

Microb Ecol (2011) 61:438–447
DOI 10.1007/s00248-010-9758-7

SOIL MICROBIOLOGY

Bacterial Communities Involved in Soil Formation and Plant Establishment Triggered by Pyrite Bioweathering on Arctic Moraines

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Received: 16 June 2010 / Accepted: 28 September 2010 / Published online: 16 October 2010
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Abstract In arctic glacier moraines, bioweathering primed by microbial iron oxidizers creates fertility gradients that accelerate soil development and plant establishment. With the aim of investigating the change of bacterial diversity in a pyrite-weathered gradient, we analyzed the composition of the bacterial communities involved in the process by sequencing 16S rRNA gene libraries from different biological soil crusts (BSC). Bacterial communities in three BSC of different morphology, located within 1 m distance downstream a pyritic conglomerate rock, were significantly diverse. The glacier moraine surrounding the weathered site showed wide phylogenetic diversity and high evenness with 15 represented bacterial classes, dominated by Alphaproteo-

bacteria and pioneer Cyanobacteria colonizers. The bioweathered area showed the lowest diversity indexes and only nine bacterial families, largely dominated by *Acidobacteriaceae* and *Acetobacteraceae* typical of acidic environments, in accordance with the low pH of the BSC. In the weathered BSC, iron-oxidizing bacteria were cultivated, with counts decreasing along with the increase of distance from the rock, and nutrient release from the rock was revealed by environmental scanning electron microscopy-energy dispersive X-ray analyses. The vegetated area showed the presence of *Actinomycetales*, *Verrucomicrobiales*, *Gemmatimonadales*, *Burkholderiales*, and *Rhizobiales*, denoting a bacterial community typical of developed soils and indicating that the lithoid substrate of the bare moraine was here subjected to an accelerated colonization, driven by iron-oxidizing activity.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-010-9758-7) contains supplementary material, which is available to authorized users.

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Introduction

Primary succession is defined as the changes over time in biological communities on newly inhabited substrates [1]. Many studies described the process of plant primary succession [2, 3], but few described the mechanisms driving the first stages of the colonization of a barren substrate, before plant establishment. Microbes have a key role in the process of primary succession in terrestrial habitats, with an active role in element cycling, contributing to soil development and fertility, and to plant establishment. Moreover, biological weathering of rock is an important process that maintains a continuous supply of inorganic nutrients for plants in barren environments [4, 5].

Newly exposed glacial moraines are increasing due to global warming and consequent glacier retreat [6]. Moraines constitute ideal sites to study primary succession, where soil

development from the mineral proto-soil released by the glacier can be studied along spatial gradients moving away from the glacier forefront. Hodkinson et al. [3] characterized a chronosequence in the foreland of the Midtre Lovénbreen glacier in the High Arctic region (Svalbard islands, 78°53'N). Along the chronosequence, covering 2,000 years of ecosystem development following glacial regression, plant establishment and soil development were demonstrated to increase for the first 100 years after the retreat. Between 100 and 2,000 years since exposition from ice cover, plant species diversity did not increase, and soil development was similar [3]. A succession in soil bacterial communities, analyzed by T-RFLP of 16S rRNA gene, was demonstrated to occur in the Midtre Lovénbreen glacier, with a significant change in population structure along the chronosequence [7]. Very few works nevertheless analytically described the microbial succession along glacier forefronts. Successions of communities of early colonizers, dominated by Cyanobacteria, followed by typical soil bacterial phylotypes have been described, occurring in a large time interval during glacial regression in several glacier forefronts [8–12].

Recently, Borin et al. [13] characterized a series of sites in the moraine of the Midtre Lovénbreen glacier, estimated to have been released by the glacier less than 30 years ago. In these sites, conglomerate rocks rich in pyrite (FeS_2) micro-nodules showed red weathering biological soil crust (BSC) strips downstream the rocks, with low pH and high content of iron oxides. At the border of the red strip, green BSC strips were observed with a blooming vegetation of mosses and vascular plants that in the surrounding gray moraines are found only in advanced stages of the succession. Geochemical, biochemical, and microbiological characterization of these sites demonstrated that chemolithoautotrophic iron–sulfur oxidation of pyrite contained in the rock triggered early soil formation and promoted primary colonization by typical moraine mosses and vascular plants, such as the species *Bryum* sp., *Ditricum flexicaule*, and *Saxifraga oppositifolia*. The rock pyrite weathering is mediated by the chemolithotrophic *Acidithiobacillus ferrooxidans*, which produces H_2SO_4 and iron oxides due to sulfide and reduced iron oxidation. The generated pH gradient corresponded to fertility gradient, where water retention, cation exchange capacity, and nutrient availability were increased. This constituted a novel and previously unrecognized soil genesis and vegetation development model, where the proximity of the iron/sulfur-based chemolithoautotrophic activity in the red strip and the cyanobacterial photoautotrophic activity in the surrounding grey moraine allowed the establishment of an oasis of life at the border between the two areas, with an enhanced plant colonization [13].

In the present work, we analytically described the diversity and composition of the bacterial communities

inhabiting BSC present in the area, only briefly presented in the previous multidisciplinary manuscript [13]. The aim of this work was to understand the role and dynamics of bacterial communities in the time-independent development of this particular ecosystem.

Materials and Methods

Sampling

Superficial soil crusts were collected from the Midtre-Lovénbreen glacier (Ny Álesund, Svalbard) on sites ML-RS1 and ML-RC1 above a pyritic rock showing a weathering downstream strip with intensely vegetated areas at the border [13] (Supplementary online material, Fig. S1). Samples were collected with a sterile spatula along parallel transects. At site ML-RS1, two transects were studied, A and B, located, respectively, 5 and 23 cm downstream the rock [13].

SEM-EDX and ESEM-EDX Analysis

Shiny thin sections of stone were visualized by a scanning electron microscope (Oxford, mod. STEREOSCAN 360) equipped with LaB6 filament. EDX analyses were performed with a link microprobe (Oxford, EDS) with acceleration tension 20 kv and 25 mm working distance. Air-dried intact BSC samples were analyzed by environmental scanning electron microscopy-energy dispersive X-ray (ESEM-EDX) spectroscopy. Samples were visualized by a scanning electron microscope (FEI Quanta mod. 200, Eindhoven, Netherlands) at an acceleration voltage of 25 kV to determine elements in soil particle surface (triplicate) at 1.0 and 0.1 bar after 100 s scanning. Mean concentrations and standard deviations of each element were calculated from three random determinations with an approximate error of 1%. The X-ray beam was 4 mm wide and penetrated to a depth of 2 μm .

Iron-Oxidizing Bacteria Counts

Most probable number-based counts of autotrophic iron-oxidizing bacteria were performed on BSCs from site ML-RC1 that remained undisturbed after extensive multidisciplinary sampling of site ML-RS1. Soil crusts were aseptically collected, stored at 4°C during transporting to the laboratory, and inoculated (1% w/v) in decimal serial dilutions in modified 9 K medium [14] supplemented with reduced iron sulfate in 3 ml microtiter plates. After 4 weeks incubation at 15°C, growth was positively scored when a rusty precipitate was observed in the wells, and bacterial cells were visible by microscope observation.

16S rRNA Gene Libraries Construction

Samples RedA and RedB from the weathered area, transects A and B, respectively, GreenA and GreenB from the same transects in the vegetated area, and GreyA and GreyB from the same transects in the external moraine area were chosen for 16S rRNA clone library screening, after an initial screening of a larger set of samples by ARISA fingerprinting [13].

The almost complete bacterial 16S rRNA genes were amplified from the total DNA extracted from BSC samples as previously described [13]. PCR products were ligated into TOPO-TA cloning vector (Invitrogen s.r.l. Milan, Italy) and transfected into *Escherichia coli* JM 109 cells according to the manufacturer's instructions. One hundred white colonies containing recombinant plasmids were randomly picked from each library and grown in Luria-Bertani (LB) plates containing 100 µg/ml of ampicillin, 40 µg/ml of X-gal, and 0.5 mM of IPTG at 37°C overnight. All the recombinant colonies have been inoculated in LB broth containing 100 µg/ml of ampicillin and 15% v/v of glycerol and incubated at 37°C overnight. Inserts were amplified using 2 µl of cell culture as template and M13F (-20)/M13R primers in the following PCR reaction: initial denaturation at 94°C×5 min, followed by 35 cycles at 94°C×1 min, 55°C×1 min, 72°C×1 min 30 s, and by a final extension step at 72°C for 10 min. Ninety of the clones containing the expected amplicon of 1.6 Kb have been partially sequenced (PRIMM, Italy). Sequences were edited in Chromas lite 2.01 (<http://www.technelysium.com.au>) and subjected to BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences 800–850 bp long retrieved from the clone libraries have been deposited in GeneBank under accession numbers AM 940440 to AM 940936.

Phylogenetic Analyses

The CHIMERA_CHECK program available from the Ribosomal Database Project (<http://rdp8.cme.msu.edu/cgis/news.cgi>) was used to exclude the presence of chimeric sequences. 16S rRNA gene sequences were aligned using the ClustalX software [15], and the output file was used to define operational taxonomic units (OTUs) and lineage per time plots using DOTUR [16]. A quantitative matrix was created basing on the absence/presence of each polymorphic OTU calculated at 95% nucleotide similarity, and detrended principal coordinate analysis has been performed with the MVSP software (Kovach Computing Services, UK). Data scored were detrended, and no species weighting

was applied. The number of axes to be extracted was calculated by Gittma–Kaiser's familiar eigenvalue rule [17]. Principal component analysis was performed on the same matrix with the NTSYS pc version 2.01 software (Applied Biostatistics Inc.).

Diversity Analyses

Number of taxa, Shannon–Weaver, and Evenness indexes of the OTUs, defined at 95% of similarity, have been calculated using the PAST software [18]. Library coverage was calculated for each library using the equation $C=[1-(n_1/N)]\times 100$, where n_1 is the number of singleton OTUs, and N is the total number of clones in the library. To compare the six 16S rRNA gene libraries, the β -Libshuff analysis has been applied using the software available at <http://whitman.myweb.uga.edu/libshuff.html> [19].

Results

16S rRNA Gene Libraries and Diversity Indexes

Six libraries of bacterial 16S rRNA gene of 90 clones each have been screened, obtained from BSCs of (1) the weathered area above the pyritic stone (libraries RedA and RedB), (2) the surrounding moraine (libraries GreyA and GreyB), and (3) the vegetated area at the border between them (libraries GreenA and GreenB). The libraries have been compared by a statistical shuffling approach. β -Libshuff analysis [19] demonstrated that the three areas are colonized by significantly diverse bacterial communities. β -Libshuff applied on replicated samples collected in each area at a distance of less than 20 cm (transects A and B) showed significant inter-sample variability in the vegetated area and in the surrounding moraine. On the contrary, libraries from the weathered area below the stone were not significantly diverse ($p<0.05$).

OTUs were defined by the alignment of all the sequences at genus level, basing on 95% nucleotide similarity (OTU₉₅). The weathered area demonstrated an average of 25 OTU₉₅, while in the vegetated area and the surrounding moraines, biodiversity was higher with, respectively, 54 and 58 OTU₉₅. The diversity indexes based on the relative abundance of each OTU₉₅ in the libraries were calculated and reported in Table 1. RedA and RedB libraries showed the lowest values of both Shannon index and Evenness, whereas the vegetated area demonstrated diversity indexes in the same range of the surrounding moraines. Detrended correspondence analyses (DCA) of a matrix constituted by OTU₉₅ relative abundance reported that the bacterial community colonizing the BSC in the weathered area was strongly peculiar, while the vegetated

Table 1 Diversity indexes and coverage calculated for the six 16S rRNA clone libraries

	RedA	RedB	GreenA	GreenB	GreyA	GreyB
Taxa ₉₅	29	20	64	44	67	48
Shannon index ₉₅	2.878	2.429	4.020	3.400	4.032	3.564
Evenness ₉₅	0.613	0.567	0.871	0.681	0.841	0.735
% Coverage ₉₅	83	91	46	69	39	64
% Coverage ₉₇	80	88	38	67	32	60
% Coverage ₉₉	76	83	29	64	24	49

Sequences have been grouped in OTUs based on nucleotide similarity. Subscripts report the percentage of similarity considered for OTU definition

BSC and the surrounding moraine were similar, differentiated only on axis 1 of DCA plot (Fig. 1). The results were also confirmed by a different ordination method (principal component analysis, data not shown).

Lineage per time plots (Fig. 2) were in accordance with diversity indexes. Plots of the weathered area demonstrated a steep initial slope, similar to what was featured in young undeveloped moraine soils [9], indicating that this site is colonized by many individual phylotypes very closely related. In the vegetated area and the surrounding moraine, lineage per time plots were less steep, indicating that the bacterial communities in these sites had greater diversity at higher phylogenetic ranks. This was confirmed by coverage value calculation (Table 1), which reported that 90 clones sized libraries were enough to explore in the weathered area up to 91% of the total bacterial diversity at genus level (OTU₉₅) and up to 88% at species level (OTU₉₇), decreasing to 83% at subspecies level (OTU₉₉). On the contrary, the high diversity of the vegetated area and the surrounding moraines was not sufficiently described by this clone size libraries, even at genus level (maximum 69% coverage for OTU₉₅).

Phylogenetic Analyses

Detailed composition of the bacterial communities inhabiting BSC on the weathered area, the vegetated area, and the surrounding moraine has been determined by identifying the phylogenetic position of the 16S rRNA gene sequences

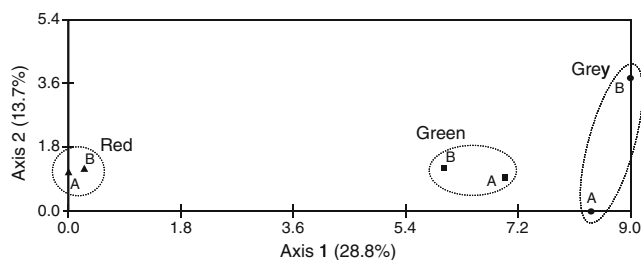


Figure 1 Detrended correspondence analysis applied on the quantitative matrix reporting OTU₉₅ relative distribution in the 16S rRNA gene libraries. *Triangles* red A, B libraries, *squares* green A, B libraries, *circles* grey A, B libraries

from the six clone libraries. Table 2 reports the percentages of sequence affiliation. Ten major bacterial phyla and two plastid lineages have been identified. Between 2% and 9% of the clones could only be classified as unknown bacteria, since they did not exhibit significant homology with any of the sequences deposited in the Ribosomal Database Project. The weathered area was colonized by an average of nine different families, while the vegetated area exhibited a significantly higher number, with an average of 26 bacterial families, similar to the surrounding moraines, containing an average of 24 families.

Phototrophs were significantly more abundant in the surrounding moraine rather than in the area affected by the weathered stone. Unidentified Cyanobacteria accounted up to 39% of the clones, while the green non-sulfur Chloroflexi reached 8% of the clones in GreenA library. On the contrary, Cyanobacteria in RedA and RedB were less than or equal to 4%, and the libraries were dominated by Acidobacteria mainly belonging to the *Acidobacteriaceae* family (up to 38% of the clones) and Alphaproteobacteria of the family *Acetobacteraceae* (up to 33% of the clones). Between 17% and 30% of the clones belonging to the latter family were in particular affiliated to the genus *Acidiphilium*.

The vegetated area demonstrated the presence of a significant fraction of Cyanobacteria (10% and 17% in GreenA and GreenB, respectively). Other abundant groups

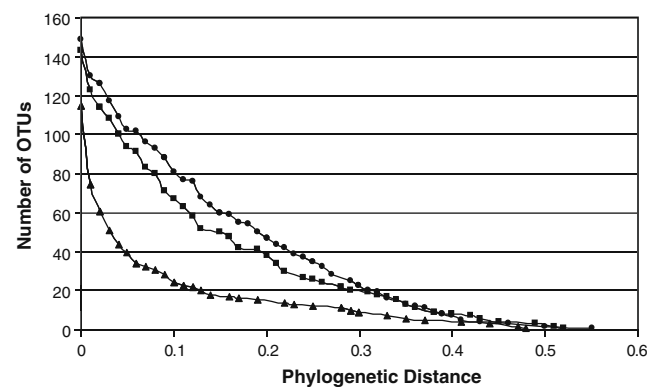


Figure 2 Lineage per time plots, calculated for each cumulative library containing the sequences obtained from the samples of each area. *Triangle* bioweathered area (RedA+RedB), *square* vegetated area (GreenA+GreenB), *circles* external moraine (GreyA+GreyB)

Table 2 Phylogenetic identification of the clones from 16S rRNA libraries

Phylum and/or class	Sites						Order and/or family and/or genus	Sites											
	Red		Green		Grey			Red		Green		Grey							
	A	B	A	B	A	B		A	B	A	B	A	B						
Bacteria unk	4	2	7	7	8	9													
Acidobacteria	38	37	4	3	6	2	<i>Acidobacteriaceae</i> unk	1	1			2							
							<i>Acidobacteriaceae, Acidobacterium</i>	19	23										
							<i>Acidobacteriaceae, Gp 1 family</i>	19	12										
							<i>Acidobacteriaceae, Gp 3 family</i>												1
							<i>Acidobacteriaceae, Gp 4 family</i>			2	1	1	1						1
							<i>Acidobacteriaceae, Gp 6 family</i>			1	2								
							<i>Acidobacteriaceae, Gp 7 family</i>												1
							<i>Acidobacteriaceae, Gp 10 family</i>												1
Bacteroidetes, Sphingobacteria			19	2	19	6	<i>Sphingobacteriales</i> unk			11		1	2						
							<i>Sphingobacteriales, Flexibacteraceae</i>			4	1	7							
							<i>Sphingobacteriales, Crenotrichaceae</i>			3	1	8	3						
							<i>Sphingobacteriales, Saprospiraceae</i>					2							
							<i>Sphingobacteriales, Sphingomonadaceae</i>					1							
Cyanobacteria	4	1	10	17	28	39	Cyanobacteria unk	4	1	10	17	28	39						
α Proteobacteria	30	34	18	24	13	24	α Proteobacteria unk			4	2	3							
							<i>Rhodospirillales, Acetobacteraceae</i>			16			3						
							<i>Rhodospirillales, Acetobacteraceae, Acidiphilium</i>	30	17										
							<i>Rhizobiales</i> unk			1	6	2	1	3					
							<i>Rhizobiales, Rhizobiaceae</i>				1		1						
							<i>Rhizobiales, Bradyrhizobiaceae</i>					3							
							<i>Rhizobiales, Hypnomicrobiaceae</i>					7	1	2					
							<i>Rhizobiales, Methylocystaceae</i>			1				1					
							<i>Sphingomonadales, Sphingomonadaceae</i>				4	4	6	12					
							<i>Rhodobacterales</i>			1	1	1	2						
							<i>Caulobacterales, Caulobacteraceae</i> unk					1							
							<i>Caulobacterales, Caulobacteraceae, Brevundimonas</i>				1	2							
							<i>Caulobacterales, Caulobacteraceae, Phenylobacterium</i>					1							
β Proteobacteria	4	1	14	23	4	8	β Proteobacteria unk	4	1	2	3	2	1						
							<i>Burkholderiales</i> unk				1	3	2						
							<i>Burkholderiales, Comamonadaceae</i>				4	6	1						
							<i>Burkholderiales, Incertae sedis 5</i>				6	10	2						
							<i>Burkholderiales, Oxalobacteraceae</i>					1							
							<i>Rhodocyclales, Rhodocyclaceae</i>												1
							<i>Methylophilales</i>				1								
							<i>Alcaligenaceae, Derxia</i>												2
γ Proteobacteria	1	6	6		3		γ Proteobacteria unk				3	1							
							<i>Xanthomonadales, Xanthomonadaceae</i>	1	6			1							
							<i>Pseudomonadales, Pseudomonadaceae, Pseudomonas</i>				2	1							
δ Proteobacteria			3	4	4	2	δ Proteobacteria unk				3	1	2						
							<i>Mixococcales</i> unk					1							
							<i>Mixococcales, Polyangiaceae</i>					3	1						
							<i>Mixococcales, Cystobacteraceae</i>						2						

Table 2 (continued)

Phylum and/or class	Sites						Order and/or family and/or genus	Sites									
	Red		Green		Grey			Red		Green		Grey					
	A	B	A	B	A	B		A	B	A	B	A	B				
Actinobacteria	7	6	6	4	3		Actinobacteria unk	6	1								
							<i>Acidimicrobiales, Acidimicrobiaceae, Acidimicrobium</i>	1									
							<i>Actinomycetales unk</i>		3	1							
							<i>Actinomycetales, Micrococcinaea</i>			1							
							<i>Actinomycetales, Microbacteriaceae</i>			1							
							<i>Actinomycetales, Actinosynnemantaceae</i>			1							
							<i>Actinomycetales, Intrasporangiaceae</i>			1	1						
							<i>Actinomycetales, Nakamurellaceae</i>		1								
							<i>Actinomycetales, Nocardiodaceae</i>				1						1
							<i>Actinomycetales, Frankineae</i>				2						
							<i>Rubrobacterales</i>										2
Planctomycetacia	1		1		1		<i>Planctomycetales, Planctomycetaceae</i>	1		1							1
Chloroflexi			1		8	1	<i>Chloroflexaceae unk</i>										2
							<i>Chloroflexales, Chloroflexaceae, Roseiflexus</i>			1							3
							<i>Herpetosiphonales, Herpetosiphonaceae, Herpetosiphon</i>										1
							<i>Caldineales, Caldilineacea, Caldilinea</i>										1
Gemmatimonadetes						4	<i>Gemmatimonadaceae, Gemmatimonas</i>										4
Clostridia			1				<i>Clostridiales, Clostridiaceae, Clostridium</i>			1							
Verrucomicrobiae			3	3	2		<i>Verrucomicrobiales, Xiphinematobacteriaceae</i>			2							2
							<i>Verrucomicrobiales, Opiritaceae</i>			1	3						
Streptophyta unk	6	9	7	11	2												
Chlorophyta unk	4	4				1											

unk unknown classification

in this area were alpha- and beta-subgroups of Proteobacteria (18–24% and 14–23% of the clones, respectively). In the vegetated area, pH is around neutrality (7.1 ± 0.3) [13], *Acetobacteraceae* are absent, and Alphaproteobacteria are represented by different families like *Rhizobiales*, *Sphingomonadales*, and *Caulobacterales*. Betaproteobacteria are significantly higher in the vegetated BSC than in the adjacent ones, mainly represented by *Burkholderiales*. Sequences belonging to the *Actinomycetales* and Verrucomicrobiae taxonomic groups were quite exclusively found in the vegetated area (4–6% and 3% of the clones, respectively).

Nutrient Weathering and Iron-Oxidizing Bacteria Counts

The area affected by pyritic stone weathering demonstrated very low pH, up to 3.5, and high reduced Fe^{2+} content, up to $60.8 \text{ mg kg}^{-1} \text{ dm}$ [13]. The weathered rock was a conglomerate, embodying microcrystals of pyrite (Fig. 3a) and apatite (Fig. 3b) containing the essential mineral nutrients Fe, S, and P, as revealed by SEM-EDX backscat-

tering analyses. The release of these nutrients was demonstrated by the higher relative abundance of Fe, S, and P elements on the surface of BSC collected below the stone, with respect to the surrounding areas, as measured by ESEM microscopic analysis (Fig. 4c).

Iron-oxidizing bacteria were counted by most probable number in BSC downstream a second more extended pyritic rock, site BC-RC1 [13], along the bioweathered strip from 0.5 to 10 m distance from the rock. Iron oxidizers demonstrated a relatively high count below the rock ($4.3 \pm 3.7 \log_{10}$), progressively decreasing to $0.5 \pm 0.1 \log_{10}$ along with the increase of the distance from the stone (Fig. 4).

Discussion

The β -Libshuff analysis has been applied to compare the composition of bacterial communities colonizing the weathered area, the vegetated area, and the barren moraine. The results of β -Libshuff analysis indicated that different

Figure 3 Mineralogical analysis of rock and BSC surface. **a, b** SEM-EDX back scattering electron micrographs of thin-layer shiny sections of the rock. EDX analyses were performed in areas indicated by the *arrows*. **c** ESEM analysis of BSC collected on the horizontal transect A, 5 cm below the rock. Percentage of Fe, S, and P elements are reported for samples collected in the *red* weathered area in proximity of the rock, the *green* vegetated areas at the border, and the *grey* external moraine. The samples that were characterized also by 16S rRNA clone libraries are indicated

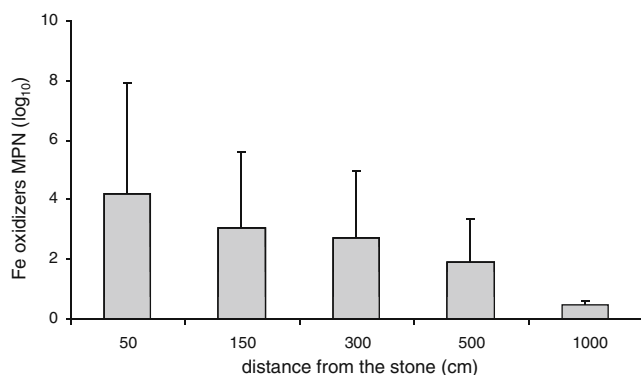
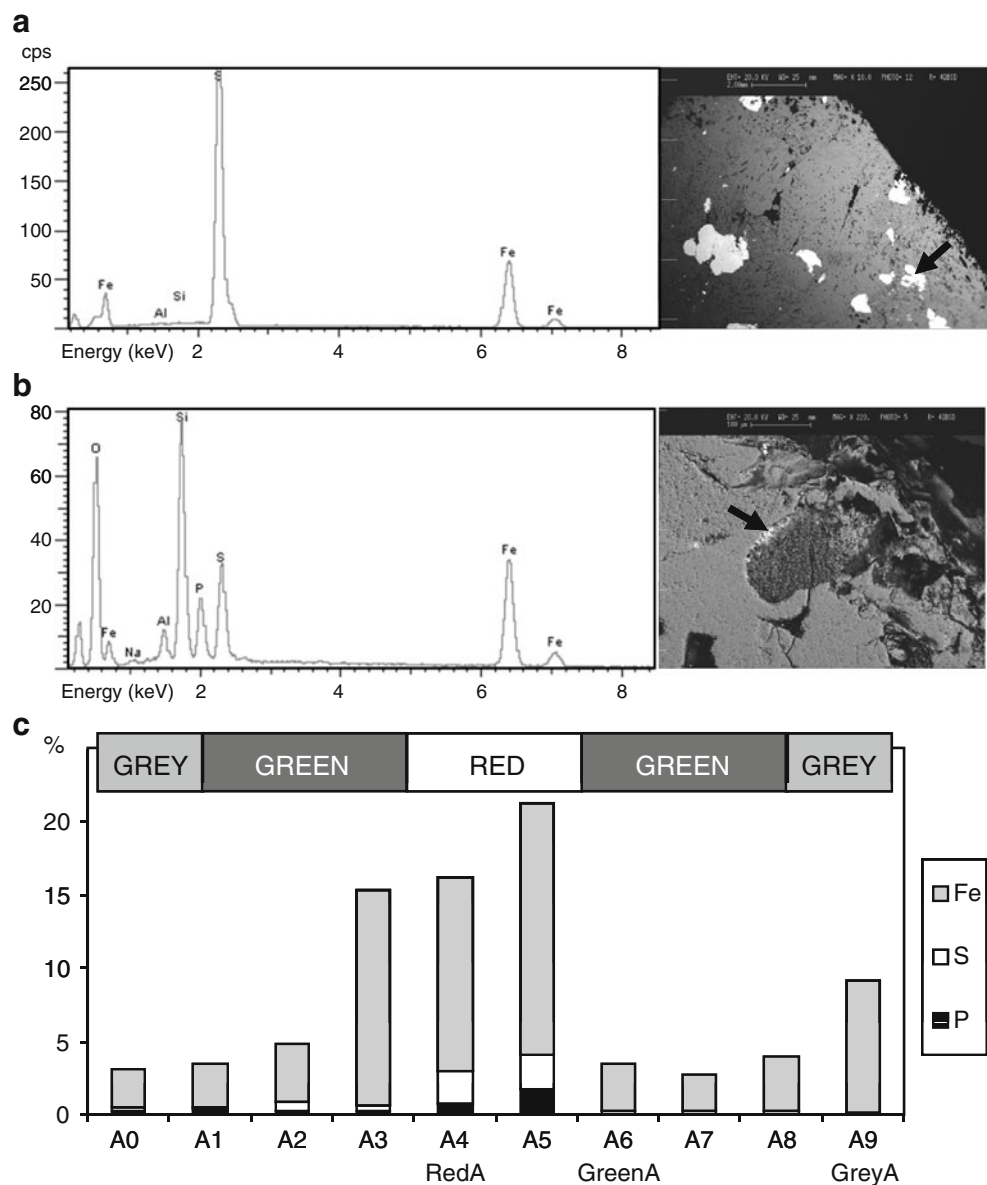


Figure 4 MPN counts of iron-oxidizing bacteria in the bioweathered area downstream the pyritic rock in the ML-RC1 site [13]

environmental factors, triggered by the presence of the weathered stone, created different ecological niches, which selected a peculiar microbiota composed by bacteria belonging to few dominant taxa. The comparison of the replicated samples collected in each area showed that among the vegetated area and the surrounding moraine, the bacterial colonization of the BSC was highly heterogeneous. Only the samples collected from the weathered area were not significantly different, suggesting that in this niche, strong selecting forces induced the flourishing of specific adapted populations. Acidic pH, as a consequence of pyrite oxidation, is the environmental parameter that mainly characterizes this area, reaching a value of 3.5 ± 0.2 below the stone [13]. This result is in accordance with previous findings that reported a strong correlation between soil pH and bacterial diversity [20]. The presence of strong

selecting forces in weathered BSC is evident also from DCA analyses of OTU₉₅ distribution, where both the libraries resulted well separated from the others (Fig. 1). The vegetated area and the surrounding moraine were more similar, being differentiated only on axis 1 of the DCA plot (Fig. 1). Young deglaciated moraines were reported to host unsuspected high bacterial diversity, probably due to a loss of competition in poorly developed environments [11], where fungal mycelia and plant roots are hampered by nutrient limitations and mechanical constraints like freeze and thaw cycles [21, 22]. On the contrary, in developed soils, high diversity in substrate sources and interactions with plants and microfauna lead to high bacterial diversity indexes [23, 24]. Despite similar values of bacterial biodiversity, the vegetated BSC was colonized by peculiar phylogenetic groups, differentiated from the surrounding moraine (Fig. 1 and Table 2).

The phylogenetic composition of the microbiota that colonized the BSC of the three different areas has been described in detail by the screening of bacterial 16S rRNA clone libraries. While in the weathered area the low biodiversity permitted a high percentage of library coverage, in the vegetated area and in the barren moraine, only a fraction of the overall diversity was described (Table 1). The interpretation of differences among bacterial communities in the different sites could in principle be affected by the not exhaustive sampling of their phylogenetic diversity. Some correlations between community phylogenetic composition and environmental factors affecting the sites can be nevertheless found.

The abundance of bacterial families retrieved both in the vegetated area and in the barren moraine was almost three times higher than in the weathered area, confirming the occurrence of strong selective environmental conditions that limit the ability of many bacteria to colonize this habitat.

Between 92% and 100% of the 540 clones retrieved in all the libraries could be grouped within Gram-negative (G^-) bacteria. G^- -dominated communities have been interpreted as initial stages of ecosystem development [12], since many chemolithotrophic bacteria are G^- . Tschérko et al. [12], basing on PLFA profiles, detected an increase in G^+/G^- ratio in the rhizosphere of *Poa alpina* during ecosystem development along a deglaciation chronosequence. The extremely low G^+/G^- ratio retrieved in the libraries would suggest that chemolithotrophy is an important energy metabolism in all the three areas of the site, estimated to be released from ice cover only 27 years ago [13]. The high percentage of Cyanobacteria and Chloroflexi detected in the barren moraine libraries is consistent with previous findings that reported photoautotrophic bacteria, and particularly Cyanobacteria, as the pioneer organisms in BSC of developing terrestrial ecosystems [25]. They are

typical of arctic soils and were detected with high prevalence along all the chronosequence in the Midtre Lovénbreen moraines up to 2,000 years of soil development out of ice cover [3]. Cyanobacteria can exploit sunlight to fix atmospheric carbon and nitrogen, having a key role during the first colonization stages by providing nutrients to the heterotrophic populations, increasing soil pH due to photosynthesis and increasing soil properties like water retention, essential for plant establishment.

Above the weathered stone nevertheless, a second autotrophic metabolism has been identified, mediated by *A. ferrooxidans*, having a key role in establishing acidity with consequent fertility gradients and plant flourishing [13]. Iron–sulfur oxidizing bacteria were counted in the BSC of ML-RC1 weathered site, and their number decreased along with the increase of the distance from the stone, demonstrating their involvement in rock bioweathering (Fig. 4). Moreover, pyrite and apatite were retrieved in the weathered stone, source of mineral nutrients like P, Fe, and S that were demonstrated to occur in higher abundance in the BSC downstream the rock, contributing to the creation of fertility gradients (Fig. 3). Rock weathering occurred by the release in the soil above the stone of Fe and S from pyrite crystals, as the result of iron–sulfur oxidation, and P from apatite crystals, due to the strong acidification occurring from pyrite bioleaching.

The phylogenetic taxa that mainly colonize the weathered area belong to Acidobacteria and Alphaproteobacteria of the family *Acetobacteraceae*. These groups are typically found in acidic environments [26, 27], consistently with the low pH of the area affected by *A. ferrooxidans* iron–sulfur oxidation. In the weathered area, 17–30% of total clones belong to the genus *Acidiphilium*, hypothesized to be in a commensal association with *A. ferrooxidans*, acting as scavenger of the pyruvate excreted by the latter species [28].

Despite the cultivation of iron-oxidizing bacteria, responsible for the chemolithoautotrophic metabolism detected in the weathered area, 16S rRNA clone libraries did not reveal the presence of *A. ferrooxidans* in the weathered area, but this could be expected, considering that iron oxidation is one of the less energy-gaining metabolisms [29]. *A. ferrooxidans* was hence present in the stone weathered area under the detection limit of the method, but despite low abundance had high pyrite oxidation activity as demonstrated by low pH values.

The microbiota inhabiting the vegetated area was primarily represented by taxa normally found in mature soils like *Rhizobiales*, *Sphingomonadales*, and *Caulobacterales* [30]. *Rhizobiales* are of particular interest since they comprise many N fixers that can have, in parallel with Cyanobacteria, a primary role in the N cycle. Moreover, several clones were affiliated to the families *Rhizobiaceae* and *Bradyrhizobia-*

ceae, known to establish symbiotic relations with plants. Among Betaproteobacteria, we found a high prevalence of bacteria belonging to the order *Burkholderiales*. This order, and especially the family *Comamonadaceae*, was retrieved in several glacial environments like ice cores [31], permanent lake ice [32], and deglaciated soil along moraine chronosequences in the first and medium stages of development [9]. Nemergut et al. [9] hypothesized that *Comamonadaceae* are typical populations of ice environment, that during deglaciation are seeded in the developing soil, decreasing in relative abundance along with soil age. Only in the vegetated area, we detected also the occurrence of *Actinomycetales* and *Verrucomicrobiae*, known as typical soil microbiota [33, 34]. *Verrucomicrobia*, *Bacteroidetes*, and *Acidobacteria* were found in late successional stages of ecosystem development on the foreland of Puca glacier (Peruvian Andes) and were considered proxy of old soil microbiota [9]. Moreover, *Verrucomicrobia* and *Acidobacteria*, known to be among the soil bacterial groups with the lower cultivability levels, can indicate a shift from *r* to *K* growth strategy along with soil age and development and increase of carbon sources complexity, yet described in Puca glacier [9] and in Dammaglacier (Switzerland) [10] forelands. Bacterial succession and development of soil ecosystem along with time after ice retreat have been reported in several glacier forefronts [7, 9, 35].

The results obtained in the present work confirmed and detailed what was previously described by Borin et al. [13], showing that in ecological niches with the same age out of ice covering, at the border between chemolithotrophic- and phototrophic-based autotrophic metabolisms, enhanced ecosystem development took place. The age-independent interaction of these microbial metabolisms increased here the availability of nutrients with the effect of triggering soil development and plant biocoenosis. The synergy between these two bacterial autotrophic processes in favoring microorganism and plant colonization is probably not restricted to the Midtre Lovénbreen glacier foreland but should be explored in other Fe(II)-rich environments. The analytical 16S rRNA gene library survey carried out in the present work demonstrated that the microbial weathering of a pyritic rock favored only few acid-loving bacterial taxa in the soil crust in close proximity of the rock. Less than 40 cm apart, in parallel with the increase in pH, the bacterial phylogenetic biodiversity in the soil crust increased, with the selection of a more complex microbial community, typical of soil and rhizosphere, with a key role in the formation of a plant oasis.

Acknowledgments The authors thank the Earth and Environment Department of CNR for the use of “Dirigibile Italia” Station in Ny-Ålesund, Svalbard, and Ev-K2-CNR committee for supporting the project “Study of primary colonisation and soil neogenesis mechanisms in deglaciating environments at high altitude and low latitude.”

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