity into vegetated portions of succession (Sigler et al., 2002; Noll and Wellinger, 2008; Schütte et al., 2009, 2010). Tscherko et al. (2005) investigated the relationship of different successional plant communities with microbial diversity and enzymatic activity patterns strongly conditioned by the successional stage, as well as the carbon and nitrogen content of the forefield soils. Studies about plant effects showed shifts in the relative abundance of different bacteria and fungi taxa, increases in microbial metabolic function, alterations in bacterial community structure, and changes in plant influence with more advanced successional stages (Ohtonen et al., 1999; Tscherko et al., 2004, 2005; Edwards et al., 2006; Miniaci et al., 2007). The most common approach to characterize communities in high-altitude and oligotrophic alpine environments are based on the analysis of the bacterial 16S rRNA gene through Denaturing Gradient Gel Electrophoresis (DGGE, Sigler and Zeyer, 2002) Terminal Restriction Fragment Length Polymorphism (T-RFLP, Mannistö et al., 2007, Noll and Wellinger, 2008) or clone libraries (Nemergut et al., 2005, Gonzalez-Toril et al., 2009) which give us a first view on major taxa. Previous investigation of alpine dry meadows (Lipson and Schmidt, 2004) and alpine and arctic soils (Nemergut et al., 2005) evidenced the different high amount of Acidobacteria and α-Proteobacteria in different seasons whereas a study on the chronosequences of two alpine glaciers showed high abundance of Actinobacteria (Philippot et al., 2011).

Although automated techniques to analyze microbial communities profiles give us large amount of information with respect to traditional techniques, they are not sufficient to describe deeply microbial diversity. In the present

study, we used next-generation sequencing, a technique able to provide deeper insights into complex microbial communities. Furthermore, almost all the previous studies about bacterial communities in unvegetated and vegetated alpine soils were conducted on 16S rRNA gene which explain the bacterial presence (Felske et al., 1997; Felske and Akkermans, 1998) rather than active lineages in the sample. To obtain an indication of the potentially metabolically active bacterial communities (Mengoni et al., 2005), bacterial 16S rRNA at both the DNA and RNA level would be compared (Watanabe et al., 2001; Lillis et al., 2009). As pioneer plants and the associated rhizobacterial communities microbes tolerate constantly strong abiotic stressors, a deeper analysis of the overall rhizobacterial community is required as well as the comprehension of the roles of biotic and abiotic factors on the active rhizobacterial community at the beginning and at the end of the plant growing season in transitional environments of two different soil age.

Moreover, the direct tracking of *nifH* key gene provide a better insight into nitrogen fixation capacity in this environment to assess the taxonomic groups and the involved ecological patterns. The temporal dynamic of the free living *nifH* population have to be assessed to better understand if the N fixation by free living bacteria is the most important process to provide N to plant.

Materials and methods

Study site and soil samples

The study site is located into the upstream sub-catchment of Saldur river (46° 46' 30"N; 10°41'46"E; 2,400 a.s.l.) in the high Matsch valley (South Tyrol, Italy) with a drainage area of 11 km^2. The main geological processes are periglacial and the streamflow is characterized by the glacier dynamics. During the 1970-2000, the valley had an average rainfall of about 550 mm per year. In 2011, the mean temperature of the plant growing season were 7.3°C in July, 10.3°C in August, 8°C in September and the mean precipitations were 2.7, 2.5, 3.6 mm per day, respectively. The dominant rock types are schist and gneiss (Habler et al., 2009) and the most common soil types are acidic leptosols, regosols and umbrisols (mean pH = 4.3), derived from carbonate-free bedrocks.

Table 1 - Geographical data, soil composition and nutrient chemical parameters.

Plot name	Altitude	Latitude	Longitude	Vegetation	Moisture July (1)	Moisture October (1)	pH	DM (2)	Sand (2)	Silt (2)	Clay (2)	Humus (2)	TOC (2)	NH4 (3)	N tot (3)	N tot (2)	C/N
201	2407	46°46'30.79"N	10°41'45.74"E	Festucetum halleri	276	682	4.4	76	74	19	6.5	4.8	2.8	4.0	4.0	0.23	12
202	2406	46°46'31.10"N	10°41'46.08"E	Cetrario-Loiseleurenion	274	684	4.3	78	75	19	5.7	3.9	2.3	2.6	2.6	0.18	13
203	2404	46°46'30.58"N	10°41'46.61"E	Cetrario-Loiseleurenion	272	706	4.2	77	73	20	6.8	6.3	3.7	2.6	2.6	0.28	13
204	2404	46°46'30.06"N	10°41'47.41"E	Nardion strictae	279	775	4.8	80	68	26	6.9	3.3	1.9	3.8	3.8	0.19	10
205	2400	46°46'30.00"N	10°41'48.11"E	Festucetum halleri	151	447	4.4	84	79	16	5.0	3.2	1.9	2.4	2.4	0.15	12
206	2398	46°46'29.71"N	10°41'48.66"E	Nardion strictae	574	956	5.0	70	65	26	8.7	5.9	3.4	5.0	5.0	0.26	13
301	2415	46°46'19.22"N	10°41'46.00"E	Festucetum halleri	344	378	4.4	81	82	15	3.6	4.4	2.6	3.1	3.1	0.21	12
302	2400	46°46'19.76"N	10°41'47.51"E	Cetrario-Loiseleurenion	763	839	4.2	66	72	21	7.4	8.0	4.6	0.8	0.8	0.3	15
303	2391	46°46'20.21"N	10°41'49.38"E	Cetrario-Loiseleurenion	796	904	4.2	63	78	14	7.6	10.7	6.2	0.8	0.8	0.49	13
304	2387	46°46'20.38"N	10°41'50.62"E	Nardion strictae	961	786	4.1	68	80	13	6.7	8.9	5.2	0.7	0.7	0.39	13
305	2386	46°46'20.41"N	10°41'50.70"E	Nardion strictae	972	820	4.1	56	65	25	11.0	13.5	7.8	1.8	1.8	0.59	13
306	2305	46°46'20.67"N	10°41'51.23"E	Festucetum halleri	758	736	4.1	72	68	23	9.7	8.1	4.7	0.7	0.7	0.37	13

(1) mV
(2) %
(3) mg/kg DM

The study site, a foreland of about 3.3 Km^2 left after a quick glacier retreat in the last 160 years (Knoll et al., 2010), was located above the tree line (2,100 m a.s.l). The analysis of the historical maps of the Third Austro-Hungarian topographic survey (the so called "Franzisco-Josephinische Landesaufnahme") dated 1850 and the aerial

photograph of 1945 and of 2006 ortophotos, allowed to reconstructed the different stages of glacier retreat, comparing the photos.

Two successional sites were sampled (Transects T2 and T3, plots from 201 to 206 and from 301 to 306, respectively; see Table 1) and all study sites are located in the almost plain center of the glacier foreland with a uniform, very slightly southern aspect.

The transition grassland stage is located at 2400 m a.s.l. and has been deglaciated for 160 years. The site is characterized by a transitional grassland community habouring 44 plant species. The dominant species are *Racomitrium canescens* (Hedw.) Brid., *Nardus stricta* L., *Potentilla aurea* L*., Festuca halleri* All*., Silene acaulis* (L.) Jacq*., Polytrichum juniperinum* Hedw. The more mature grassland stage (>160 years) is located in front of the terminal moraine of 1850 outside the glacier foreland at 2380 m a.s.l. The number of plant species is 46 and the dominant ones are *Loiseleuria procumbens* (L.) Desv., *Nardus stricta* L*., Potentilla aurea* L*., Festuca halleri* All*., Ligusticum mutellina* (L.) Crantz, *Cetraria islandica* (L.) Ach., *Veronica bellidioides* L. Sp. Pl.

Rhizospheres were carried out at the beginning (July 2012 - J) and at the end (October 2012 - O) of the plant growing season. Six plots of 100 × 100 cm were chosen inside and outside the glacier foreland per each transect. The rhizosphere samples within the plot were collected following a 'W' soil sampling pattern to ensure that the sample was representative of the entire plot. Plants were carefully pulled out the soil, without damaging the root system and the rhizosphere soil strictly adhering to the roots was collected with a pair of tweezers and the five samples within each plot were mixed together.

From each plot, 200 g of soil was collected in plastic bags and in aliquots in 2 ml tubes which were immediately put on dry ice and transported in refrigerated boxes to the laboratory as soon as the logistic constraints permitted. In the laboratory, the aliquots were stored at −80 ∘C for DNA and RNA extraction and subsequent molecular analysis. The remaining fresh soil in the plastic bags was stored at 4 ∘C for geochemical characterization. All soil chemical measurements were performed within two days after sampling.

Table 2 - Metal and other ions concentration of rhizosphere soil samples.

Plot name	P Tot (1)	K Tot (1)	Ca Tot (1)	Mg Tot (1)	Fe Tot (1)	Al Tot (1)	Mn Tot (2)	Cu Tot (2)	Zn Tot (2)	Cr Tot (2)	Ni Tot (2)	Pb Tot (2)	Co Tot (2)	Hg Tot (2)	Cd Tot (2)
201	0.68	7.20	2.73	12.80	42.41	26.16	411	30	85	49	37	24	14	0.019	0.11
202	0.57	6.73	2.68	13.42	45.53	27.90	422	36	90	45	38	23	15	0.02	0.12
203	0.71	8.31	3.23	14.62	48.77	33.00	470	41	99	52	45	24	17	0.023	0.13
204	0.64	8.62	3.56	14.93	53.43	36.15	530	64	108	53	59	20	25	0.01	0.04
205	0.83	7.48	3.80	10.29	33.52	20.70	455	26	69	38	26	17	13	0.013	0.15
206	0.75	8.29	4.36	14.24	48.83	32.65	497	53	113	51	50	29	22	0.018	0.35
301	0.81	6.69	3.67	12.76	42.23	26.37	453	34	76	45	37	18	15	0.017	0.12
302	0.72	5.40	3.31	12.39	38.73	23.92	381	29	78	42	35	16	14	0.026	0.88
303	0.81	5.58	3.17	11.31	36.46	24.18	396	19	73	41	26	17	13	0.038	0.93
304	0.74	5.46	2.79	12.74	41.55	26.96	374	23	76	48	27	19	12	0.033	0.88
305	0.88	6.76	2.72	12.53	41.07	29.88	393	19	83	50	24	25	12	0.052	0.82
306	0.76	5.54	2.78	12.49	41.07	26.00	384	24	77	41	28	16	11	0.026	0.92

(1) g/kg DM
(2) mg/kg DM

Soil chemical analysis

The geochemical characterization of soils collected in July 2012 was carried out (Tables 1, 2). Soil samples for chemical analysis were oven-dried at 105°C until constant weight and then acid digested (HNO$_3$ concentrated 65% and H$_2$O$_2$ 30%) in a Milestone high performance microwave oven (MLS Mega, Gemini BV Laboratory, Apeldoorn, The Netherlands). To determine the total organic carbon content, soil samples were also acidified with HCl (6M) to eliminate all carbonates. C was quantified with an Elemental Analyzer (Flash 2000, Thermo Scientific). The pH$_{H2O}$ was measured using an Accumet AP85 pH (Fisher Scientific Ltd., Pittsburgh, PA). Metals and total phosphorous were determined by inductively coupled plasma–optical emission spectroscopy (ICP-OES, Spectro Ciros CCD, Spectro GmbH, Kleve, Germany; Table 2). Total nitrogen concentration was measured using the Dumas combustion method with a TruSpec N analyzer (LECO Corporation, St. Joseph, MI). Soil water content (m^3 m^{-3}) at a depth of 6 cm was measured in triplicate per each plot using a Theta Probe (Type ML2x, Delta-T devices Ltd., Cambridge UK). A Principal Component Analysis (PCA) on normalized environmental and chemical data was done with PAST software (Hammer et al., 2001).

DNA and RNA extraction

Soil DNA (D) and RNA (CD) were extracted according to Griffith et al. (2000). RNeasy Mini Kit (Qiagen, Milan, Italy) was used according to the manufacturer's instructions to remove DNA contamination, and Optional on-column DNase digestion was carried out by using RNase-Free DNase set (Qiagen). For reverse transcription QuantiTect Rev. Transcription Kit (Qiagen) was used, and all the reverse transcription steps were carried out on ice according to the manufacturer's instructions. To test whether purification had worked and whether cDNA was amplifiable, 16S rRNA gene PCR were carried out with 27F and 1494R primers. Controls without the addition of RNA and without the addition of the enzyme were run in parallel.

Molecular analysis of the ammonia-oxidizing bacterial communities

Denaturing Gradient Gel Electrophoresis (DGGE; Muyzer et al., 1993) of 16S rRNA of β-Proteobacteria to preliminarily assess the diversity of ammonia-oxidizing bacteria (AOB) was performed adopting a nested-protocol as previously described (Offre et al., 2009). The nested PCR approach was demonstrated to capture a wide range of diversity of ammonia oxidizers bacteria (Kowalchuk et al., 1997) yielding AOB phylogenies that are similar to those based on the AmoA gene (Purkhold et al., 2003). DGGE was run in a BioRad DCode Universal Mutation Detection System (Biorad, Milan, Italy). Polyacrylamide gels were done according to Muyzer et al. (1993). Gels were stained for 30 min in 1× TAE buffer containing SYBR® Safe - DNA Gel Stain (Invitrogen, Milan, Italy). DGGE bands were excised and reamplified (Muyzer et al., 1993) and sequencing was performed by Macrogen Inc. (Korea).

Identification of 16S rRNA genes was done by comparison with EMBL/Genebank/DDBJ database and RDP database using BLASTN and Classifier respectively. All sequences were submitted to the RDP-Ribosomal Database Project (Cole et al., 2009) web server to assign taxonomy.

Non-Metric Multidimensional Scaling (NMDS) was carried out to explore similarities between sites, based on the resemblance matrix generated using Bray–Curtis similarity on the matrix for DGGE. The same set of

environmental data was used in the distance-based multivariate analysis for a linear model (DistLM) to determine which significant environmental variables explained the observed similarity among the samples. The Akaike Information Criterion (AIC) was used to select the predictor variables. The contribution of each environmental variable was assessed, firstly using "marginal tests" to assess the statistical significance and percentage contribution of each variable taken alone, and secondly a "sequential test" was employed to evaluate the cumulative effect of the environmental variables explaining biotic similarity. A distance-based redundancy analysis (dbRDA) was used for graphical visualization of the DistLM results. Significant differences in microbial community composition were investigated by permutational analysis of variance (PERMANOVA; Anderson, 2001), considering the sampling area as a fixed and orthogonal factor. Ecological diversity indices were calculated from the matrix of ARISA OTUs. All the statistical tests were performed by PRIMER v. 6.1, PERMANOVA+ for PRIMER routines and PAST software.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA fingerprint was performed as described by Cardinale et al. (2004) to assess the structure of the rhizobacterial communities with the ITSF/ITSREub primer set. Denatured ARISA fragments were run by STAB-Vida Inc. The data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems, Monza, Italy), and a threshold of 40 fluorescent units was used, corresponding to two times the highest peak detected during the negative control run. Output matrix was obtained according to Rees et al., 2004. ARISA matrix was normalized with the formula $(x/\sum x)*1000$ where "x" is the fragment height in units of fluorescence, then transformed on a logarithmic scale for multivariate analysis. Log-transformation was used to stabilize the sample variance, to reduce the interaction effect and to normalize the distribution of data. Moreover, log-transformation can combine the information of a binary matrix with the those of a non-transformed data matrix, hence preserving the relative abundance information and down-weighing dominant groups. In order to assess changes in rhizobacterial community structure between floristic consortia, a NMDS was applied with Bray-Curtis algorithm. NMDS does not need of the assumption of linear associations among variables, being described as the most efficient ordination method for microbial ecology (Clarke, 1993). Bray-Curtis is not influenced by recurrent absent values into the matrix, a characteristic very common in ARISA (Clark and Warwick, 2001). ANOSIM (based on Bray-Curtis similarity) was performed to test significant differences in the profile composition of the different sites. ANOSIM is a nonparametric statistical test, based on permutation, which uses rank similarity matrix of an ordination plot to calculate an R test statistic on the null hypothesis H^0 that there are no differences among groups. When R is near to 0, H^0 is true, while when R is reaching 1, H^0 can be rejected and there is a discrimination between groups. When ANOSIM statistics approaches 1, the similarities within groups are larger than similarity between groups. We rejected H^0 when significance *P* value was < 0.05.

Roche 454 16S rRNA gene pyrosequencing

All the DNA and cDNA obtained for each transect and each sampling season were used for 454 pyrosequencing analysis. Genomic DNA and cDNA were shipped to the Molecular Research LP (MR DNA^{TM}) (www.mrdnalab.com). DNA and cDNA were used for PCR amplification of environmental 16S rRNA genes for

bacteria with a primer set amplifying the V4-V6 variable regions (primers 518F 5'-CCAGCAGCYGCGGTAAN-3' and 1046R 5'-CGACRRCCATGCANCACCT-3'). Samples were sequenced using the Roche 454 GS-FLX system, titanium chemistry, accordingly to the protocols of that company. Pyrosequencing data were analyzed with proper analysis pipeline as suggested by the company. Sequences with length < 200 bp, or with ambiguous bases and homopolymer runs exceeding 6 bp were removed before chimera checking. 16S rRNA gene pyrotags were defined after removal singleton sequences, by clustering into 97% similarity. Representatives from each OTU were classified using BLASTN against a curated GreenGenes database.

Raw sequences were processed using the software Mothur (Schloss, 2009). The pipeline was the following: trimming the raw data using the trim.seqs function, setting the max of homopolimer to 6, no ambiguous basecall was allowed together with an average quality score of 35 within a window of 50 bp. After this screening sequences shorter than 200 bp were removed. Sequences were then checked for chimeras with the chimera.uchime function. Then they were classified and aligned on the SILVA database setting a confidence threshold of 60%. The OTUs were formed with the standard cutoff of 3%. The matrix was exported for the downstream statistical analysis.

A redundancy control has been performed in order to obtain a single file containing only unique sequences. The sequences contained in the output file were clustered using Usearch (Edgar, 2010) with an identity cutoff value of 90%. After this step, all the centroid sequences were collected from the Usearch output and classified using the RDP Classifier (Wang et al., 2007). In particular, a confidence threshold of 80% was used in order to obtain only classification hits with high confidence. Finally, a frequency table of taxonomic assignments was reconstructed. Raw sequences have been submitted to the EMBL/NCBI/DDBJ Short Read Archive under accession number to be confirmed.

PAST software was used to perform the statistical analysis. Chao1 index was calculated on OTU assessed with 454 pyrosequencing. ANOSIM and NMDS was performed by using default settings. Cluster analyses were performed on pyrotags samples with Bray-Curtis measure. Similarity Percentage (SIMPER) analysis was performed according to the groups of samples as defined after the cluster analysis. SIMPER visualization of the average values was done through Correspondence Analysis (CA). Rarefaction curves of pyrosequencing data were done with R software (http://www.r-project.org/). NMDS on pyrotags matrix data was performed by using Bray-Curtis dissimilarity distance, and the environmental variables were overlaid on the ordination plots with the specific function. Moreover a PCA was performed with default setting on the transformed data of the environmental variables. Heat map of the most important genera was done with Excel Software. The other statistical analyses such as Kruskal-Wallis test on soil C and N percentages or on NMDS sample position were performed using PAST program. Finally, rarefaction curves of pyrosequencing data were done with R software.

Roche 454 nifH gene pyrosequencing

Primers PolF and PolR (Poly et al., 2001) were used because of their reliable amplification of DNA extracted from soil without amplification of non-specific products. The pyrosequencing reads processing started using tools available on the Ribosomal Database Project's (RDP) Fun Gene pipeline website. Reads without barcodes were filtered by using Mothur script. Sequences shorter than 200 bp were delete. Then FrameBot utilities was used for aminoacidic translation and frameshift correction. Referenced *nifH* database from RDP pipeline was used.

Sequencing data were then clustered into OTU by using FrameBot utilities. A 5% of aminoacidic diversity was used to identify different OTU clusters. SIMPER analysis and diversity indices were calculated with RDP utilities and PAST software. Moreover, identification of most important aminoacidic reads was done with BLASTP. Sequences were deposited in the Short Read Archive under accession numbers to be confirmed. All the statistical analysis, rarefaction curves calculation and NMDS were done as above explained.

Results

Environmental variables

Soil moisture did not vary among seasons and between sites: water content was between 378-956 mV in the samples collected in October while in July it ranged from 151 to 972 mV. The soil pH ranged from 4.1 to 5 in the soils of both the transects. Total organic C and humus were significantly higher in samples of T3, whereas % of total N, which represented the whole amount of NH_4^+, decreased (Table 1). Mineral content in terms of metal and other ions concentration was similar for all the samples (Table 2). PCA has been done in order to assess the main variability axes of the environmental parameters (Fig. 1). The first two axes explained 59.0% and 19.3% of the variance respectively. PCA ordination plot showed two distinct groups according to soil age except for samples 205 and 301. T2 was influenced predominantly by the soil mineral composition whereas T3 was influenced by TOC, humus, P content, Cd, Hg and C/N ratio. Sample 301 seems to be influence by DM only, while 205 by none environmental parameter. The loading values, which correspond to the relative contribution of each environmental variable on the axis, showed high positive values for the parameters linked to the soil mineral composition on the first axis, whereas high negative values were connected to a more mature soil.
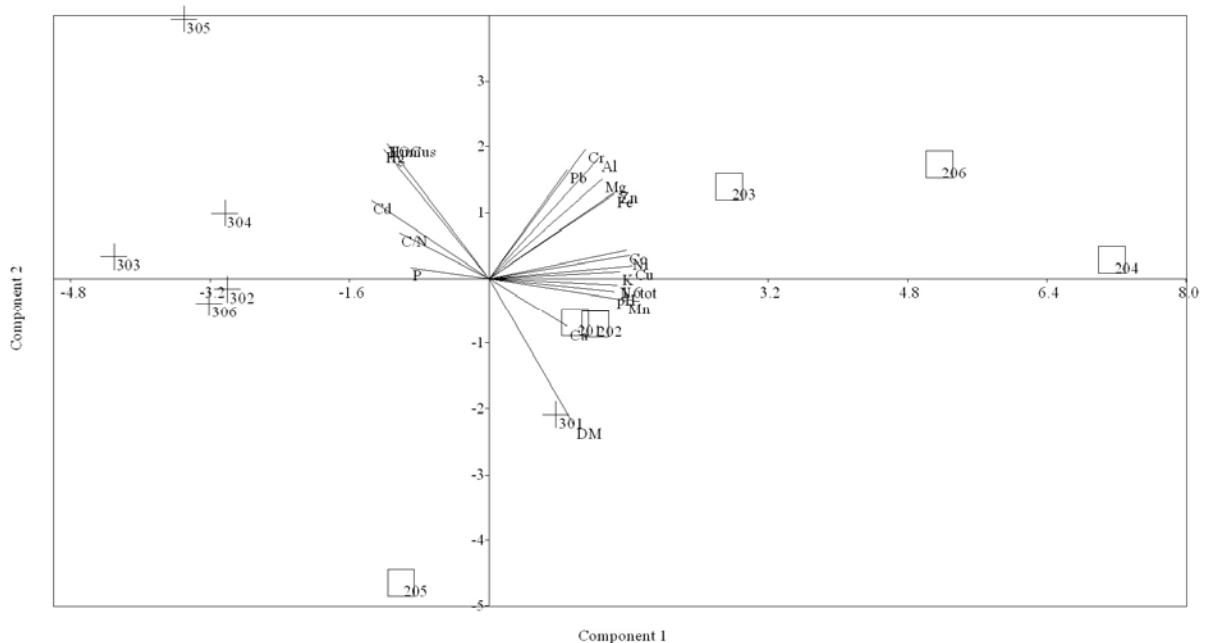

Figure 1 - Principal Component Analysis ordination plot of the environmental parameters among the two transects. Samples of transect 2 are shown by squares, while transect 3 by crosses.

ARISA

ARISA fingerprints provided spatial patterns of both the overall and active rhizobacterial communities associated to the vegetation plots. Complex profiles were produced with peaks ranging from 150 bp to 1459 bp and the 16S-23S rRNA internal transcribed spacer region (ITS) richness varied from quite low peaks abundance of the active populations (33 peaks of average) to higher peaks abundance of the overall communities (78 peaks of average). The electropherograms, characterized by distinct peaks number and intensity, revealed shifts in rhizobacterial community. On the NMDS plot of the overall rhizobacterial communities (D), samples from transect two (T2) and from transect three (T3) collected in July (DJ2, DJ3) and in October (DO2, DO3) showed four separate clusters based on microbial community structure (Fig. 2) and explained a temporal dynamic of the bacterial communities of the two transects.
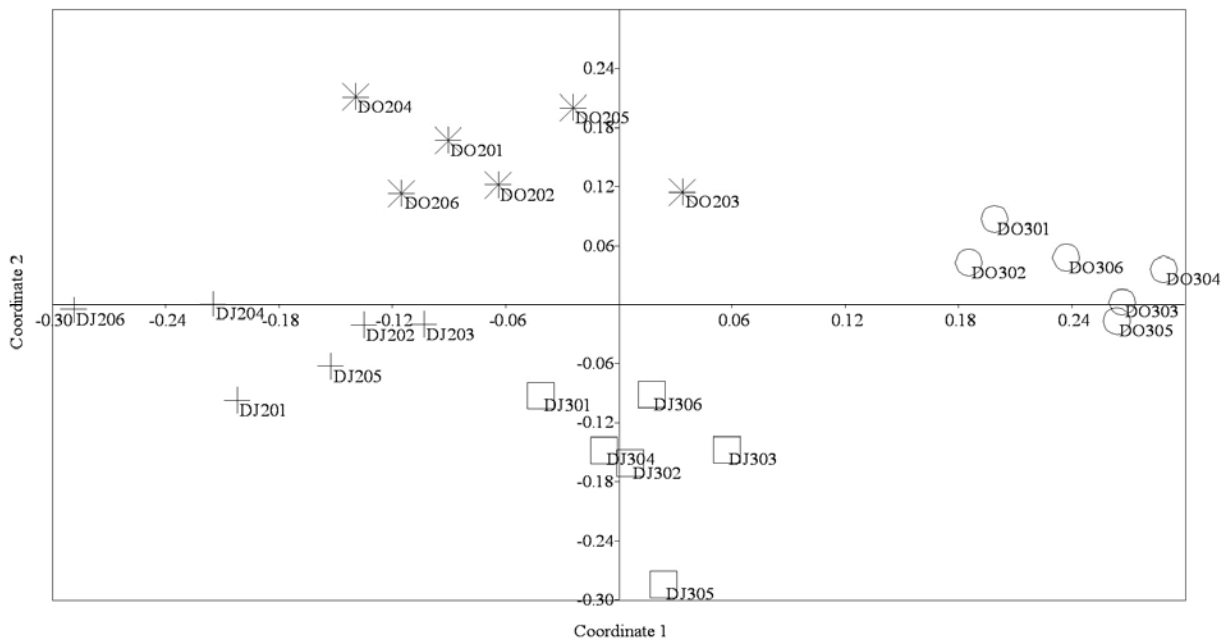
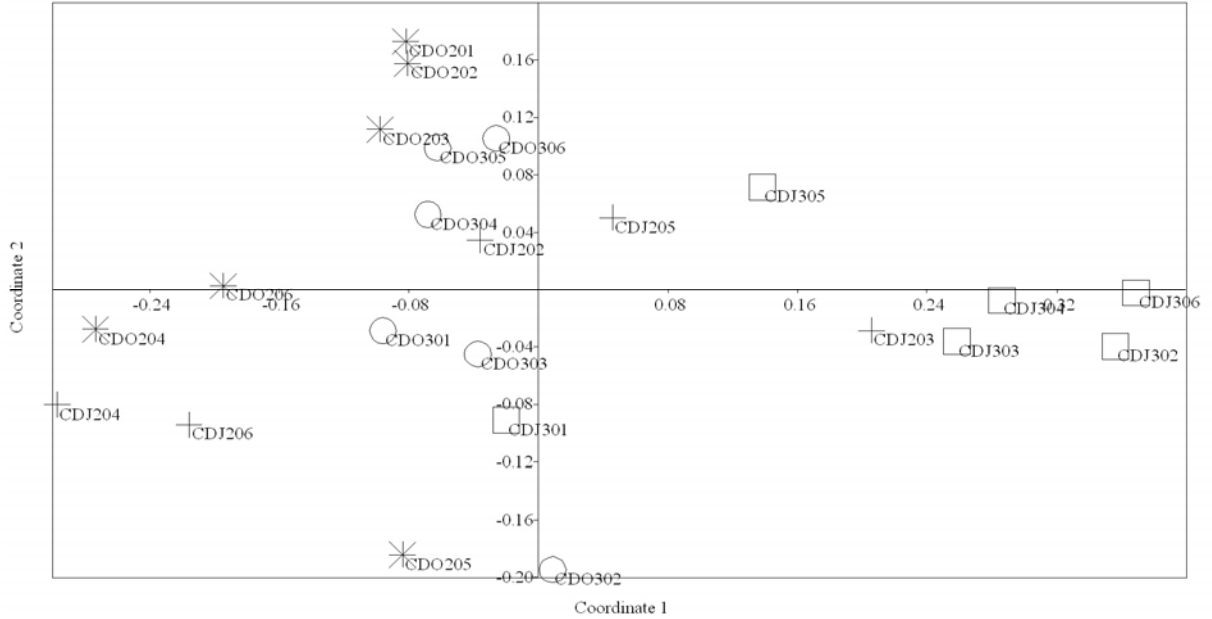Figure 2 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of DNA samples of both the sampling seasons and transects. Cross: T2 July; square: T3 July; star: T2 October; circle: T3 October.

ANOSIM analysis confirmed a highly significant difference among the four overall community structures (R=0.96; *P*=0.0001) and the performed test showed significant differences in the pairwise comparisons of the transect sampled in different season with R values reaching 1 in most of the cases: DJ2 vs DJ3 (R = 0.89), DJ2 vs DO2 (R = 0.84), DJ2 vs DO3 (R = 1), DJ3 vs DO2 (R = 0.99), DJ3 vs DO3 (R = 1), DO2 vs DO3 (R = 0.98). On the other hand, the NMDS plot of the active rhizobacterial communities (CD) did not showed well-defined clusters. According to axis 1, almost all the samples collected in July are separated from the ones collected in October. Samples from T3 collected in July clustered together, except for sample 301. Samples 204 and 206 collected both in July and in October clustered together according to vegetation type. Finally samples from T3 collected in October clustered together with almost all the samples of T2 collected both in October and the others

of T2 July (Fig. 3). ANOSIM analysis showed highly significant differences among the four active community structures (R=0.46; *P*=0.0001) except for the pairwise comparisons of CDJ2 vs CDO2 and CDO2 vs CDO3 which showed *P*=0.08 and *P*=0.07 respectively.

Figure 3 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of cDNA samples of both the sampling seasons and transects. Cross: T2 July; square: T3 July; star: T2 October; circle: T3 October.
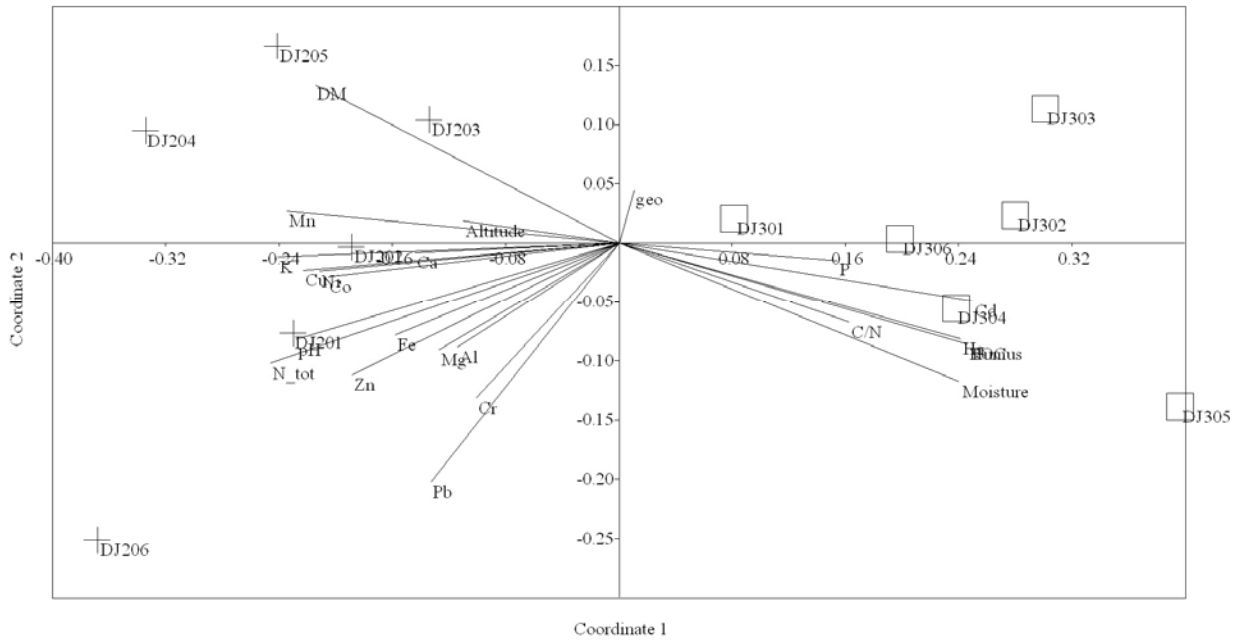
Figure 4 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of DNA samples of both transects in July. Cross: T2 July; square: T3 July.
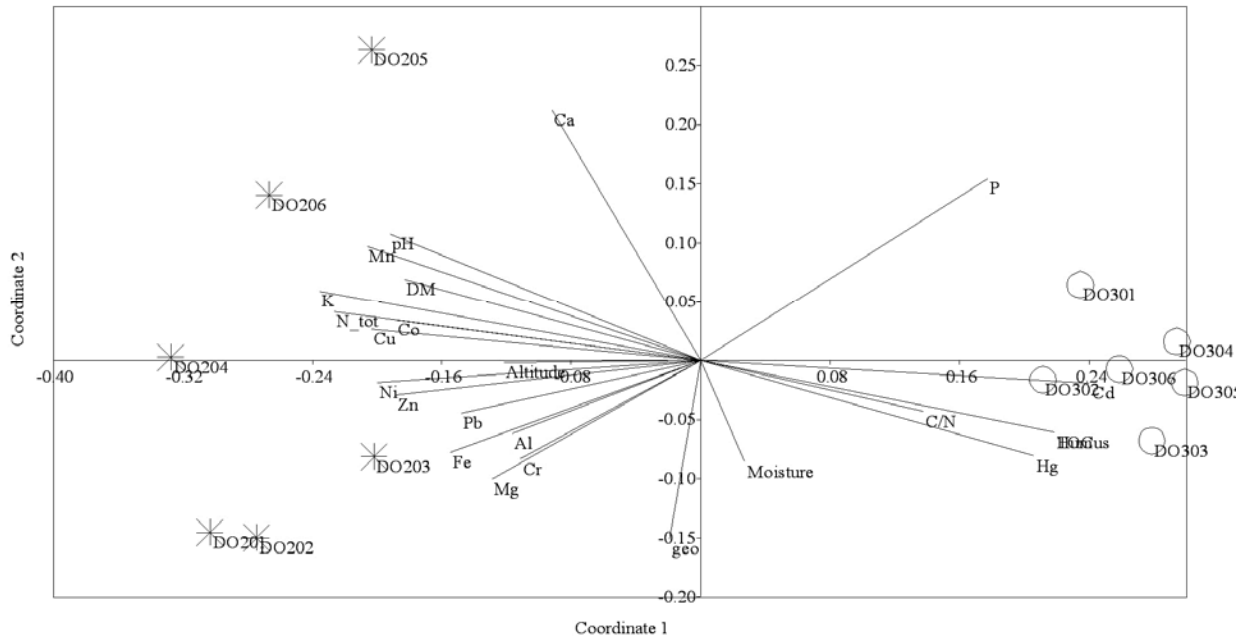
Figure 5 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of DNA samples of both transects in October. Star: T2 October; circle: T3 October.

The NMDS analyses of overall and active bacterial communities T2 and T3 in both the sampling season were done to show the influence of the environmental parameters on samples distribution in the plots. DJ2 samples clustered separately by DJ3 samples (Fig. 4). In July, samples 201, 202, and 206 were positively influenced by all the metals, total N and pH, which conversely influenced negatively samples 301, 302, 303 and 306. Samples 203, 204 and 205 were positively influenced by dry matter amount. P amount, C/N ratio, TOC, humus content, and soil moisture were positively related to samples 304 and 305. The influence of the environmental parameters in October was quite different (Fig. 5). Samples 201, 202, 203 clustered separately from the other samples of T2, being influenced by metal composition only. Plots 204, 205 and 206 were influenced by total N, K, dry matter, pH and occasional ions. Samples of T3 clustered more strictly together, being all influenced by humus content, TOC, C/N ratio, except for sample 301 which seemed weakly influenced by P.

The active rhizobacterial populations were differently influenced by the environmental parameters. In July (Fig. 6) samples of T3, except 301 and 305, and sample 203 were strictly related to humus, TOC and moisture. Sample 305 seemed to be not influenced by environmental parameters, whereas 301 was plotted close to samples 201, 202, 205 which were influenced by altitude, geographical location and weakly by DM. Samples 204 and 206 were strongly influenced by pH and by metal ions in the soil. In October (Fig. 7), samples 204 and 206 were also strongly influenced by total N. Samples 201, 202, 203, 305, and 306 were not influenced by environmental parameters except by moisture which showed a weak influence. Samples 302, 303, 304 are affected by the same parameters as above, and more clearly also by C/N ratio. Also samples 301 and 205 seem to be affected by other parameters rather than those measured.
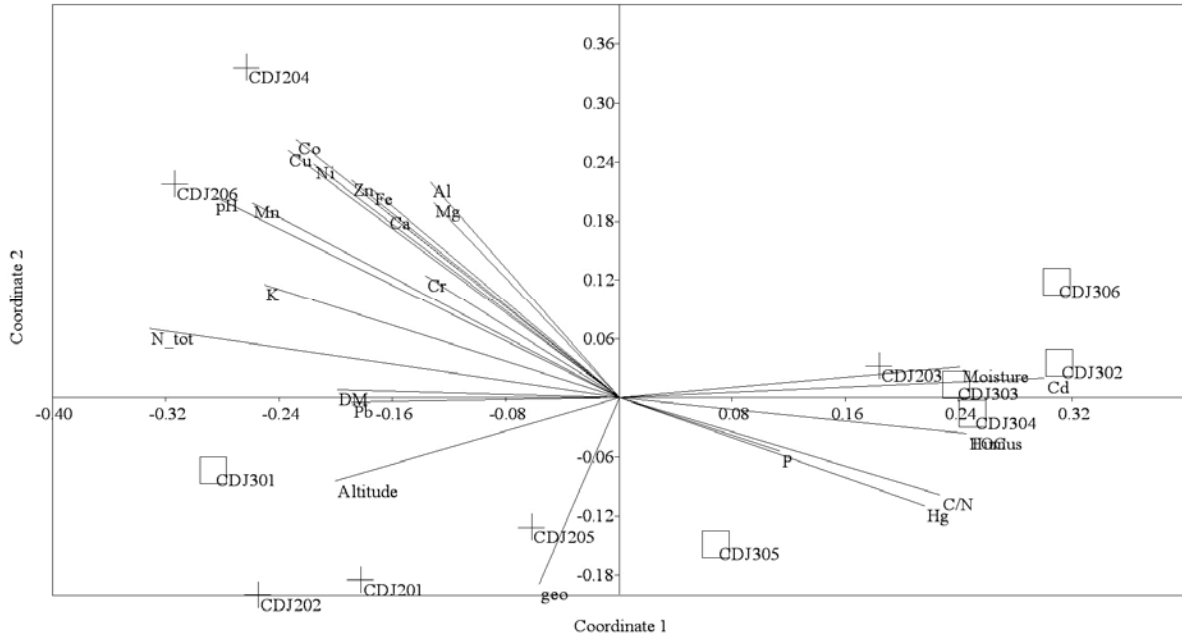
Figure 6 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of cDNA samples of both transects in July. Cross: T2 July; square: T3 July.
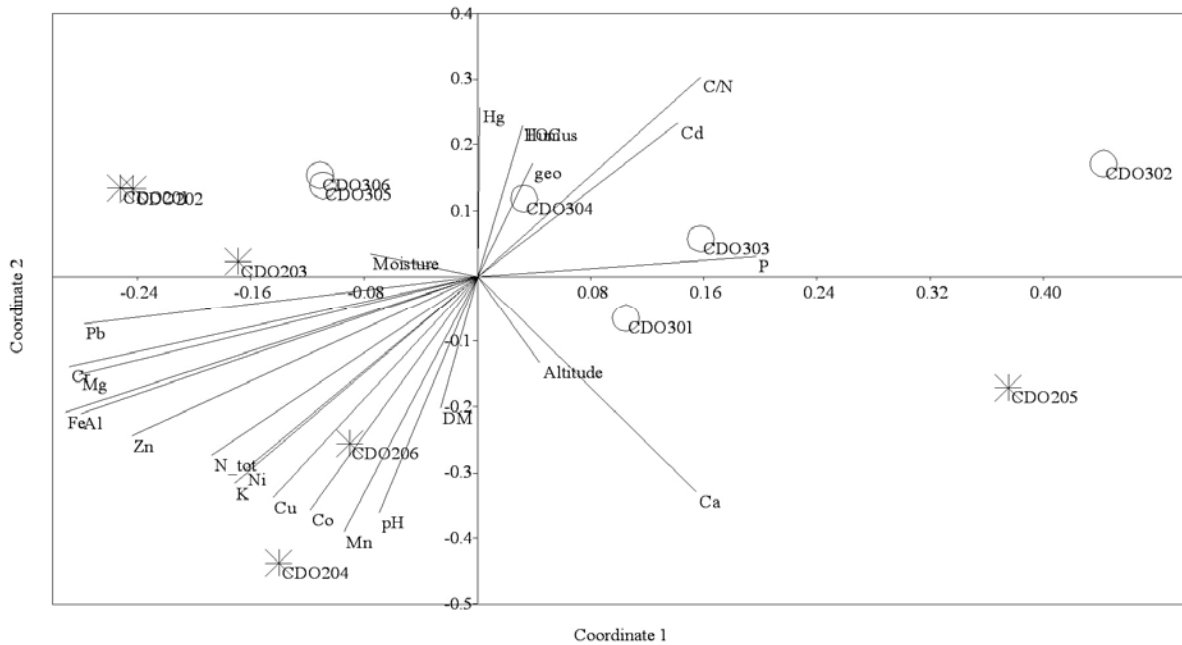

Figure 7 - Non-Metric Multimensional Scaling plot of the ARISA peak pattern of cDNA samples of both transects in October. Star: T2 October; circle: T3 October.

Ammonia oxidizing bacteria

16S fingerprinting of Ammonia oxidizing β-Proteobacteria was carried out analyzing DGGE gel patterns of the overall rhizobacterial communities associated to the vegetation plots in both the sampling seasons. Per each sample complex profiles were produced and were characterized by distinct bands number and intensity which revealed shifts in rhizobacterial community. NMDS analysis with Bray Curtis similarity measure, showed samples from transect two collected in July and in October strictly together and with samples 301 collected in July and in October and sample 302 collected in October. The other samples of transect three were scattered in the plot and only five samples collected in both the sampling seasons clustered together. PERMANOVA Monte Carlo test analysis confirmed a highly significant difference among the two transects (P=0.0087). Moreover, the diversity of AOB communities associated to the samples was studied. *Nitrosospira* and other AOB genera were identified and most of the sequences were affiliated to *Nitrosospira multiformis* lineage previously described (Kowalchuk and Stephen, 2001) and to *Nitrosospira*-like sequences as previously reported by Purkhold et al., 2003.

Structure and diversity of the overall bacterial communities

After filtering the low quality reads with the RDP Initial Process in Pyrosequencing Pipeline (PP) and trimming the adapters, barcodes and primers, we obtained 62,977 and 138,863 effective reads for the DNA and cDNA samples respectively. After denoising, filtering out chimeras, and removing the archaeal sequences, the library size of each sample was normalized to conduct the analyses for different samples at the same sequencing depth. The OTU numbers, certainly affected by sequencing noise, ranged from 388 to 3,298 for DNA samples and from 1,043 to 13,161 for cDNA samples, as shown by rarefaction curves (data not shown). The numbers of OTUs, Chao 1 and the main diversity indices for DNA and cDNA are summarized in Table 3.

Table 3 - OTUs number and main diversity indices of the 16S rRNA gene pyrosequencing of DNA and cDNA samples of the two transects.

Indices	DJ2	DJ3	DO2	DO3	CDJ2	CDJ3	CDO2	CDO3
OTU N.	5865	6766	7950	7828	8492	23840	9118	13090
Dominance	0.0032	0.0028	0.0023	0.0023	0.0019	0.0006	0.0018	0.0008
Shannon	7.90	8.05	8.21	8.20	8.31	9.10	8.45	8.71
Evenness	0.46	0.46	0.46	0.47	0.48	0.37	0.51	0.46
Chao	13950	15880	18200	17700	19030	47250	21450	28830

On the basis of the OTUs and reads number at cutoff levels of 3%, the DNA samples of both the transects in July had little less richness, while the DNA samples of the transects in October displayed the highest diversity. Chao 1 patterns were very similar to the OTU and reads numbers. All the indices, demonstrated that the richness values were quite constant in different seasons and among the two transects. Although the similar evenness values between the four cases (about 0.46) showed oligarchic bacterial communities, the dominance values exhibited quite low values and the bacterial communities were also characterized by high value of Shannon indices. About cDNA samples of both the transects collected during to different seasons, the highest richness was shown by cDNA samples of T3 in both the sampling seasons. The other indices confirmed the richness differences between the two transects. The effective bacterial sequences were assigned to different taxa levels (from genus to phylum)

using the RDP Classifier at 50% threshold, as suggested by the RDP pipeline. Although the V4 region captured the highest extent of correctly classified sequences (Claesson et al., 2009), quite large amounts of bacterial sequences at different taxa level could not be assigned to any taxa at the 50% threshold. The number of unclassified sequence increased from the phylum level to the genus level. From 6.7-8.4% and 5.4-6.8% of sequences in DNA and cDNA samples respectively up to 42.5-45.2% and 34.2-43.1% of sequences in DNA and cDNA samples respectively could not be assigned to any taxa at phylum and genus levels. Without the unclassified sequences, Proteobacteria resulted to be the most abundant phylum in all the DNA and cDNA samples, accounting for 36.7-62.4% of DNA sequences and 36.5-60.3% of cDNA sequences. This is similar to the results of bacterial communities in alpine soil, in which Proteobacteria was the most dominant taxa (Lipson and Schmidt, 2004; Lazzaro et al., 2012). They were followed by other phyla including Acidobacteria and Verrucomicrobia which were significantly more abundant in cDNA samples (9.2-46.5% and 1.9-17.6% respectively) than in DNA samples (8.7-22.4% and 0.6-5.7% respectively). Other phyla like Actinobaceria (5.3-23.3% in DNA, 2.4-21.5% in cDNA), Bacteroidetes (1.2-9.7% in DNA, 1.2-14.7% in cDNA), Cloroflexi (1.3-7.7% in DNA, 0.8-2.2% in cDNA), Planctomycetes (4.5-10.4% in DNA, 0.3-4.2% in cDNA) presented larger amounts in DNA samples than in cDNA samples whereas Firmicutes, Armatimonadetes, Cyanobacteria were occasionally present with quite low percentage in both the samples types. α-Proteobacteria, the most dominant class within Proteobacteria, occurred at high levels of total bacterial effective sequences (averaging 36.5% in DNA and 28.5% in cDNA samples) whereas β-, γ-, and δ-subdivisions occurred at levels quite lower than 10%. ε-Proteobacteria were rarely found. While some studies (Xia et al., 2010) showed that in agricultural soils, α-subdivision was the most abundant group of Proteobacteria by analyzing samples with microarrays, other studies that used pyrosequencing (Roesch et al., 2007) demonstrated that in most soils, the β-subdivision was the most abundant. Our findings on alpine soils seem to confirm this latter result. Other dominant classes included Acidobacteria Gp1, Sphingobacteria, Opitutae which averaged 18.9%, 6%, 5.8% respectively in cDNA samples, were more abundant than in DNA samples (Acidobacteria Gp1 8.1%, Sphingobacteria 2.3%, Opitutae 2.2%). On the other hand, Actinobacteria, Planctomicetaceae, Ktedonobacteria were present in DNA samples at higher levels than the cDNA samples (17.9%, 6.7%, 3.7% vs 8.5%, 1.3%, 0.7% respectively). Interesting Thermomicrobia class which was very rarely found in all the samples, were significantly present (average 3%) in DNA samples of plot 202, collected both in July and in October and in DNA sample 301 collected in October. At the order level, over 68 orders, the 15 most shared accounted for 92.8% of the sequences. Rhizobiales represented the most abundant order in both DNA and cDNA samples (38.7% and 25.9%) reaching level of 61% in 201 DNA sampled in October followed by Actinomycetales with percentages of about 9.4% in bath cases. Myxococcales, Sphingobacteriales, Opitutales, Rhodospirillales, Burkholderiales, were more common in cDNA samples whereas Planctomycetales, and Solirubrobacterales were higher in DNA samples. At the family level, Bradyrhizobiaceae represented 28.7% of effective sequences in DNA samples and 17% in cDNA samples. The top 10 families, which explained the 60% of the diversity, comprehended also higher level of Opitutaceae, Sphingobacteriaceae, Acetobacteriaceae, Caulobacteraceae, Polyangiaceae, Chitinophagaceae in cDNA samples whereas Planctomycetaceae, Hyphomicrobiaceae, Rhodospirillaceae were more abundant in DNA samples. The analysis at the genus level, gave us an idea about the crucial role of the rhizobacterial communities in the studied environment. The Gp1 and *Granulicella* genera, members of Acidobacteria phylum, represented 10.3% and 1.4%

respectively in DNA samples, 14.9% and 8.4% in cDNA samples. Although they are widely distributed in soil ecosystems (Janssen, 2006), their ecological role is not clearly understood. Recent studies on few cultivable *Acidobacteria* explained their role in carbon and nitrogen cycles and their tolerance to oligotrophic condition and to highly variable condition in the soil (Eichorst et al., 2011). Together with the symbiontic nitrogen fixating *Bradyrhizobium*, the nitrate reducing *Opitutus* and *Afipia*, the five genera represented the 37.3% of all. The evidence was confirmed by SIMPER analysis which reported sequence affiliated to the genera *Afipia*, *Bradyrhizobium* as the most determinant in shaping the overall bacterial communities whereas sequences of the genus *Bradyrhizobium* shaped the active rhizobacterial communities in both the transects and Acidobacteria of the genera *Granulicella* and Gp1 had a crucial role in shaping rhizobacterial communities of transect 3. Other less represented genera involved in nitrogen cycle i.e. *Caulobacter*, *Agromonas*, *Gemmatimonas*, *Rhodopila*, *Acidisphaera*, *Hyphomicrobium*, *Ideonella*, were detected. These results confirmed a scenario distinctive of harsh environmental conditions where the rhizobacterial communities supplied plant of nitrogen. Other genera i.e. *Mucilaginibacter*, *Zavarzinella*, *Aciditerrimonas*, *Singulisphaera* were generally detected in acidic habitat and the genus *Aciditerrimonas* seems to be involved in the oxidation of H_2S to sulfur with atmospheric oxygen followed by oxidation of sulfur to sulfuric acid by sulfur-oxidizing bacteria. The taxa involved in carbon cycles and cellulose degradation were rarely detected.
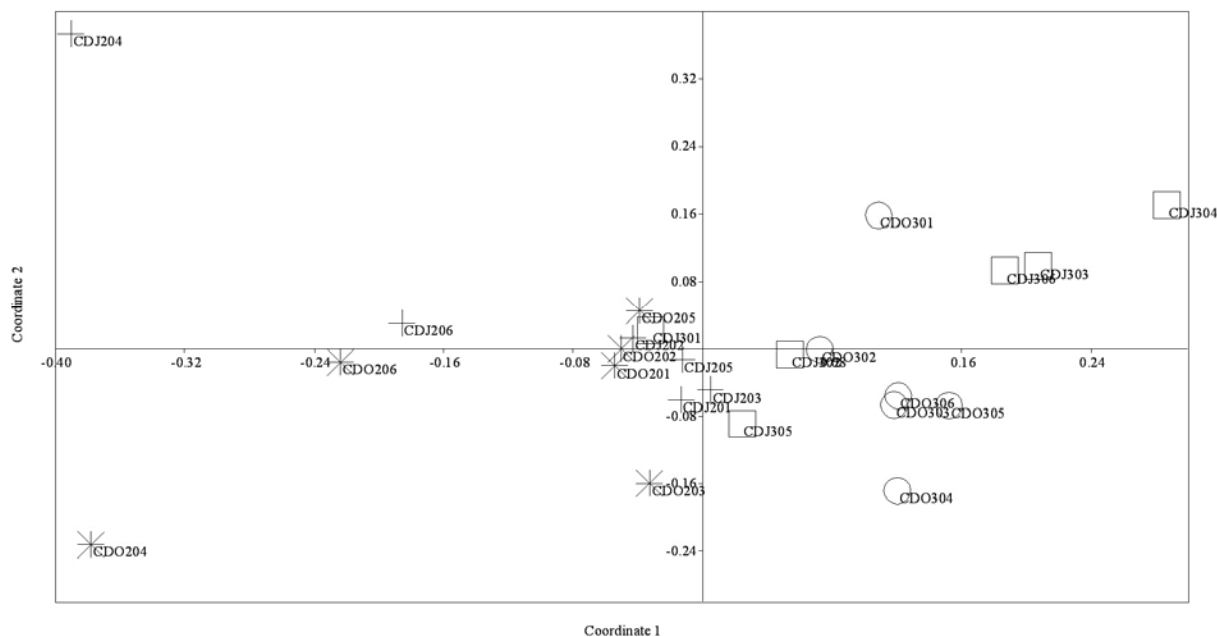

Figure 8 - Non-Metric Multimensional Scaling plot of the OTUs pattern of cDNA pyrosequenced samples of both the sampling seasons and transects. Cross: T2 July; square: T3 July; star: T2 October; circle: T3 October.

Influence of environmental parameters on viable bacterial communities

One of our aims was to find possible changes in the composition of the active bacterial communities along the transects and during the sampling seasons, and relate them to nitrogen cycle. Previous investigations aimed to

identify the dominant physiologically viable microorganisms in unvegetated soils of two glacier forefields (Damma and Tsanfleuron) highlighted the shared presence of α-Proteobacteria, especially *Methylobacterium* species, Actinobacteria and Firmicutes (Lazzaro et al., 2012). These dominant lineages had been previously recognized in the Damma glacier forefield and in other similar habitats (Skidmore et al., 2005; Duc et al., 2009; Philippot et al., 2011). Moreover, Lazzaro et al. (2012) detected seasonal shift of bacterial communities favoring bacterial taxa (i.e. Actinobacteria) able to degrade recalcitrant substrates in autumn than in summer. However, even if our approach was based on cDNA analysis, which detect only the viable community members, our results confirmed other studies of high altitude environments. Conversely of those researches, done on unvegetated soils, our vegetated samples were richer in Acidobacteria, β- Proteobacteria, Verrucomicrobia and Bacteroidetes than the alpine Damma and Tsanfleuron forefields (Lipson and Schmidt, 2004; Nemergut et al., 2005; Mannistö et al., 2007; Nemergut et al., 2007). One of the most representative group detected in Damma glacier (Lazzaro et al., 2012; Duc et al., 2009) was the type II methanotrophs (*Methylocystis* and *Methylobacterium* sp.) which are able to survive with oxidation of atmospheric methane (Dunfield et al., 1999) or with alternative C sources (Theisen and Murrell, 2005) in oligotrophic upland soils (Knief and Dunfield, 2005). However, our study did not detected high levels of methanotrophs, rarely detected in all the samples in both summer and autumn time.

A NMDS analysis was conducted to evaluate similarities of active population samples using the RDP Classifier taxa and the OTUs. For the two approaches, taxa or OTUs are regarded as equally related and the analyses were conducted at the different taxa levels. Although there are slight variances among the results, the same general trend was observed. The cDNA-based analysis of bacterial communities is not free of biases which may determine erroneous conclusions about diversity. Firstly, rRNA synthesis vary rapidly in response to nutrient and environmental variables. Consequently, a cDNA-based analysis represents an instant picture of the diversity of the viable bacterial community: this complex description is valid only for the moment chosen for sampling, and it is affected by the overall environmental variables. Secondly, soil extraction of labile RNA is often difficult, and contamination from stable DNA could occur. PCR-based downstream analysis could be also a crucial step due to the preferential amplification of certain sequences (Acinas et al., 2005). Moreover, organisms involved in particular ecological functions often exist in low abundance and may not be detectable (Harrington et al., 2001; Zwolinski, 2007). Despite these limitations, our pyrosequencing results showed an increased dominance of certain OTUs among the transects and the two seasons. NMDS analysis, based on abundances of OTUs, revealed that active bacterial communities in all the samples clustered differently according to the transect ($P = 0.008$), while no differences were found due to sampling season (Fig. 8). ANOSIM analysis confirmed significant differences between CDJ2 vs CDO3 and CDJ3 vs CDO2, CDO2 vs CDO3. Taking account the twenty-four environmental parameters measured in the present investigation, we could understand their influence on the active rhizobacterial communities. Samples collected in July (Fig. 9) and in October (Fig. 10) from T2 were mainly influenced by soil mineral composition except for samples 203 collected in July, which was more influenced by geographical composition. Samples from T3 were influenced especially by parameters concerned the soil fertility except sample 301 which cluster more strictly together with T2 samples. Samples 301 in almost all the analyses showed a behavior quite similar to the samples from youngest soil collected in both the sampling seasons. Its geographical

position, in a very elevated slope could have been avoided the soil maturation processes because of water percolation and soil instability.
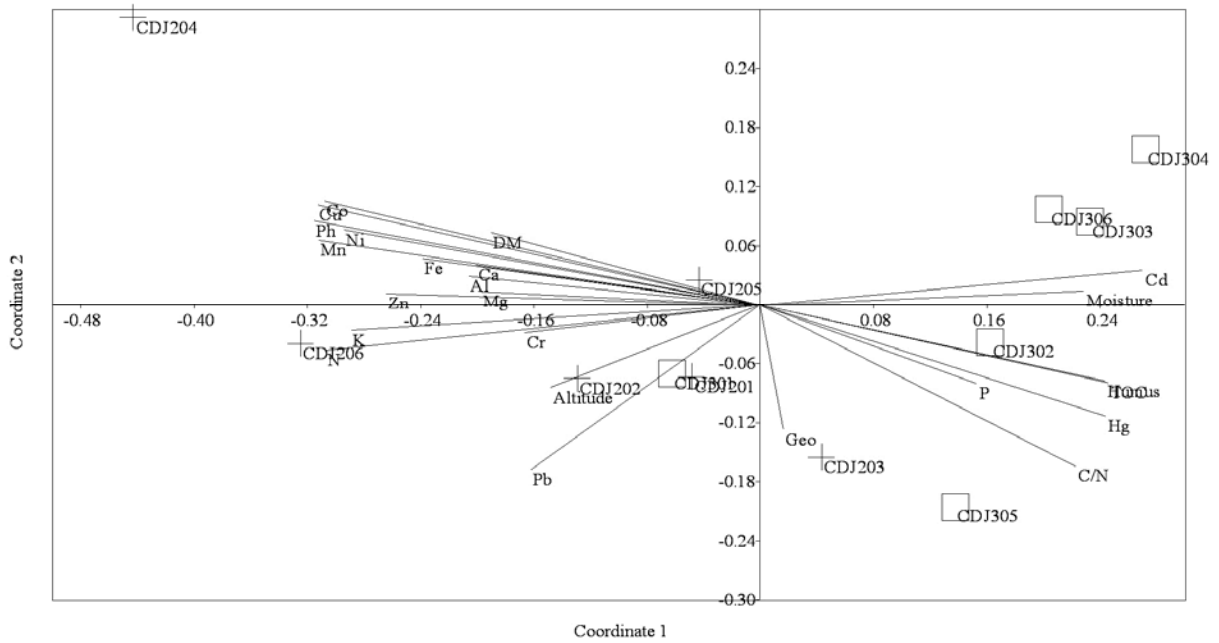

Figure 5 - Non-Metric Multimensional Scaling plot of the OTUs pattern of cDNA pyrosequenced samples of both transects in July. Cross: T2 July; square: T3 July.
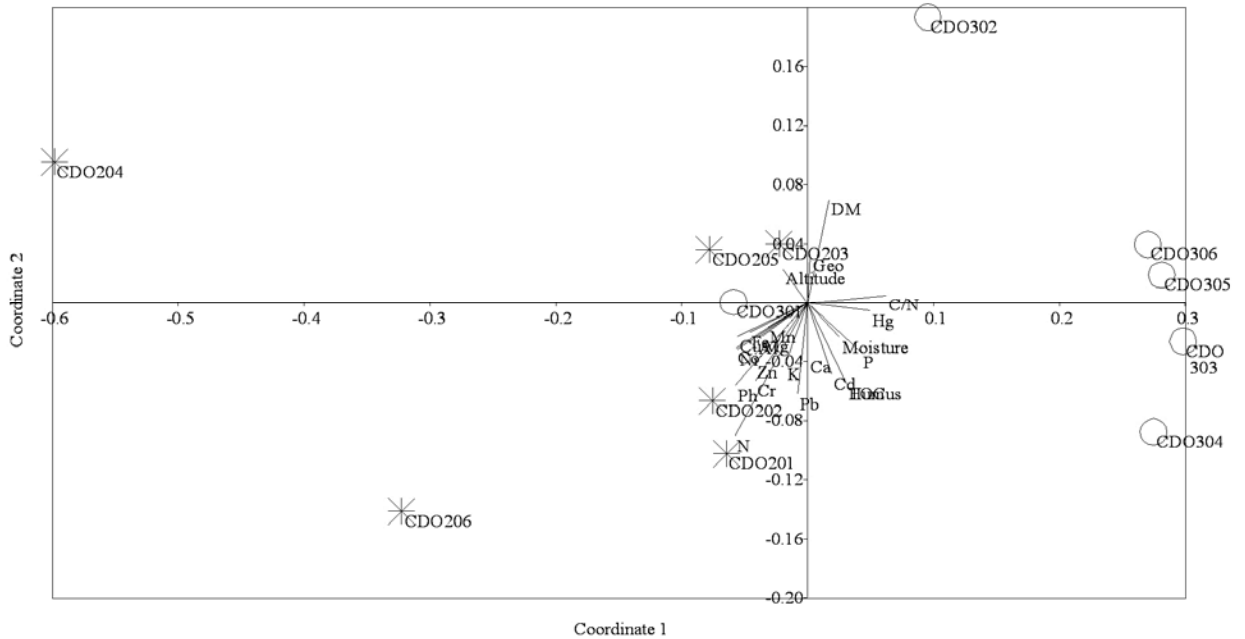

Figure 10 - Non-Metric Multimensional Scaling plot of the OTUs pattern of cDNA pyrosequenced samples of both transects in October. Star: T2 October; circle: T3 October.

Structure and diversity of the nifH genes in the overall and the viable bacterial communities

Biological nitrogen fixation is a pivotal driver of primary productivity in a primary successional chronosequence and is carried out exclusively by nitrogen-fixing Bacteria and Archaea. The *nifH* gene, one of the best-studied functional genes, has been mostly used as a marker for the nitrogen fixation pathway. However, this gene could be subjected to horizontal gene transfer, being often carried by plasmids. To analyze our complex *nifH* pyrosequencing data, we benefit from the novel FrameBot pipeline of RDP. This helpful tool provides of a reference set of many functional genes, including *nifH*. At the time of our analysis (December 2013) the *nifH* dataset encompassed 33449 sequences, being very useful in determining chimeras and ambiguous sequence identification. Moreover, our choice to delete sequences shorter than 200 bp, although eliminating a high percentage of short *nifH* sequences, assured the best identification possible even of those *nifH* genes affiliated to uncharacterized bacteria.

After sequence cleanup, the total number of reads of the *nifH* gene was 32,954: the OTUs number was 12,308 and 5,581 at 3% and 5% of aminoacidic phylogenetic distance respectively. Chao index varied from 50 to 5628 at 3% and from 35 to 1961 at 5%, Shannon index from 3.27 and 7.47 at 3% whereas at 5% from 2.62 to 6.5, while evenness from 0.885 to 0.961 and from 0.823 to 0.939 at 5%. While no significant differences of diversity indices were observed between the two transects or between the two growing seasons, we noticed that the diversity index values of each plot remained constant in time.

In a recent work, Wang et al. (2013) investigated four sites quite different in soil and climatic conditions, to study the nitrogen fixation-capable bacterial communities using high-throughput sequencing analysis of the *nifH* functional gene. Different nitrogen-fixing microbial communities were found per each site characterized by over 90% of reads most similar to proteobacterial reference sequences and high variations in the percentages of best matches to the individual reference sequences. Even if the samples were collected in an unique environment characterized by quite similar soil and climatic conditions, they were significantly (p<0.005) well separated according to soil age and not according to the sampling season by NMDS analysis on OTUs at 5% of phylogenetic diversity except for samples 201 and 301 collected in October which clustered together with samples of T3 and T2 respectively (Fig. 11). Samples from T2 were influenced by N content, pH values, and mineral contents. In particular, samples DO204, DJ204, DJ206 clustered together close to the left side of coordinate one significantly related to active population fraction affiliated to Rhizobiales. Among T3, samples collected in October were strongly related to moisture and C/N ratio whereas almost all the samples collected in July were related to TOC, Humus, P, Cd and Hg. The first 16 most important OTUs in the SIMPER analysis of the NMDS clusters explained 11.3% of the entire *nifH* pyrosequencing results. The crucial role of Rhizobiales was confirmed since 7 out the 16 *nifH* OTUs belonged to Rhizobiales, whereas 4/16 were affiliated to Cyanobacteria, 4/16 to unspecified uncultured bacteria and 1/16 to Opitutaceae. Figure 12 showed the presence of these 16 OTU in our samples. The relative presence of the OTUs was greatly different between all the plots except between samples DJ304, DJ305 and DJ306 or between samples DO303 and DO304 which could be occasionally quite similar. The majority of sequences were identified as α-Proteobacteria. The primers used in this work were originally designed to amplify

nifH sequences from Proteobacteria, Firmicutes, and Actinobacteria, but a significant number of Cyanobacteria and Verrucomicobia sequences were detected as well.
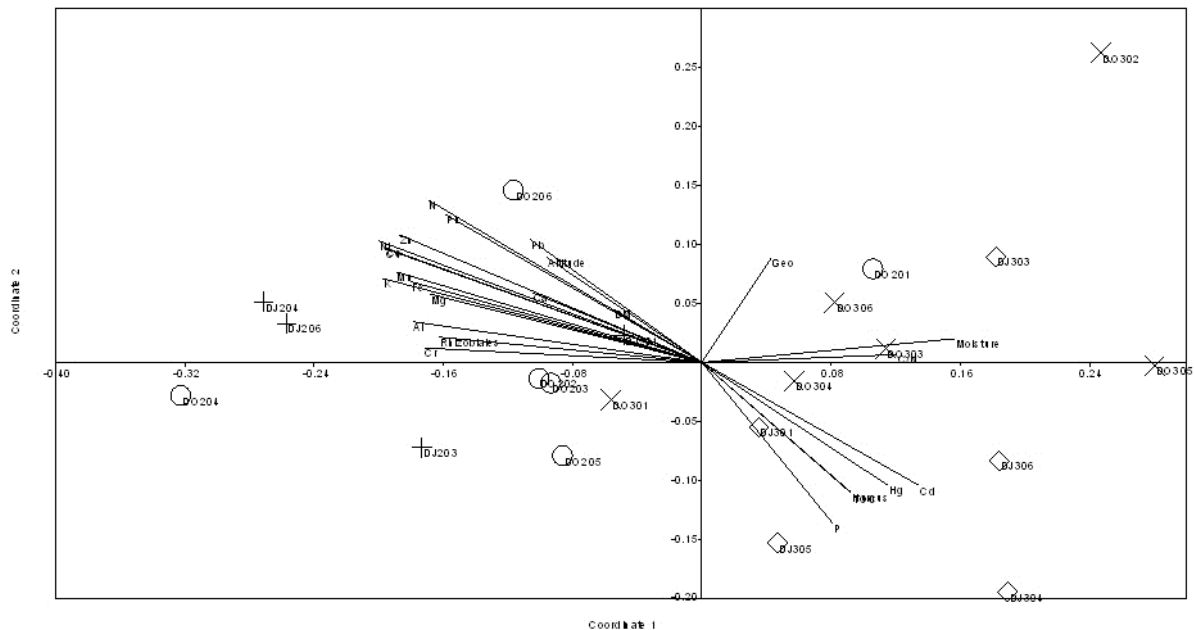

Figure 11 - Non-Metric Multimensional Scaling plot of the OTUs pattern of *nifH* gene-DNA pyrosequenced samples of both the sampling seasons and transects. Cross: T2 July; diamond: T3 July; circle: T2 October; X: T3 October.

The largest number of reads were about 75% amino acid identity (AAI) to the *nifH* gene of *Bradyrhizobium*. Rhizobiales and unclassified bacteria were widely distributed in all the samples. Actually, there are no evidence that the reads affiliated to Rhizobiales come from plant symbionts. Despite the lack of presence of Fabaceae in our plots, the high presence of *Bradyrhizobium* and of other bacteria belonging to *Rhizobia*, as well as the high incidence of *nifH* genes associated to this taxon suggest that rhizobia in Matsch valley soils are mostly free-living and maybe specialized in different ecological relationships with the other organisms. Moreover, the possibility of *nifH* to be horizontally transferred between several taxa open the chance that this gene can be hosted by other taxa rather than Rhizobiales. Beyond the Proteobacteria, the phylum Cyanobacteria was represented in almost all the samples of transect 2 collected in October. This results is quite difficult to understand, because the light exposure of all the samples was uniform and lichen cover, which could be often associated to Cyanobacteria, was uniformly distributed. Our Cyanobacteria-like *nifH* was mainly affiliated to the genus *Nostoc*, unfortunately not found in our 16S rRNA gene pyrosequencing libraries. Maybe this result represent a pyrosequencing amplification bias, or maybe it enlightens another contribution of the gene transfer. Finally, additional *nifH* genes were mainly related to members of the phylum Verrucomicrobia (mostly detected in samples of transect 3, collected both in July and in October, and in sample DO201), but also of uncharacterized bacterial representatives. Approximately 25% of the sequences were similar to environmental sequences that could not be classified to any known taxon, and may therefore represent novel sequences. An exploratory attempt to better identify them by aligning to known *nifH*-
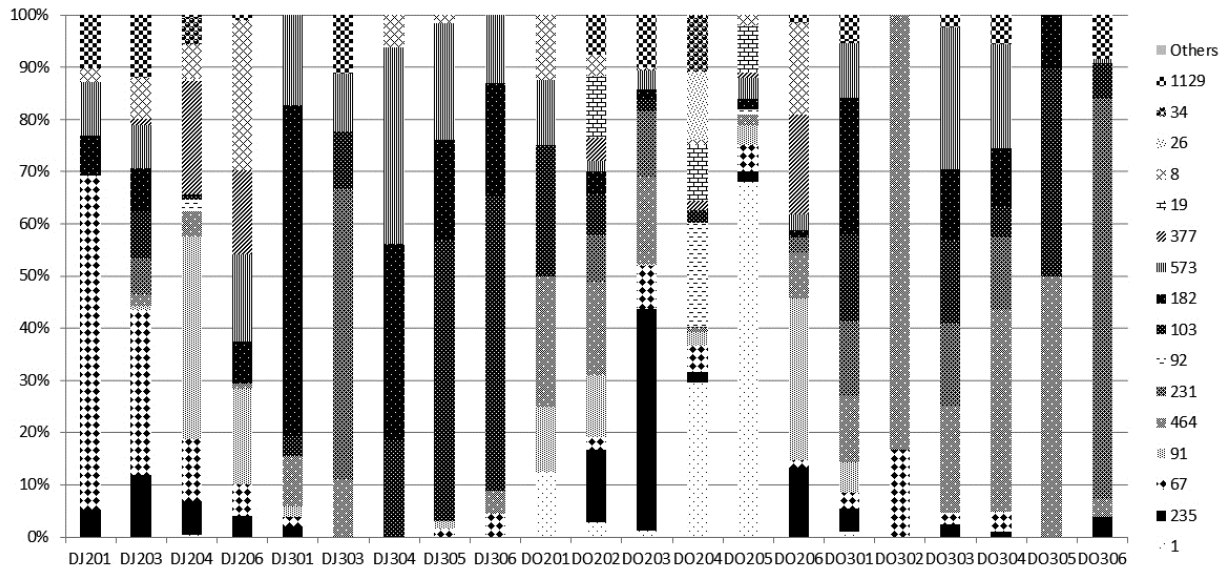
Figure 12 - Barchart of the 16 most representative *nifH* gene OTUs as resulted after SIMPER analysis. OTU sequences were identified by BLASTP analysis and the closest relative accession number and its similarity percentages was recorded. Phylogenetic affiliation was evaluated by checking the first 20 closest matches. OTUs identification legend is as follows: OTU1: *Nostoc*-like *nifH* YP_001869139.1 98%; OTU235: *Bradyrhizobium*-like *nifH* ABW87068.1 94%; OTU67: *Bradyrhizobium*-like *nifH* BAC07281.1 96%; OTU91: Uncharacterized bacterium *nifH* ABG80873.1 99%; OTU464: *Bradyrhizobium*-like *nifH* ACT67985.1 95%; OTU231: *Mesorhizobium*-like *nifH* CAR57832.1 93%; OTU92: *Nostoc*-like *nifH* AGG40740.1 97%; OTU103: Opitutaceae bacterium *nifH* WP_009512762.1 84%; OTU182: Uncharacterized bacterium *nifH* ABG80678.1 98%; OTU573: Uncharacterized bacterium *nifH* ABY25321.1 96%; OTU377: Uncharacterized bacterium *nifH* AGI96605.1 94%; OTU19: *Cyanobacterium*-like *nifH* ABG80759.1 97%; OTU8: *Bradyrhizobium*-like *nifH* AAO48649.1 97%; OTU26: *Nostoc*-like *nifH* AGG40740.1 98%; OTU34: *Rhizobium*-like *nifH* ADI61730.1 93%; OTU1129: *Mesorhizobium*-like *nifH* CAR57832.1 91%.

carrying genera such as *Frankia*, *Azospirillum*, *Azoarcus*, *Afipia* or *Burkholderia* failed. On the other side, our library showed a high incidence of uncharacterized *nifH*-OTUs, genetically far from the known types. This leads to the conclusion that in such severe environment N-fixing genera are maybe much more as supposed, and perhaps unknown representative of the dominant soil phylum Acidobacteria that obtained horizontally *nifH* genes are yet to be discovered (Wang et al., 2007). The hypothesis of an existence of a "bacterial seed bank" that can help bacterial communities to efficiently answer to rapid environmental changes (daily common in such kind of environments) can be an interesting explanation of our data (Wang et al., 2013), opening important perspectives in the microbial ecology of high mountain habitats, and in considering such kind of extreme environments a novel hot spot of genetic biodiversity.

Acknowledgments

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PUBLICATIONS

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- Esposito A., <u>Ciccazzo S.</u>, Borruso L., Zerbe S., Daffonchio D., Brusetti L. 2013. A three scale analysis of bacterial communities involved in rocks colonization and soil formation in high mountain environments. **Current Microbiology**, 67: 472-479.

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- <u>Ciccazzo S.</u>, Esposito A., Rolli E., Zerbe S., Daffonchio D., Brusetti L. Pioneer plants species select peculiar rhizosphere bacterial communities in a high mountain environment. Submitted to **Microbes and Environments**.
- <u>Ciccazzo S.</u>, Esposito A., Rolli E., Zerbe S., Daffonchio D., Brusetti L. Safe-site effects on rhizosphere bacterial communities in a high-altitude alpine environment. Submitted to **Microbes and Environments**.

Papers in preparation
- <u>Ciccazzo S.</u>, Rolli E., Daffonchio D., Brusetti L. Diversity and role of bacterial communities in soil formation and primary succession in alpine environments.
- Rolli E., <u>Ciccazzo S.</u>, Marasco R., Mapelli F., Scaglia B., Salati S., Schubotz F., Brusetti L., Adani F., Daffonchio D., Borin S. Microdiversity in the earliest stages of ecosystem succession in a high elevation mountain moraine in Himalaya.
- <u>Ciccazzo S.</u>, Borruso L., Esposito A., Rolli E., Marasco R., Pujic P., Daffonchio D., Fernandez M., Normand P., Brusetti L. Dynamics of active and total rhizobacterial communities involved in nitrogen cycle in a deglaciated alpine environment.

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- *<u>Ciccazzo S.</u>, Sen A., Esposito A., Rolli E., Marasco R., Pujic P., Daffonchio D., Fernandez M., Normand P., Brusetti L. "Biodiversity, structure and role of rhizosphere bacterial communities in a deglaciated alpine environment". 2nd International Conference Microbial Diversity: Environmental Stress and Adaptation. October 24th-26th 2013, Turin (Italy).
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- *Rolli E., F. Mapelli, <u>S. Ciccazzo</u>, L. Brusetti, R. Marasco, B. Scaglia, F. Tambone, F. Adani, S. Borin, D. Daffonchio. "Primary colonization and soil neo-genesis in deglaciating environments at high and low latitudes". 1st International Conference Microbial Diversity: Environmental Stress and Adaptation. June 26th-28th 2011, Milan (Italy).

Posters* and Oral[†] presentation in national conferences.

- *<u>Ciccazzo S.</u>, E. Varolo, D. Daffonchio, S. Zerbe, L. Brusetti. "Rhizobacterial and pioneer plant communities in a 150 years old alpine moraine". XXII Congresso Società Italiana di Ecologia, September 10th-13th 2012, Alessandria (Italy).
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Other publications

- Toscano G, Cavalca L, Letizia Colarieti M, Scelza R, Scotti R, Rao MA, Andreoni V, <u>Ciccazzo S,</u> Greco G. (2013) "Aerobic biodegradation of propylene glycol by soil bacteria". **Biodegradation**, 24: 603-613.

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