


Diversity and Assembling Processes of Bacterial Communities in Cryoconite Holes of a Karakoram Glacier

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Abstract Cryoconite holes are small ponds that form on the surface of glaciers that contain a dark debris, the cryoconite, at the bottom and host active ecological communities. Differences in the structure of bacterial communities have been documented among Arctic and mountain glaciers, and among glaciers in different areas of the world. In this study, we investigated the structure of bacterial communities of cryoconite holes of Baltoro Glacier, a large (62 km in length and 524 km² of surface) glacier of the Karakoram, by high-throughput sequencing of the V5-V6 hypervariable regions of the 16S rRNA gene. We found that Betaproteobacteria dominated bacterial communities, with large abundance of genera *Polaromonas*, probably thanks to its highly versatile metabolism, and *Limnohabitans*, which may have been favoured by the presence of supraglacial lakes in the area where cryoconite holes were sampled. Variation in bacterial communities among different sampling areas of the glacier could be explained by divergent selective processes driven by variation in environmental conditions, particularly pH, which was the only environmental variable that significantly affected the structure of bacterial communities. This variability may be

due to both temporal and spatial patterns of variation in environmental conditions.

Keywords Bacterial communities · Baltoro glacier · Cryoconite · Cryosphere · Dispersal

Introduction

Glaciers and ice sheets represent the largest part of the cryosphere (i.e. those portions of Earth's surface where water is frozen) on the continents and include most of the Earth's freshwater [1, 2]. These environments are an underexploited reservoir of biological functions with high biotechnological potentials [3], and the study of biological processes acting in them gained interest in the recent years owing to increasing evidences that they can strongly affect the energy flows of glaciers and, consequently, the climate [4, 5]. Furthermore, these environments are particularly sensitive to on-going global warming [4].

Cryoconite is a dark wind-borne debris deposited on glacier surface [6]. The presence of a layer of cryoconite, whenever thinner than a (locally variable) critical thickness [7], may lead to the formation of small depressions called cryoconite holes. The sediment, heated by solar radiation, melts the underlying ice, thus forming a depression that can be filled by meltwater [8, 9]. Cryoconite holes range in diameter from a few centimetres to more than a metre, can cover 1 to 10% of the ablation zone of glaciers [1] and, consequently, can have a role in the darkening of the glaciers, thus potentially influencing glacier melting rate [10]. They are considered the most biologically active environments on the glaciers, and thus their study has gained interest among ecologists and microbiologists [11, 12]. Finally, cryoconite holes on mountain glaciers are connected to down-valley ecosystems by water flows

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(superficial and sub-superficial) during the ablation season [6]. The functionality and chemistry of down-valley systems can therefore be influenced by the metabolic activities present in cryoconite holes that can enrich meltwater in organic matter and nutrients [5, 13].

Cryoconite holes on low-latitude mountain glaciers show different features from those on polar glaciers, mainly because glaciers on low-latitude mountains generally flow faster and are subject to larger diurnal temperature fluctuations than polar ones [9]. In addition, contrasts in the microbial ecology of cryoconite holes seem to exist, not only between polar and Alpine glaciers [14], but also among cryoconite holes on different mountain glaciers. Indeed, bacterial communities in the cryoconite of Rotmoos Ferner Glacier (Austrian Alps) were dominated by Proteobacteria [15]. In contrast, Cyanobacteria dominated communities on Forni Glacier (Italian Alps) after snowmelt, while Bacteroidetes became more abundant during the ablation season [16]. Finally, on Ürümqi Glacier No. 1 (Tien Shan Mountains, China), cryoconite bacterial communities were dominated by Betaproteobacteria, Bacteroidetes and Cyanobacteria [13]. Cyanobacteria were also identified as the main constituents of the dark-coloured cryoconite that accelerated the melting rate of Himalayan and Tien Shan Glaciers [17, 18]. Further studies are therefore needed to gain insight into the processes assembling the microbial communities of cryoconite in temperate mountain glaciers, the geographical variation in the structure of bacterial communities living in these environments and the potential impact of surface bacterial communities on glacier mass balance [9, 18].

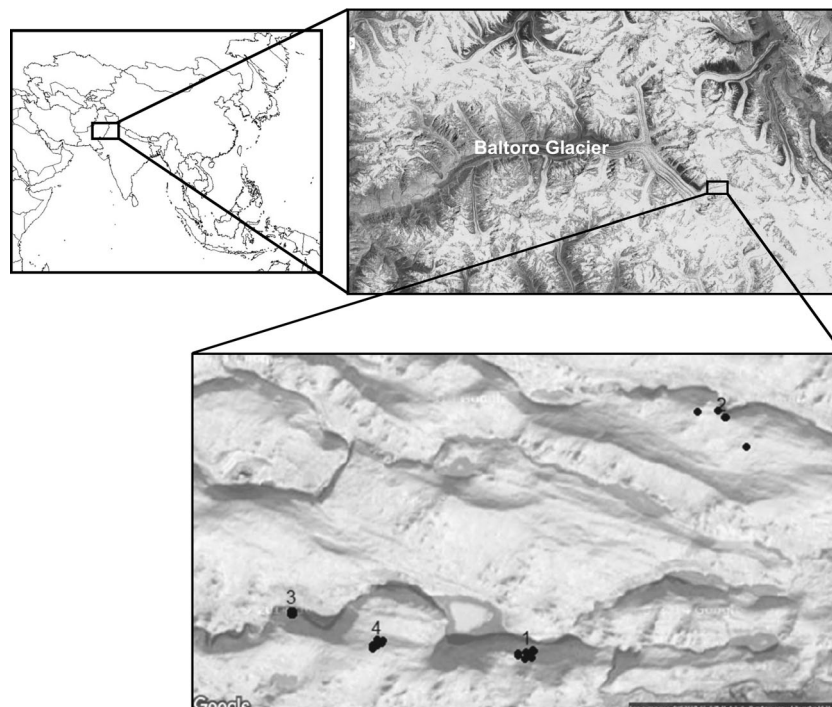
In this study, we aimed at contributing to filling this gap of knowledge by describing the structures of the microbial communities of 30 cryoconite holes sampled on Baltoro Glacier (Karakoram) and by investigating the ecological processes explaining the spatial diversity of these bacterial communities. The Karakoram is one of the major mountain systems in the world, with four peaks rising more than 8000 m a.s.l. Karakoram is also one of the largest glacierized regions outside the Arctic and the Antarctic, and among the very few areas of the world where glaciers showed possible slight mass gain in the last decade [19]. Importantly, to the best of our knowledge, microbial communities of cryoconite holes on Karakoram glaciers have been investigated in one previous paper only [20].

Materials and Methods

Study Area, Field Methods and Environmental Data

Cryoconite holes were sampled on the surface of Baltoro Glacier close to Gasherbrum Base Camp (GBC) (approximate coordinates 35°41'07" N, 76°38'52" E; Fig.1). Baltoro is one of the largest glaciers in the Karakoram Range, with a maximum length of 62 km and an area of 524 km², including all connected tributaries [21]. Baltoro surface in the GBC area is characterized by a high superficial roughness, with undulations of about 10–20 m. Cryoconite holes occurred in flat areas on ridges, while small supraglacial lakes often occurred

Fig.1 Distribution of the 30 cryoconite holes sampled on Baltoro Glacier. Areas were numbered according to the order of samples collection



between ridges (R. Ambrosini and U. Minora, personal observations). Cryoconite holes were therefore naturally spatially clustered, with groups of holes close to one another on flat areas on ridges, well separated from those in other flat areas on the same or on a different ridge.

We sampled cryoconite from holes in four separated areas (Fig. 1). Due to difficulties in moving on the surface of Baltoro, holes in different areas were samples on different days (Fig. 1). Areas 1, 2 and 3 were sampled on 29 and 30 June and 1 July, respectively, while area 4 was sampled on 9 July 2013. This sampling design, forced by logistic constraints, implied that we could not disentangle the effects of spatial disposition of holes with those of short-term (days) temporal variation in the structure of bacterial communities. We, therefore, decided to consider the area as a categorical variable accounting for both spatial location of samples and temporal variation of sampling, and we further analysed spatial variation of microbial community structure within each area, because each area was sampled in the same day or in two consecutive days.

Cryoconite samples were kept at the lowest possible temperature allowed by the logistics constraints. During the permanence at the base camp, samples were kept in a small ice cave carved into the glacier. They were stored at +4 °C during transfer days at Islamabad and then shipped by plane to the final storage at -20 °C in the laboratory. We did not use any chemical preservative for sample storage because the results of a previous study showed that the structure of microbial communities, as described by DNA-based methods, did not change when samples were stored for a rather long time without any chemical preservative even at temperature up to 20 °C higher than their ambient temperature [22]. In contrast, bacterial communities of samples stored with a chemical preservative significantly differed from those analysed after sampling and from those of frozen samples [22].

We also estimated oxygenic phototrophic activity and oxygen respiration in cryoconite holes on the days of sampling using the light and dark bottle technique [23]. We put a 0.1–1-cm deep layer of cryoconite in two 50-mL Falcon™ tubes filled with water from the same cryoconite hole. In preparing the light and dark tubes, we took care to put in each tube an amount of sediment such that the thickness of the sediment was approximately equal to that of the sediment in the hole in order to better simulate in situ conditions [9]. Oxygen content was measured by a portable oximeter/pH meter (HACH LANGE HQ40D, Loveland, CO, USA) immediately after the preparation of the bottle and after 24 h of incubation into the cryoconite hole. Organic matter content of cryoconite was measured with the loss-on-ignition method by heating the samples at 400 °C overnight [24]. We also measured oxygen concentration in the pool, pH and temperature with the same portable oximeter/pH meter as above, while maximum hole depth was recorded with a ruler (precision 1 mm). Debris depth was measured by a different

ruler (precision 0.5 mm). Hole area was also estimated by an automatic method of cryoconite holes delimitation where a picture of the hole with a reference ruler was taken and analysed by the ImageJ software [25] to estimate major and minor hole axes. Hole area was finally calculated as the area of an ellipse with corresponding axes.

16S rRNA Gene Fragment Sequencing, Sequence Processing and Data Analysis

Total bacterial DNA was extracted from samples using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified and sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 250 bp × 2 paired-end protocol. Details on PCR conditions are reported in the Supplementary Information. After the amplification, DNA quality was evaluated spectrophotometrically and DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). The sequencing was carried out at Science for Life Sequencing facility (Stockholm, Sweden).

Reads from sequencing were demultiplexed according to the indices. Uparse pipeline was used for the following elaborations [26]. Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Suspected chimeras and singleton sequences (i.e. sequences appearing only once in the whole data set) were removed. OTUs were defined on the whole data set clustering the sequences at a 97% of similarity and defining a representative sequence for each cluster. A subset of 10,000 random sequences was chosen from each sample, and the abundance of each OTU was estimated by mapping the sequences of each sample against the representative sequence of each OTU at 97% of similarity. Taxonomic classification of the OTU representative sequences was obtained by RDP classifier [27].

Sequence data were submitted to European Nucleotide Archive (ENA), study accession number PRJEB16145 (<http://www.ebi.ac.uk/ena/data/view/PRJEB16145>).

Statistical Methods

Variation of Bacterial Community Structure

The number of sequences of each OTU was used as an estimate of the relative abundance of the OTU in a sample. OTUs found in one sample only (singletons) were removed because they may inflate variance explained by models [28]. Figure S1 shows the relative abundance of OTUs classified at order level.

The analyses of variation in the structure of bacterial communities in cryoconite holes according to ecological variables were based on the relative abundance of OTUs. We used

redundancy analysis (RDA) and variation partitioning [29] to determine the amount of variation in community structure that can be explained by spatial disposition of holes (sampling area) and local ecological conditions at each hole. OTU relative abundances were Hellinger-transformed before the analyses. The framework of analysis we used is exposed in Borcard et al. (pages 257–263) [30]. First, we ran separate RDAs including as predictors: the area of the glacier where the cryoconite holes occurred, which accounted for the temporal and broad-scale spatial variation of bacterial community structures [A] and environmental conditions at each hole [E], namely pH, oxygen concentration, water temperature, amount of organic matter, net productivity, hole depth and hole area. Environmental variables that significantly contributed to explain variation in bacterial community structure were selected from the total set of environmental variables by means of a forward selection procedure using the double stopping criterion [31]. Second, we used variation partitioning to quantify the variation in the bacterial community structures explained separately and jointly by [A] and [E], and partial RDA to assess significance of the pure contribution of each source of variation while controlling for the possible covariation with the other sources. These analyses were performed with the VEGAN and STEPFOR packages in R 3.1.2 [32].

Variation of Alpha-Biodiversity and of the most Abundant Bacterial Taxa

The number of OTUs found in each hole and the Shannon diversity index [33] were used to measure alpha diversity, while the Gini inequality index was used to measure evenness of community composition. The Gini index is a measure of statistical dispersion [34]. It was developed as a measure of inequality of income in economics, while in ecology it is used to measure community evenness. Its values range from 0 to 1, with increasing values indicating lower evenness [35].

We used Generalized Linear Models (GLMs) assuming a Poisson error distribution and corrected for overdispersion or Generalized Least Squares (GLS) models assuming inequality of variance among areas, to investigate variation in the number of OTUs and in the Gini index according to environmental variables.

We also aimed at investigating variation in the abundance of Burkholderiales, Enterobacteriales, Sphingobacteriales, Pseudomonadales, Sphingomonadales and Cyanobacteria (the most abundant bacterial taxa, see “Results” and Fig. S1) according to area and environmental variables. To this end, we ran separate Generalized Linear Mixed Models (GLMMs) for each taxon and variable. OTU identity was entered as a random grouping factor in each model, while coefficients of environmental variables were allowed to vary within OTU (random intercept and slope model [36]). If the model did not converge, we removed the random slope but always included OTU identity as

a random effect (random intercept model [36]). We assumed a Poisson error distribution in all GLMMs and included the total number of sequences (10,000) as an offset. With this parameterization, the GLMMs modelled the variation in the relative number of sequences according to the predictor. Since several models were run, we corrected significance of each predictor by the FDR procedure [37]. When we detected variation in the structure of bacterial communities among areas, we also performed post hoc tests (Tukey method). These analyses were performed with the NLME, LME4, LMERTTEST and MULTTEST in R 3.1.2 [32]. We also checked whether significant differences among areas detected by RDA (see “Results”) arose because of within-area variation in the composition of bacterial communities [38]. To this end, we performed an analysis of homogeneity of multivariate variance of OTU composition (dispersion) among areas [39] by using the function *betadisper* implemented in the VEGAN package [40] of R. This test is a multivariate analogue of Levene’s test for homogeneity of variance. Large dispersion within an area indicates that it hosts heterogeneous bacterial communities.

Dispersal of Bacteria Between Cryoconite Areas

We aimed at investigating potential dispersal of bacteria among the four areas of the glacier where we sampled cryoconite. To this end, we conducted an indicator species analysis to identify taxon-area association patterns. This analysis was used to identify not only OTUs associated to one area but also OTUs associated to two or three areas. This analysis was done with the *multipatt* function (with 99,999 permutations) implemented in the INDICESPECIES package of R [41]. This procedure returns an IndVal statistics that is a measure of the strength of the association between one OTU and an area or one OTU and a combination of areas. OTUs indicator of one area are typically found in that area only, while OTUs indicator of e.g. a combination of two areas are typically found in those two areas, but not in the other ones. A larger number of OTUs typical of a pair of areas may thus suggest a larger dispersal between the areas, or that these areas share a common source of bacteria.

A large number of statistical tests were run also in this case, so we corrected *P* values according to the FDR procedure as above. Indicator OTUs with a $P_{\text{FDR}} < 0.05$ were considered significantly associated with a habitat or a combination of habitats. Indicator taxa were then represented in a network by using the IGRAPH package of R 3.1.2, where habitats were connected with their indicator OTUs.

Small Scale Spatial Variation within Sampling Areas

We investigated variation in the structure of bacterial communities at small, within-area, spatial scale according to spatial trends (i.e. turnover of bacterial communities along and across

the ridge) and according to spatial patterns (i.e. patches of holes close to one another). These analyses were restricted to holes in areas 1 and 4 only, because 10 holes were sampled in each of these areas. Areas 2 and 3, where only five holes were sampled in each area, were discarded.

We used the Principal Coordinates of Neighbourhood Matrices (PCNM), a multivariate statistical technique that generates spatial variables able to capture the spatial structure of the data at all spatial scales [42, 43]. PCNM was applied to UTM coordinates of holes sampled in areas 1 and 4 separately. Only PCNM variables with a positive eigenvalue and accounting for positive spatial autocorrelation were retained [30]. We then ran separate RDAs on Hellinger-transformed OTU relative abundances for each area by including as predictors: (1) pH, which was the only environmental condition that significantly affected the structure of bacterial communities of cryoconite holes (see “Results”) [E], (2) UTM coordinates accounting for spatial trends [T] through the whole area, and (3) PCNM variables accounting for spatial patterns at smaller spatial scales than the whole area [P]. Finally, we used variation partitioning to quantify the variation in the bacterial community structures explained separately and jointly by [E], [T] and [P].

Results

Composition of Bacterial Communities in Cryoconite Holes

The number of sequences obtained from each sample ranged between 32,912 and 470,288. Overall, we observed 491

OTUs in 30 cryoconite holes. The number of OTUs in each hole ranged from 136 to 292. In Fig. 2, the structure of bacterial communities based on the abundance of OTUs classified at order level is reported. Burkholderiales was the dominant taxon in all the areas, with relative abundance ranging from 45.58% (area 4) to 77.28% (area 3). Other abundant orders were Enterobacteriales (0.88 to 9.36%), Sphingomonadales (1.68 to 9.36%), Sphingobacteriales (1.68 to 8.58%) and Pseudomonadales (0.55 to 4.82%). Cyanobacteria (class) ranged from 1.71% (area 2) to 3.71% (area 4). Overall, the five most abundant bacterial orders and Cyanobacteria represented more than 70% of bacteria in each area. Among Burkholderiales, *Limnohabitans* and *Polaromonas* represented the most abundant genera, with abundances exceeding 60% of Burkholderiales in all areas (Fig. S1). Betaproteobacteria represented 18–94% of sequences at each hole, while Alphaproteobacteria represented only 0.42–15%.

Variation of Bacterial Communities in Cryoconite Holes

RDA indicated that the structure of bacterial communities changed significantly among the four areas (Table 1). However, post hoc tests also indicated that no pairwise difference between areas was significant (Pseudo- $F_{1,15} \leq 5.462$, $P_{FDR} \geq 0.089$). In addition, dispersion (multivariate variance) of bacterial communities did not differ significantly among areas ($F_{3,26} = 2.149$, $P = 0.118$), thus indicating homogeneity of multivariate variance of OTU composition. When we repeated the RDA analysis by removing area 4, we found no significant difference among areas (Pseudo- $F_{2,17} \leq 1.223$, $P = 0.255$).

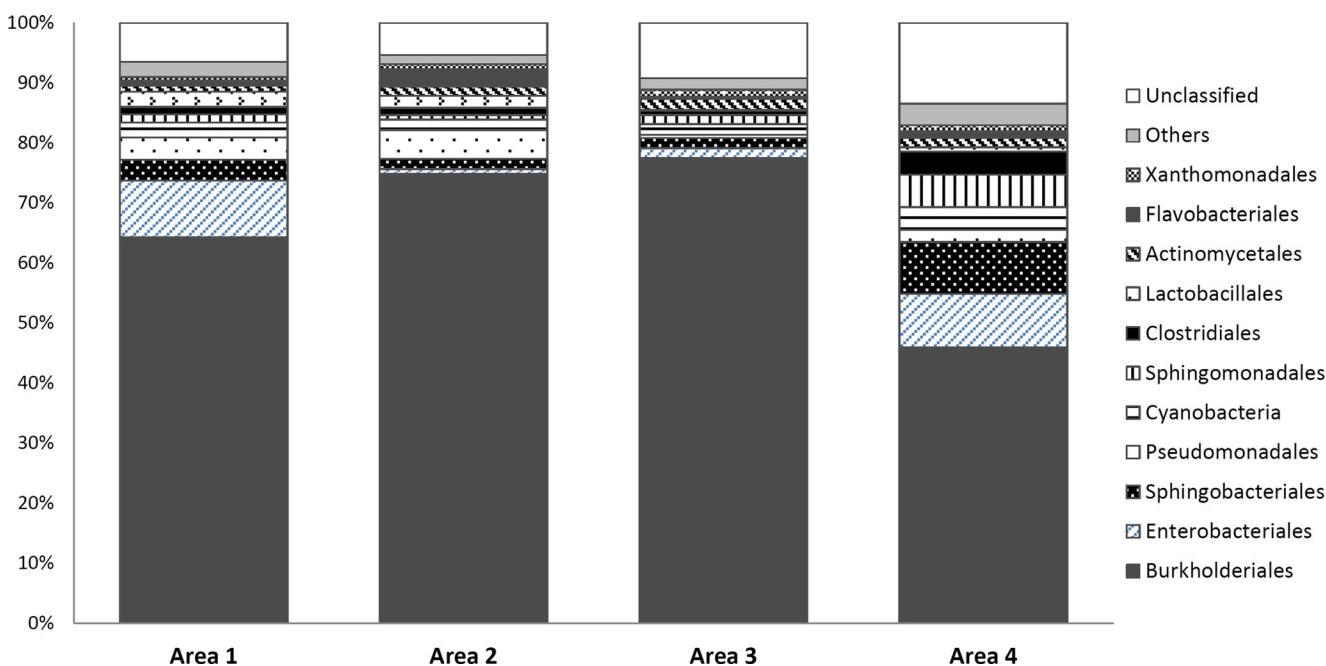


Fig. 2 Mean relative abundance of OTUs classified at order level in the four sampling areas

Table 1 Results from RDAs, partial RDAs and variation partitioning analyses

Model/variable	<i>df</i>	Variance	<i>Pseudo-F</i>	<i>P</i>	Adjusted <i>R</i> ²
Sampling area [A]					
Area	3	0.066	2.908	0.004	0.165
Residual	26	0.197			
Environment [E]					
pH	1	0.035	4.352	0.007	0.104
Residual	28	0.228			
[A E]	3	0.047	2.193	0.011	0.102
[E A]	1	0.017	2.318	0.058	0.040
[A∩E]				0.063	

[A] and [E] are the fractions of total variation in community structure explained by variables accounting for sampling area [A] and environmental variables [E]. First lines report the results of final RDA models after forward selection with the double stopping criterion. Following lines report marginal effects for each variable included in the model. [A|E] and [E|A] are variation fractions identifying, respectively, pure effects of spatial clusters and environmental conditions calculated by partial RDAs. Significance of RDAs and partial RDAs was assessed by a randomization procedure (Legendre and Legendre 1998). [A∩E] is the fraction of variation shared between spatial clusters and environmental variables calculated according to variation partitioning analysis. Significance of this latter fraction is not testable (Legendre and Legendre 1998)

Among environmental variables, only pH significantly affected the structure of bacterial communities in cryoconite holes (Table 1; Fig. 3). Variation partition analysis revealed that, after accounting for the differences in pH, the individual effect of area [A|E] explained a significant fraction (10.2%) of variance in bacterial community structure. Conversely, pH per se [E|A] explained a small (4%) non-significant fraction of variance, while 6.3% was explained by variation of pH among areas [E∩A] (significance of this latter fraction is not testable, see [44]) (Table 1). Indeed, a GLS model indicated that pH differed significantly among areas ($F_{3,26} = 7.20$, $P = 0.001$), with cryoconite holes in area 4, which were sampled 8–10 days after those in the other areas, significantly more alkaline than those in area 1 (post hoc tests: $z = 4.306$, $P < 0.001$; $|z| \leq 1.852$, $P \geq 0.232$ in all the other cases; Fig. 3a).

Variation of Alpha-Diversity and Order Abundance in Relation to Area and pH

Number of OTUs and Shannon diversity index did not differ significantly among areas ($F_{3,25} = 2.513$, $P = 0.082$) but increased significantly with pH (number of OTUs: coef = 0.158 ± 0.047 SE, $F_{1,25} = 11.475$, $P = 0.002$; Fig. S2b; Shannon index: coef = 0.502 ± 0.206 SE, $F_{1,25} = 5.973$, $P = 0.022$; Fig. S2c). Gini inequality index differed significantly among areas ($F_{3,25} = 5.18$, $P = 0.006$) but did not vary according to pH ($F_{1,25} = 3.27$, $P = 0.083$). Post hoc tests showed that Gini index of holes in area 4 was significantly larger than that of holes in areas 2 and 3 ($z \geq 3.021$, $P \leq 0.013$; $|z| \leq 1.724$, $P \geq 0.301$ in all the other cases; Fig. S2d). Consistently with the results of post hoc tests, we saw no difference among areas when we removed area 4 from the analysis ($F_{2,16} = 1.02$, $P = 0.382$).

We also investigated variation in the abundance of OTUs belonging to Cyanobacteria and to the five most abundant bacterial orders (i.e. Burkholderiales, Enterobacteriales, Sphingobacteriales, Pseudomonadales and Sphingomonadales) according to area and pH. We found that the abundance of both Sphingobacteriales and Sphingomonadales increased significantly with pH ($t_{28} \geq 3.452$, $P_{FDR} \leq 0.017$; Fig. S3) and varied significantly among areas (main test: $\chi^2_3 \geq 20.511$, $P_{FDR} < 0.019$; Fig. S4). Sphingobacteriales were more abundant in area 4 than in areas 2 and 3 (post hoc tests: $z \geq 3.427$, $P \leq 0.003$; Fig. S4), while Sphingomonadales were significantly more abundant in area 4 than in all other areas (post hoc tests: $z \geq 3.503$, $P \leq 0.003$; Fig. S4). No significant difference among areas was observed when we removed area 4 from the analyses ($\chi^2_2 \leq 4.808$, $P \geq 0.090$).

Spatial Patterns within Study Areas

The analyses conducted within areas 1 and 4 revealed no variation in the structure of the bacterial communities according both to spatial trends ($F_{2,7} \leq 2.089$, $P \geq 0.073$) and to spatial patterns assessed by PCNM ($F_{4,5} \leq 1.309$, $P \geq 0.272$). These analyses also revealed that pH significantly affected the structure of bacterial communities of area 1 ($F_{1,8} = 2.490$, $P \geq 0.037$) but not those of area 4 ($F_{1,8} = 1.053$, $P = 0.337$).

Indicator Taxa

The indicator taxa analysis identified only 12 OTUs significantly associated to one area or to a combination of areas (Table S1). This suggests that most OTUs could be found in holes in all areas with similar abundance and were not typically associated to any area. In particular, no OTU was significantly

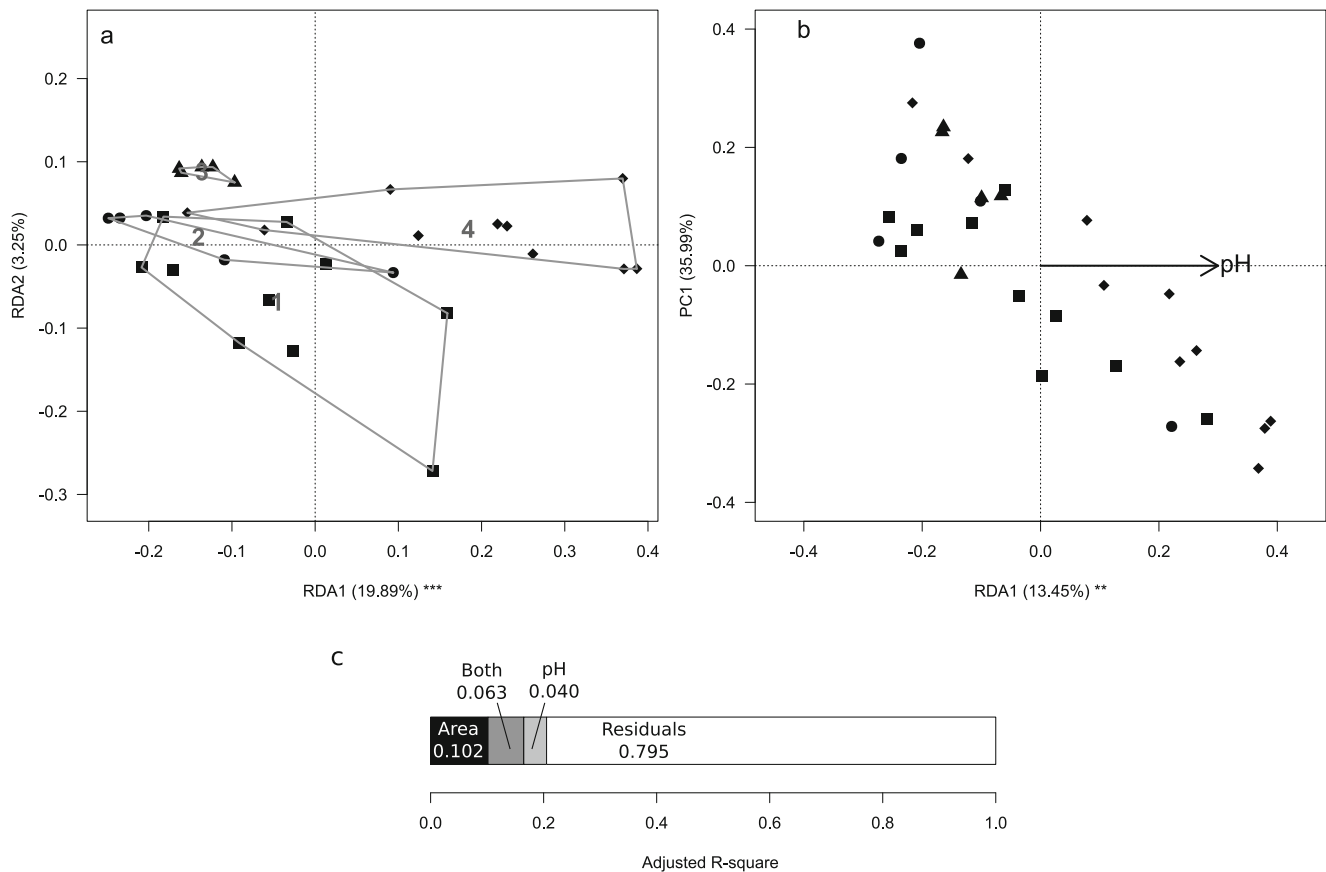


Fig. 3 Biplots of first and second components from separate RDAs of bacterial communities in cryoconite holes according to **a** sampling area and **b** pH. Each symbol represents the bacterial community in a cryoconite hole. Different symbols represent different areas (squares = area 1; dots = area 2; triangles = area 3; diamonds = area 4). In **a**, polygons include cryoconite holes sampled in each area and numbers denote the centroid of bacterial communities sampled in each

area. The arrow in **b** represents the constraining variable. The amount of variance explained by each axis is shown as well as the significance of each constrained (RDA) axis as assessed by a randomization test (** $P < 0.01$; *** $P < 0.001$; no asterisk: $P > 0.05$). **c** Results of variation partitioning analysis showing the proportion of variance in bacterial community structure explained by area and pH per se, and by both area and pH

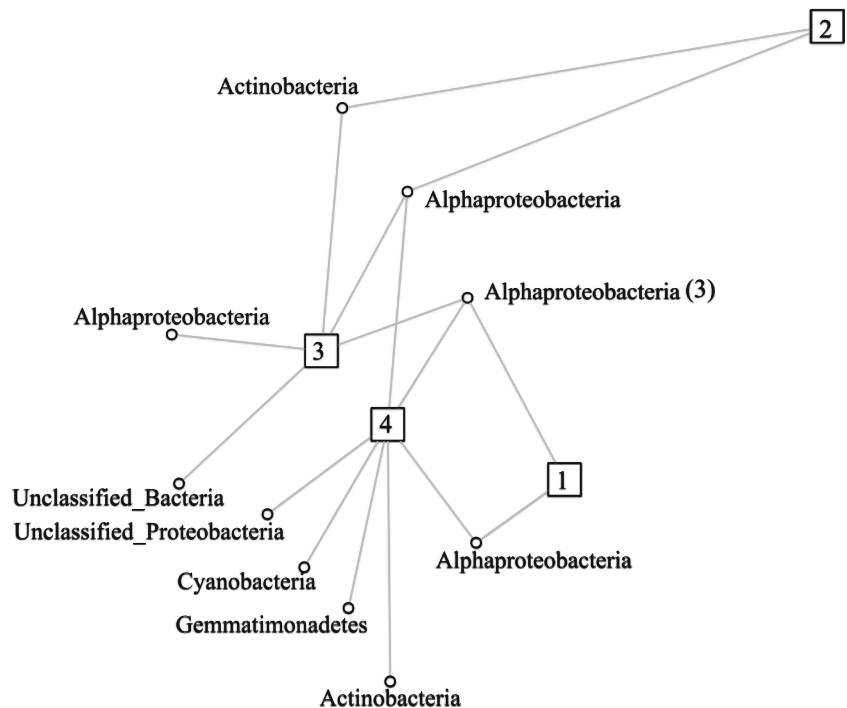
associated to areas 1 or 2, two OTUs were significantly associated to area 3, and four to area 4 (Fig. 4). In addition, three OTUs were associated to areas 1, 3 and 4, and two to areas 2, 3 and 4. One OTU was associated to areas 1 and 4, and one to areas 2 and 3. No OTU was significantly associated to areas 1 and 2 and to areas 3 and 4. Thus, the network representing the results from the Indicator Taxa analysis showed few connections among areas, with most OTUs rather ubiquitous (Fig. 4), as also indicated by the fact that 225 OTUs (51.9%) were found in all areas.

Discussion

In this study, we collected cryoconite samples from holes in four areas physically separated on elevations that occurred on the surface of the Baltoro Glacier and described the structure of their bacterial communities and the ecological processes that may have contributed to shape them.

Cryoconite of Baltoro Glacier was dominated by Betaproteobacteria and showed higher Beta- to Alphaproteobacteria ratios compared to previous studies on mountain glaciers outside the Polar regions [14]. The presence of Betaproteobacteria has been extensively reported in cryoconite and high altitude glacial environments. For instance, Edward et al. [15] described the bacterial metagenome obtained from cryoconite sampled in the Rotmoos Ferner Glacier (Austrian Alps) and found that 62% of all bacteria contigs belonged to Proteobacteria, mainly represented by Betaproteobacteria (52% of all the sequences from Proteobacteria). Betaproteobacteria also dominated cryoconite on Ürümqi Glacier No. 1 (Tien Shan Mountains, China) [13]. High abundance of Betaproteobacteria was also found in Arctic snow and ice communities, where this taxon represented up to the 56% of the microbial communities [45]. Similar results were found by Edwards et al. and Musilova et al. [14, 46]. Higher ratios between Alphaproteobacteria and Betaproteobacteria have been associated with community development in glacial and non-glacial environments [47, 48], and to high stability of cryoconite holes typical of Arctic environments

Fig. 4 Association network showing significant ($P_{\text{FDR}} < 0.05$) associations between indicator OTUs (circles) and areas (squares). Numbers within squares identify the area. Square position represents spatial position of the areas. The class to which each OTU belongs is shown. Number in brackets indicates the number of OTUs. OTUs were connected to the areas to which they were significantly associated according to the Indicator Species analysis



[14]. Conversely, dominance of Betaproteobacteria is associated to alpine glaciers [14, 15].

At lower taxonomic rank, *Polaromonas* and *Limnohabitans* (order Burkholderiales) were the dominant Betaproteobacterial genera. *Polaromonas* genus is emerging as ubiquitous in glacial environments from polar and high-elevation environments, mainly in glacial ice and sediment samples [13]. It has been hypothesised that its wide distribution across the globe depends on air-dispersion processes and *r*-strategy lifestyle [49–51]. However, its wide distribution may also depend on the ability of this taxon to use alternative energetic pathways. Indeed, annotation of *Polaromonas* genomes reconstructed from Forni and Baltoro cryoconite metagenomes showed the presence of carbon monoxide dehydrogenase genes, whose coverage was particularly high in Baltoro metagenomes, and the lack of a complete CO_2 fixation pathway [20]. These features have already been reported in other complete genomes available for *Polaromonas* and suggest that this taxon might use carbon monoxide (CO) as an energy source in the presence of an organic carbon source (i.e. mixotrophy) [52–54].

Limnohabitans is known to inhabit a broad range of freshwater habitats within at least three continents and can constitute up to 30% of free-living bacteria in freshwater systems [55]. The several small supraglacial lakes present between the ridges of the Baltoro Glacier in the area where we collected cryoconite may have facilitated the diffusion of this genus, which has been described as dominant photoheterotrophic aerobic bacteria in high altitude lakes on Pyrenees [56]. Abundance of taxa with this metabolism is consistent with the high *pu*fML genes (photosynthetic reaction centre L and M subunits) found in Baltoro

cryoconite metagenomes [20]. In addition, the presence of genes encoding ribulose-1,5-bisphosphate carboxylase (RuBisCO), *sox* gene cluster, which contains sulphur-oxidizing-related genes, *cox* genes, encoding carbon monoxide dehydrogenase clusters, and genes encoding ammonia monooxygenase, indicates a high metabolic versatility of this genus [57]. Hence, the genomic features of highly abundant Burkholderiales in cryoconite suggest that their high metabolic versatility might contribute to their successful colonization of cold environments.

Cryoconite on the Baltoro Glacier showed low abundance of Cyanobacteria (< 4%), which, in contrast, were among the dominant taxa in many previous investigations of cryoconite [13, 14, 58] including one conducted on the Forni Glacier (Italian Alps) during the same months when we collected cryoconite on the Baltoro Glacier and with identical methods [16]. However, other studies reported low abundances of Cyanobacteria both in Arctic [14] and Alpine cryoconite [15]. In addition, abundance of Cyanobacteria obtained by 16S rRNA sequencing is not consistent with the high-gene coverage levels of *rbcL* genes obtained in a previous metagenomics study on the same samples [20]. Unlike other aquatic environments, taxonomic and functional data showed that Aerobic Anoxygenic Phototrophs (AAPs) were more abundant than Cyanobacteria in Baltoro cryoconite. AAPs have been found in many aquatic environments and were particularly abundant in high altitude oligotrophic lakes [56]. Among AAPs, the Betaproteobacteria populations, which were dominant in our samples (*Limnohabitans*), were particularly abundant in environments with very low salinity like melting water [59, 60].

Unfortunately, we did not record nutrient concentration and salinity on Baltoro to verify whether the higher abundance of AAPs on Baltoro is consistent with the values of these parameters. Sphingobacteriales were among the most abundant orders on Baltoro where they increased with increasing pH. High abundance of Sphingobacteriales was observed also in Forni cryoconite, where they increased along the ablation season [16], while previous studies reported that their abundance was influenced by the availability of organic carbon [50, 61]. These results might suggest that this taxon is able to respond quickly to changes in environmental conditions in glacier environments.

Before further discussing the ecological processes that may have determined the structure of bacterial communities, a point deserves consideration. Samples were transported with no chemical preservative and simply kept as cool as possible during transport because one previous study focusing on soil sample preservation showed that a chemical preservative significantly altered the structure of bacterial communities, as described by DNA-based methods, while storing samples for a rather long time without any chemical preservative, even at warm temperature, did not [22] (see also “Materials and Methods”). However, recent studies, published after we sampled cryoconite, reported consistent results with fresh frozen vs. chemically preserved samples from cryoconite [62, 63]. These contrasting results open the possibility that the low-temperature adapted communities of cryoconite may respond differently to those of soils to preservation. In particular, metabolically flexible bacterial heterotrophs like those belonging to Burkholderiales may have increased in abundance during transport. Therefore, a posteriori, we cannot totally rule out the possibility that post-sampling changes in community occurred. We stress that we based our choice of storage method on the best available information at the time we collected samples, and future studies should very carefully address this problem. As a general indication, we can argue that a safe choice for future studies should be to preserve samples in parallel by a range of means to allow cross-validation of community composition.

With this caveat in mind, we can now discuss the ecological processes driving community assemblies. Our results revealed that differences among the four areas explained a significant fraction (10.2%) of variance in bacterial community structure. Variation in bacterial communities is explained by divergent selective processes driven by variation in environmental conditions, particularly of pH, which was the only environmental variable that significantly affected the structure of bacterial communities. However, the only area that differed from the other ones was area 4, which was sampled 8–10 days after the other areas. Given that different areas of the glaciers were sampled in different days due to logistic constraints, we could not assess whether the structure of bacterial communities of area 4 differed from that of the other areas due to spatial or temporal variability. For example, area 4 may have hosted

different bacterial communities because of different pH values due to different mineral inputs in that area. Alternatively, bacterial communities may have changed during the 10 days elapsed between the sampling of area 1 and area 4, because of e.g. seasonal changes of bacteria communities [16]. Alternatively, input of allochthonous materials may have altered cryoconite pH and, consequently, bacterial community structures. For instance, a snowfall occurred on 1–2 and 6 July in the study area, i.e. between the sampling of areas 1–3 and 4, and may have deposited acidic material to the glacier surface.

Thus, we have no clue to assess whether area 4 hosted different bacterial communities because of spatial or temporal effects. Furthermore, indicator OTUs and their association network indicated that most of the OTUs were not typically associated to any area (Fig. 4).

In conclusion, our results suggest that microbial communities of cryoconite holes on Baltoro Glacier were dominated by Betaproteobacteria probably owing to their highly versatile metabolism. Although we cannot totally rule out the possibility that these bacteria population have increased in abundance during transport, available genomic information of this taxon suggests that light supports bacterial metabolism in this environment by providing complementary energy to photoheterotrophic aerobic bacteria [20]. The high abundance of these taxa make these communities similar to those inhabiting high altitude ultraoligotrophic lakes [56]. Supraglacial lakes, which occurred in the area where we sampled cryoconite, may therefore be sources of cryoconite bacteria. Variability in the structure of bacterial communities among different areas of the glacier may be due to both temporal and spatial patterns of variation in environmental condition, particularly pH.

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Compliance with Ethical Standards

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