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Prokaryotic Diversity and Distribution in Different Habitats of an Alpine Rock Glacier-Pond System

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33 **Abstract**

34 Rock glaciers (RG) are assumed to influence the biogeochemistry of downstream
35 ecosystems because of the high ratio of rock:water in those systems, but no studies have considered
36 the effects of a RG inflow on the microbial ecology of sediments in a downstream pond. An alpine
37 RG-pond system, located in the NW Italian Alps has been chosen as a model, and Bacteria and
38 Archaea 16S rRNA genes abundance, distribution and diversity have been assessed by qPCR and
39 Illumina sequencing, coupled with geochemical analyses on sediments collected along a distance
40 gradient from the RG inflow. RG surface material and neighbouring soil have been included in the
41 analysis to better elucidate relationships among different habitats.

42 Our results showed that different habitats harboured different, well separated microbial
43 assemblages. Across the pond, the main variations in community composition (e.g. Thaumarchaeota
44 and Cyanobacteria relative abundance) and porewater geochemistry (pH, DOC, TDN and NH_4^+)
45 were not directly linked to RG proximity, but to differences in water depth. Some microbial
46 markers potentially linked to the presence of meltwater inputs from the RG have been recognised,
47 although the RG seems to have a greater influence on the pond microbial communities due to its
48 contribution in terms of sedimentary material.

50 **Keywords**

51 Rock glacier; alpine pond; sediments; microbial ecology; 16S rRNA

53 **Introduction**

54 Active rock glaciers (RGs) are slow-flowing mixtures of rocks and ice, representing one of
55 the most common geomorphological features in high mountain environments [1], and are
56 considered indicators of permafrost presence [2, 3]. Permafrost degradation has been reported to
57 impact chemical characteristics of surface freshwater across the globe [4, 5] and RGs have shown
58 the capability to influence waters passing through and originating from them, releasing cold
59 meltwaters enriched in solutes [6-8]. Given the widespread distribution of RGs worldwide [1], the
60 high concentrations of heavy metals [9] and nutrients [8, 10] in their meltwaters, and their lower
61 ice-melting rate in comparison to glaciers [11, 12], their importance in shaping headwater
62 biogeochemistry is probably going to increase in the future, in a climate change scenario.

63 Several studies (reviewed in [13]) have investigated how the presence of glacial-meltwater
64 inputs can affect downstream freshwater ecosystems in terms of physical and chemical properties,
65 with subsequent effects on their biological structure and function. Far less information is available
66 on RG meltwaters, and particularly on their influence on the ecology of associated water bodies.
67 For instance, Thies et al. [9] compared the diatom population of RG-influenced and reference
68 streams in the Austrian Alps, highlighting that both diatom species composition and diversity can
69 be negatively affected by the increase in water acidity commonly associated with RG runoff. In the
70 NE Italian Alps, Ilyashuk et al. [14, 15] reported high concentrations of metals in the waters of RG
71 lakes, which seem to explain the high rate of morphological abnormalities in chironomid population
72 inhabiting lake sediments. Focusing on microbial community, Fegel et al. [8] described the bacterial
73 community composition in stream sediments collected close to the terminus of several glaciers and
74 RGs, distributed across three North America mountain ranges. They found that RG stream
75 sediments were generally characterised by higher alpha diversity, and harboured a more
76 cosmopolitan bacterial population, including genera more commonly found in soil. A similar
77 approach was followed and similar results were obtained in the NE Italian Alps by Tolotti et al.
78 [16]. However, to our best knowledge, the potential impact of RG meltwaters on both Bacteria and
79 Archaea communities of a complex freshwater ecosystem has not been investigated yet.

80 In this study, we focused on a RG-pond system located in North-western Italian Alps, whose
81 structural settings and hydrological dynamics have been previously described by Colombo et al.
82 [17]. This pond represents for many reasons a suitable model system to investigate the influence of
83 RGs on downstream aquatic ecosystems. The oligotrophic conditions, as well as the lack of algal or
84 macrophyte cover on the bottom and the absence of direct anthropogenic disturbance linked to
85 human activities carried out in the pond reduce the number of factors influencing the microbial
86 community inhabiting sediments. Moreover, the limited dimensions potentially increase its
87 susceptibility to external environmental pressures [18, 19]. The presence of a localised inflow from
88 the RG has been confirmed [17], but it is important to specify that these meltwater inputs have been
89 described as intermittent, concentrated only in the snow-free season and mainly linked to
90 precipitation events [17, 20]. Meltwaters also showed only a moderate increase in solutes
91 concentration if compared with the pond waters [20], differing in this sense from other RG-
92 lake/stream systems affected by natural acid rock drainage phenomena [9, 14, 15]. Nevertheless,
93 beside RG inputs can be limited in frequency and magnitude, microbial communities can quickly
94 react to environmental pressures acting directly or indirectly on them [21], becoming a powerful
95 instrument for the detection of ecosystem perturbations.

96 Our hypothesis was that the peculiar physicochemical properties of RG meltwaters and their
97 potential to carry microorganisms of subglacial origin, may influence the sediments in terms of
98 geochemistry, microbial abundance and microbial diversity. However, due to the intermittent nature
99 and the limited magnitude of RG meltwater inputs, and to the presence of a localised inflow, we
100 supposed that such an influence would not be equally extended over all the pond but would act
101 mainly on the sediments closest to the RG inflow. Therefore, we hypothesized the occurrence of a
102 horizontal gradient in RG influence, corresponding to a horizontal distance gradient from the RG
103 inflow. In order to test this hypothesis, we firstly assessed archaeal and bacterial assemblages
104 present in three main habitats (RG, surrounding soil and lacustrine sediments) of the RG-pond
105 system. We then investigated whether the sediment microbial community and geochemistry varied
106 across the pond, reflecting possible relationships with neighbouring compartments. Based on this
107 information, we attempted to recognise microbial markers potentially symptomatic of the influence
108 of RG on downstream pond sediments.

109

110 **Materials and Methods**

111 **Study Site**

112 The Col d'Olen Rock Glacier ("Corno Rosso 2 Rock Glacier" in the Aosta Valley rock
113 glacier cadastre,
114 <http://geonavsct.partout.it/pub/GeoNavSCT/index.html?metadato=MTD010N0001>) is a talus-
115 tongue shaped RG [2], covering an area of about 37,500 m². It is located in the NW Italian Alps, at
116 the boundary between the Aosta Valley and the Piedmont regions (45°52'8.22''N, 7°51'46.98''E,
117 WGS84; Fig. 1a). The surface is covered by clasts (calcschists and serpentinites) varying from
118 pluri-decimetric to metric size, with outcrops of fine-grained, serpentinitic material at the terminus
119 and along the lateral scarps. The Col d'Olen Rock Glacier Pond is situated at the RG terminus, on
120 the right side of the tongue (Fig. 1b), at an elevation of 2722 m a.s.l.. The pond has an area of 1,600
121 m², with maximum length and width of approximately 60 x 40 m, and reaches a maximum depth of
122 about 3 m. It is a clear-water pond, characterised by oligotrophic conditions and by the lack of
123 macroalgal and macrophyte cover on the bottom, with the shallower areas close to the shore
124 undergoing seasonal freezing. More details on the catchment structural settings and hydrological
125 dynamics of the pond are described in Colombo et al. [17, 20]. The research site is a node of the
126 Long-Term Ecological Research (LTER) network in Italy (<http://www.lteritalia.it>).

127

128 **Sampling Procedures**

129 For this study, different typologies of sample were considered in order to assess the
130 microbial assemblages present in the different habitats associated with the Col d'Olen RG (Fig. 1b-
131 1c, Table 1). Sediment samples from the downstream pond were collected in triplicate at three
132 points (S1, S2, S3) along a distance gradient starting from the RG front and crossing the pond in
133 NE-SW direction. Points S1 and S3 were at approximately 1 m of water depth, while point S2 was
134 positioned in the deepest point of the pond, at 3 m of water depth. The surface sediments (top 10
135 cm) were collected with a core sampler, transferred in sterile plastic jars without preserving
136 sediments stratification, and kept covered with water during the transportation. Three aliquots of
137 fine-grained serpentinitic material (top 10 cm) were collected on the RG front. A triplicate soil
138 sample was collected from the North vegetated slope. Only the top 10 cm, after removal of
139 vegetation and roots, were considered. All the samples were kept refrigerated and in the dark during
140 the transport. In the laboratory, material for chemical analysis was stored at 4 °C, while aliquots for
141 molecular analysis were preserved at – 20 °C prior to further processing.

142 The complete sampling procedure was repeated for two consecutive years, always during
143 the advanced snow-free season (September 9th 2015, July 30th 2016), in order to avoid the
144 additional effect of snowmelt processes on the system.

146 **Samples Physicochemical Characterization**

147 *Pond sediments, RG material and soil*

148 Solid samples were homogenized and sieved to 2 mm. A fresh aliquot of 20 g was extracted with
149 100 ml KCl 1M. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were
150 determined on 0.45 µm filtered extracts with a total organic carbon analyser (Vario TOC,
151 Elementar, Hanau, Germany). Ammonium and nitrate concentration in KCl extracts were measured
152 spectrophotometrically (U-2000, Hitachi, Tokyo, Japan). For extractable ammonia determination a
153 Berthelot method based on reaction with salicylate in presence of alkaline sodium
154 dichloroisocyanurate [22] was used while, for extractable nitrate, the Gneiss reaction [23] was
155 applied. Total carbon (TOC) and nitrogen (TN) in the solids were determined on samples dried at
156 105°C by elemental analysis (NA2100, CE Instruments, Milan, Italy).

157 *Porewater*

158 Porewater was extracted from aliquots of fresh sediment by centrifugation and 0.20 µm filtered. pH
159 was measured using a Basic 20 pH meter (Crison, Modena, Italy). Anions (NO₂⁻, NO₃⁻, Cl⁻, SO₄²⁻

160 and PO_4^{3-}) were analysed by ionic chromatography with a Dionex 500 (Thermo Scientific). Major
161 cation (Ca^{2+} , Mg^{2+} , Na^+ , K^+) concentrations were measured by Atomic Absorption
162 Spectrophotometry (Perkin Elmer AAnalyst 1400), while Si was determined spectrophotometrically
163 according to standard methods [24].
164

165 **DNA Extraction**

166 Genomic DNA from sediments, soil and RG fine-grained material was extracted from 0.5 g
167 of sample using the FastDNATM SPIN Kit for Soil and the FastPrep[®] Instruments (MP Biomedicals)
168 in accordance with the manufacturer's instructions. Extracted DNA was quantified using Qubit[®]
169 (Life Technologies), quality was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo
170 Fischer Scientific) and DNA integrity was evaluated by agarose gel electrophoresis.
171

172 **Quantitative PCR Analysis**

173 Quantification of 16S rRNA genes was performed by quantitative PCR (qPCR) using primer
174 pairs 340F-1000R for Archaea [25] and 338F – 518R [26] for Bacteria. The reaction mixture
175 contained 10 μl of SsoAdvancedTM SYBR[®] Green Supermix, 0.3 μM of each primer, and 2 μl of
176 DNA diluted 1:10 (sediment, RG samples) or 1:100 (soil samples), in a total volume of 20 μl .
177 Plasmids including a single copy of a single bacterial or archaeal 16S rRNA gene, amplified with
178 the same primers used for qPCR, were serially diluted and used as standard for DNA quantification.
179 qPCR reactions were performed using a Chromo4TM Real Time PCR Detection System (Bio-Rad
180 Laboratories), and data were analysed with the MJ Opticon Monitor software (version 3.1). Both
181 samples and standards were analysed in triplicate in PCR strip tubes (Bio-Rad Laboratories), under
182 cycling conditions reported in detail in Online Resource 1. PCR specificity was verified by melting
183 curves analysis and bands visualisation on agarose gel. Standard curves R^2 values were always
184 higher than 0.997, and all the reactions showed efficiencies higher than 85%.
185

186 **16S rRNA Gene Fragment Sequencing and Data Processing**

187 The V4-V5 regions of bacterial and archaeal 16S rRNA genes were PCR-amplified using
188 primer pair 515F-Y and 926R [27]. PCR reactions were performed in triplicate in a 25 μl reaction
189 volume containing 1x KAPA HiFi HotStart Ready Mix, 0.2 μM forward and reverse barcoded
190 primers and 12 ng of DNA. Cycling conditions were as described by Parada et al. and are resumed
191 in Online Resource 1.

192 After the amplification, PCR products were checked for presence and intensity by electrophoresis
193 on 1.5% agarose gel, and replicates of the same sample were pooled.
194 Amplicon purification, 16S library preparation and sequencing by MiSeq Illumina (Illumina, San
195 Diego, CA) using a 2 x 300 bp paired-end protocol were performed at the Bristol Genomics Facility
196 (Bristol, UK).

197 Raw Illumina reads quality was checked with Prinseq [28], and terminal portions with an
198 average quality score below q15 across a 5 bp sliding window were trimmed. Forward and reverse
199 priming regions were removed and sequences resulting shorter than 230 bp after the quality
200 trimming were discarded. Paired-end reads were merged using FLASH [29] and processed using
201 QIIME pipeline [30]. Chimeric sequences were detected and removed using UCHIME2 in mode
202 sensitive and Gold database as reference [31]. Sequences showing >97 % similarity were grouped
203 in OTUs using uclust [32], and for every cluster the most abundant sequence was chosen as
204 representative. The taxonomic affiliation of the OTUs was assessed by RDP classifier [33] against
205 the SILVA 128 database [34] at a 97% confidence cut-off, and only representative sequences able
206 to align to SILVA database were conserved and used for OTU table construction. Sequences
207 appearing one time in a single sample were discarded, and one of the soil samples collected in 2015
208 (G3_15) excluded from the analysis due to the remarkably lower number of available sequences.
209 OTU tables containing bacterial and archaeal sequences were obtained and analysed separately, and
210 sequences deriving from mitochondria or chloroplasts were filtered from the bacterial OTU table.
211

212 **Diversity and Statistical Analysis**

213 Rarefaction curves were constructed without applying any normalisation on the number of
214 sequences per sample, while for alpha and beta diversity evaluation, the number of sequences per
215 sample was normalised according to the less abundant sample. Alpha diversity estimators (number
216 of OTUs, Chao1, Shannon and Simpson indexes) were calculated on multiple rarefied OTU tables
217 using QIIME [30]. The same program was used to align bacterial or archaea OTU sequences [35],
218 to build the corresponding phylogenetic trees [36], and to compute Bray-Curtis, Weighted and
219 Unweighted UniFrac distance matrixes [37] based on similarities at OTU level. Ordination of
220 samples according to the different distance matrixes was performed by non-metric
221 multidimensional scaling (NMDS), and the significance of the separation among environmental
222 matrixes and sampling points was evaluated by PERMANOVA (permutational multivariate
223 analysis of variance) using the adonis function of the 'vegan' package in R [38].

224 In order to better elucidate taxon-habitat relationships, an indicator species analysis was
225 performed. Archaeal and bacterial OTUs showing relative abundance higher than 0.1% were
226 analysed separately. The number of OTUs per sample was normalised based on the less abundant
227 sample by using the `rrarefy` function of the ‘vegan’ package. IndVal statistics were calculated with
228 the `multipatt` function (with 99,999 permutations) of the ‘indicspecies’ package in R [39]. P-values
229 were corrected for multiple testing according to the FDR procedure, and only associations with
230 $PFDR > 0.05$ were retained as significant. Moreover, in order to focus on the strongest associations,
231 only OTUs showing IndVal values higher than 0.90 were considered. Indicator OTUs were then
232 grouped into phyla/classes and their association with different habitats were represented in bipartite
233 networks by using the Gephi software [40].

234 Geochemical data, 16S rRNA genes abundance, alpha diversity estimators and the relative
235 abundance of the most abundant bacterial and archaeal classes were compared among the different
236 sampling points, by analysing separately 2015 and 2016 datasets. Data were transformed if
237 required, normal distribution of residuals and homogeneity of variances were tested and, when
238 appropriate, ANOVA was applied. In presence of significant differences, pairwise comparisons
239 were performed using Tukey’s honest significance difference (HSD) test, and differences were
240 considered significant for $P < 0.05$. When ANOVA assumptions were violated, Kruskal-Wallis
241 nonparametric test coupled with Dunn’s test as *post hoc* was applied. In this case, P-values were
242 adjusted for multiple comparisons by Holm-Šidák method.

243 All the plots (diversity bar plots, boxplots and NMDS plots) were drawn with ‘ggplot2’
244 package [41].

246 **Data Accessibility**

247 Raw sequencing data were deposited in NCBI SRA database under accession number
248 SRP126235.

250 **Results**

251 **Sediments, Soils and Porewater Geochemistry**

252 All the considered geochemical parameters showed a certain degree of variation in relation
253 to the environmental matrix (Fig. 2, Online Resource 3). Total C and N, as well as DOC and TDN
254 were significantly lower in RG samples, intermediate in the pond sediments and higher in soil (G).
255 RG samples were also characterised by the lowest levels of ammonia, while the highest levels were

256 detected in sediment samples. The C/N ratio in RG was significantly higher than in sediments and
257 soil. For nitrates, homogeneous values were reported for all the samples but G, RG and S3 in 2015.

258 Porewater characterisation of the lacustrine sediments is reported in Table 2. When
259 comparing sediments collected at different sampling points (Fig. 2, Online Resource 3), several
260 geochemical properties appeared to follow a similar trend in both the sampling times. For instance,
261 DOC, TDN, NH_4^+ concentrations and porewater pH were higher in the central, deepest samples
262 (S2), and lower in S1 and S3.

263

264 **Bacterial and Archaeal 16S Abundance**

265 16S rRNA genes abundance showed the lowest values in RG samples, with an average of
266 9.33 ± 0.24 and 7.51 ± 0.07 Log copies per g of dry weight for Bacteria and Archaea, respectively
267 (Fig. 2, Online Resource 3). Soil was characterized by higher abundances: between 9.96 and 11.15
268 Log copies per g of dry weight for Bacteria, and between 7.99 and 8.88 Log copies per g of dry
269 weight for Archaea. A similar range in 16S genes abundance was also reported for pond sediments,
270 with bacterial abundance ranging between 9.64 and 11.01 Log copies per g of dry weight, and
271 Archaea between 6.97 and 8.89 Log copies per g of dry weight. In general, in all the environmental
272 matrixes 16S bacterial markers outnumbered their Archaea counterpart by at least 1 or 2 orders of
273 magnitude.

274

275 **Bacterial and Archaeal Community Diversity**

276 A total of 6,131,017 high-quality sequences were obtained after trimming and filtering
277 procedures. 96.5 % of the sequences was classified as Bacteria (63,962 OTUs), 3.5 % as Archaea
278 (2478 OTUs), and a negligible number remained unclassified at domain level.

279 Analysis of rarefaction curves (Online Resource 2) confirmed that a satisfactory level of
280 coverage was achieved for bacterial sequences (with Good's Coverage estimator values higher than
281 94%) in all the samples, while lower coverage values were reported in sediment samples for archaea
282 (average 80%). This is coherent with trends in alpha diversity estimators (Fig. 3, Online Resource
283 3) applied to the archaeal community, showing a decrease in richness (OTUs number, Chao1
284 index), Shannon and Simpson diversity indexes from sediment to soil, to RG communities.
285 However, comparing between sediment samples only, lower diversity characterized samples S3,
286 particularly in 2015. For Bacteria, shallow sediments (S1, S3) showed higher Chao1 values if
287 compared with RG and G samples, but they did not differ significantly in terms of observed OTUs,

288 Shannon and Simpson indexes. Conversely, sample S2 always reported lower richness and diversity
289 relative to all the other samples.

290 For beta diversity, NMDS ordination plots based on different distance estimators all showed
291 a strong separation among communities inhabiting different environmental matrixes, while no
292 apparent differences in terms of sampling year were detectable (Fig. 4). In particular, for archaeal
293 community, Bray-Curtis and Unweighted UniFrac distances defined a sharp separation among pond
294 sediment, RG and soil samples, while Weighted UniFrac suggested higher similarity between soil
295 and RG communities. For bacteria, all the tested distances produced separate clusters corresponding
296 to different sample typology and were also able to divide shallower sediment samples (S1, S3) from
297 the deeper sediment sample S2. PERMANOVA analysis on Bray-Curtis, Unweighted and Weighted
298 UniFrac distance matrixes confirmed the presence of significant differences ($\text{Pseudo-}F_{(4,28)} \geq 8.0$,
299 $P < 0.001$; Online Resource 3) in archaeal and bacterial community structure among different
300 environmental matrixes.

301

302 **Composition of Archaeal and Bacterial Communities**

303 The taxonomic composition of the archaeal community was clearly differentiated based on
304 sample type (Fig 5a, Online Resource 4). Terrestrial ecosystems were dominated by
305 Thaumarchaeota, representing approximately 90 % and 80 % of RG and G sequences respectively.
306 However, while RG surface material community included mainly members of the Soil
307 Crenarchaeotic Group (SCG), a pool of additional classes (e.g. FHMa11terrestrialgroup,
308 SAGMCG-1) was also present in the soil samples. Moreover, by examining in detail the sequences
309 affiliated with SCG, a separation between RG and soil samples in terms of dominant OTUs
310 emerged. Thaumarchaeota were also detected in sediment samples, but only represented by the
311 Marine Group I class, with one dominant OTU identified as *Cand. Nitrosoarchaeum* accounting for
312 80-99% of total Thaumarchaeota sequences.

313 The most represented phyla in the pond sediment samples were Woesearchaeota (49-63 % of
314 sequences per sample) and Euryarchaeota (16-38 %), the latter including mainly Thermoplasmata
315 and Methanomicrobia classes. Despite the presence of Thermoplasmata in all the samples,
316 communities associated to different environmental matrixes showed a clear separation at family
317 level.

318 For bacterial community (Fig. 5b, Online Resource 4), all the samples across all habitats
319 harboured essentially the same classes, but variations in their relative abundance distinguished
320 communities belonging to different environmental matrixes. In particular, soil community differed

from RG and sediment community due to significantly higher proportions of Alphaproteobacteria, Acidobacteria (Acidobacteria, Solibacteres) and Planctomycetes (Planctomycetacia) and lower proportions of Betaproteobacteria (including mainly Burkholderiales, Nitrosomonadales and SC-I-84 classes). Distinctive features were also detectable by comparing RG and sediment samples: sediment community was characterised by higher Cyanobacteria, Deltaproteobacteria and Ignavibacteria relative abundances, while RG samples showed higher proportions of Actinobacteria (Thermoleophilia, Actinobacteria belonging to Frankiales, Micrococcales and Propionibacteriales), Acidobacteria (Blastocatellia) and Bacteroidetes (Sphingobacteriia). By comparing only sediment samples, some microbial groups appeared to follow peculiar trends. Cyanobacteria proportion, for instance, appeared to be higher in central (S2) relative to shallower sediment samples (S1, S3), showing also a differentiation in terms of dominant OTUs, while an opposite trend was reported for Thaumarchaeota and Acidobacteria. Moreover, some classes (e.g. Gemmatimonadetes, Blastocatellia) exhibited a reduction in percent abundance along a distance gradient moving from the RG front.

Finally, samples S3 collected in 2015 displayed anomalous characteristics in terms of community composition, if compared with all other sediment samples, including the three collected in the same point in 2016. For instance, they harboured lower proportions of Bathyarchaeota and Methanomicrobia, and higher proportions of Thermoplasmata (dominated by a single OTU) and Betaproteobacteria belonging to the Oxalobacteraceae family.

Indicator Species Analysis

A total of 55 and 780 OTUs significantly associated to a single sampling point or to a combination of sampling points was retrieved for Archaea and Bacteria, respectively (Online Resource 5).

In the case of Archaea, the analysis showed (Fig. 6a) a sharp separation between indicator OTUs associated with terrestrial and lacustrine habitats, without indicator OTUs shared between the two groups. In the pond sediments, the highest number of indicator OTUs was reported for the sampling point S2 (12 OTUs), followed by the combination S1+S2 (7 OTUs).

A more complex network of associations among different sampling points and habitats was reported when considering bacterial OTUs (Fig. 6b). A considerable number of indicator OTUs was found to characterise the different habitats ($G = 121$; $RG = 103$; $S1+S2+S3 = 163$). Investigating the relationships between the sediments (S1+S2+S3) and the terrestrial habitats, the highest number of indicator OTUs was found to be shared with RG (69 OTUs), while only 8 OTUs were shared

with G, and no OTUs were shared with both RG+G. Moreover, comparing different groups of sediment samples, the combination S1+S3 was the one with the highest number of indicators shared with all the terrestrial habitats. Finally, focusing on single sediment samples, sampling point S3 showed the highest number of indicator OTUs shared with both RG (24 OTUs) and the combination RG+G (10 OTUs). The majority of them was closely related to indicator OTUs found for the S1+S2+S3 and S1+S2+S3+RG combinations, but OTUs affiliated to Intrasporangiaceae and Methylophilaceae were exclusively found to be representative of the combination S3+RG (Online Resource 5).

362

363 Discussion

Despite the increasing interest on the effects of RG thawing, only few studies have considered its consequences on the ecology of downstream ecosystems [8, 9, 14, 15]. In this study, we sampled different compartments of an alpine RG-pond system to: 1) investigate their microbial community structure and identify the key microbial groups that characterise each habitat; 2) assess the potential links between microbial diversity and geochemistry; 3) propose microbial markers potentially indicating the presence of RG influence on connected freshwater ecosystem.

370

371 Microbial Community Structure in the Different Habitats of an Alpine RG-Pond System

In general, beta diversity analysis highlighted a sharp separation among communities associated with different environmental matrixes, for both archaea and bacteria.

Soil bacterial community was dominated by Acidobacteria and Proteobacteria, followed by Planctomycetes, Chloroflexi, Actinobacteria and Verrucomicrobia, as previously reported for several alpine and high latitude tundra soils [42-44]. However, while these studies included Cyanobacteria among the most represented phyla, in our case cyanobacterial sequences represented less than 1% of the total soil sequences. The archaeal community from soil also corroborated with other studies where a prevalence of Thaumarchaeota sequences, all affiliated with terrestrial groups, was found, followed by Euryarchaeota of the class Thermoplasmata [45-47].

Considering lacustrine sediments, a recent survey on 13 freshwater lakes of the Yunnan Plateau (southwestern China) highlighted that bacterial community composition can remarkably vary in terms of dominant phyla among different high elevation freshwater lakes, although factors governing the intra-lake distribution of sediment bacterial and archaeal communities remain unclear [48]. However, some common traits appear to be conserved among the abovementioned lakes and

386 the Col d'Olen Rock Glacier pond bacterial community structure, with Proteobacteria
387 (Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria), Planctomycetes (in Olen
388 sediments mainly Phycisphaerae and Planctomycetacia) and Chloroflexi being the dominant groups.
389 Nevertheless, other phyla often poorly represented in the Zhang et al. study, like Nitrospira,
390 Gemmatimonadetes and Ignavibacteria had higher relative abundances in the Col d'Olen Rock
391 Glacier pond.

392 Freshwater ecosystems have been reported to harbour, on average, more rich and diverse archaeal
393 communities if compared with soil [49], and this was also the case of Olen pond system, and
394 differences in dominant archaeal taxonomic groups among alpine lacustrine ecosystems exist. For
395 instance, sediment communities dominated by Bathyarchaeota and methanogenic Euryarchaeota
396 (Methanomicrobia, Methanobacteria and Thermoplasmata classes) have been described in lakes of
397 the Yunnan and Tibetan Plateau in China [48, 50, 51], or in a number of water systems in the
398 Iberian Peninsula [52, 53]. However, even if 16-38% of total archaea sequences affiliated to
399 Euryarchaeota (mainly Methanomicrobia), Olen pond showed a quite different situation, with only
400 small proportions of Bathyarchaeota (0.2-4%) and a prevalence of Woesarchaeota (49-63%).
401 Analogous archaeal community composition has previously been described in waters of high-
402 altitude Pyrenean lakes [54].

403 The RG fine-grained surface material offered the harshest conditions for life of all the
404 investigated compartments, probably due to a combination of physical instability and lack of
405 vegetation cover. The low TOC and TN and the high C/N ratio, potentially indicating limited rates
406 of mineralization, seem to confirm the impact of such environmental constraints on resident
407 microbial communities. However, these challenging conditions seem to influence in different ways
408 Bacteria and Archaea. Bacterial abundance was significantly lower in the RG samples than in soil
409 or sediments, but community richness and evenness were comparable to the other studied habitats.
410 Conversely, for Archaea no significant differences were detected between RG and sediments for the
411 16S copy number, but alpha diversity was lower in RG than in sediments. This might suggest the
412 presence of a less diverse but highly specialized archaeal population, able to proficiently colonise
413 the RG surface. For instance, Thermoplasmata in RG samples included mainly representatives of
414 Marine Group II plus two highly abundant unidentified OTUs, while soil was dominated by
415 sequences of the Terrestrial Miscellaneous Group (TMEG) and sediments showed more complex
416 assemblages. This is further corroborated by the indicator species analysis.
417 The comparison of the bacterial community between different habitats showed that the RG
418 community was more closely related to sediment than soil, where proportions of phyla typically

419 associated to terrestrial habitats like Acidobacteria, Actinobacteria and Alphaproteobacteria were
420 highly represented. If the separation between RG and soil communities may be partially attributed
421 to differences in lithology (serpentine vs calcschist), as demonstrated in other alpine, cryoturbated
422 soils [55], the different stage of habitat evolution is also likely to play an important role. The
423 presence of Sphingobacteriales, Actinobacteriaceae affiliated to Micrococcales and
424 Propionibacteriales, and Comamonadaceae in the RG matrix resembled other high elevation, poorly
425 evolved soil ecosystems, such as unvegetated glacier forefields [56] and debris-covered glaciers
426 [57].

427

428 **Trends in Geochemistry and Microbial Community Structure**

429 In this study, we also aimed to describe variations in microbial community composition and
430 abundance across the pond, in relation to sediment geochemistry. The choice of only three sampling
431 points at two different depths in the pond system and the focus on just one sampling time in the
432 snow-free season, albeit repeated for two consecutive years, prevented detailed definition and
433 verification of temporal, horizontal and vertical trends. However, the obtained data still offered
434 some insights on the overall ecosystem relations between geochemistry and microbial community
435 composition. For instance, our initial hypothesis was that the presence of RG hydrological inputs
436 into the pond documented by Colombo et al. [17, 20], although limited in time and magnitude,
437 would have led to a differentiation of sediments closest to the RG front from the farther ones.
438 Instead, we found out that the strongest differences in terms of both geochemistry and microbial
439 diversity exist between shallow (S1, S3) and deep (S2) sediments, regardless their distance from the
440 RG front, and this trend seems to be conserved for the two seasons covered by this study. Water
441 depth has been previously reported as one of the factors involved in community shaping in
442 lacustrine and marine sediments [58], but it is interesting to find similar significant differences at a
443 small scale (2 m of variation in water depth, about 30 m of horizontal distance), in a well-mixed,
444 not stratified lacustrine ecosystem. Among the Archaea, the reported separation pond centre-borders
445 is limited to the Thaumarchaeota phylum, which showed higher relative abundance in shallow than
446 deep sediments. The absolute prevalence in sediments of sequences affiliated to Marine Group I and
447 identified as *Candidatus Nitrosoarchaeum*, commonly found in freshwater ecosystems [52, 59],
448 excludes the hypothesis of an accumulation of microorganisms or DNA from surrounding
449 compartments (richer in Thaumarchaeota sequences, but mainly affiliated to others classes),
450 suggesting that factors regulating their distribution must depend on lake intrinsic dynamics. This is
451 also in accordance with the absence of indicator OTUs shared between any pond sediment and G or

RG samples. Another picture of intra-pond variation is offered by the analysis of the bacterial community structure and composition. On the one hand, the shallow sediment samples were clearly separated from the deepest ones in terms of overall community structure (as shown by NMDS analysis based on both phylogenetic and not phylogenetic distances) and showed the highest number of indicator OTUs shared with G and RG samples, potentially indicating stronger relationships with the terrestrial habitats. On the other hand, the deepest samples harboured higher proportions of cyanobacterial sequences than the shallow ones, mainly corresponding to a single OTU showing 100% sequence similarity with *Cyanobium* species and uncultured Cyanobacteria detected and isolated from the Baltic Sea [60] and lacustrine sediments [61]. Interestingly, the relative abundance of such OTU decreases in shallower sediments, where other phylotypes occupy higher proportions of the cyanobacterial sequences pool. The presence and activity of Cyanobacteria in marine and lacustrine sediments at depths far higher than 3 m, where the penetration of solar radiation is limited, has been widely reported in literature [62-65]. Moreover, several studies described how microalgal and cyanobacterial community structure and diversity in freshwater lake sediments vary in relation to water depth, often as a consequence of disturbance events affecting shallow sediments, i.e. drying-rewetting or exposure to freezing [66-68]. Therefore, the variations observed in Olen Pond in terms of Cyanobacteria relative abundance and community composition may be consistent with the potentially more stable conditions offered throughout the year by the pond deepest zone, apparently not affected by water freezing phenomena that regularly occur in the lateral, shallower areas.

472

473 **Microbial Markers and Localised Anomalies in Alpine RG-Pond Systems**

474 In the last part of this study, we have considered possible candidates for microbial markers
475 that can be used to indicate the presence of RG inputs in a connected alpine freshwater ecosystem.
476 Both the indicator species analysis and the community composition data showed the existence of a
477 stronger link between sediment and RG habitat than between sediment and soil, at least for the
478 bacterial community. However, when formulating our hypothesis, we pointed out that the
479 hydrochemical influence of the RG on the overall Olen pond is limited in frequency and magnitude
480 and, as demonstrated by previous studies [17, 20], it is not apparently affecting the entire pond in
481 terms of hydrology and water chemistry. Therefore, a different explanation for the links between
482 pond sediment and RG bacterial communities should be looked for, and to considerate the issue
483 under a geomorphological point of view may be useful in this sense. From an exclusively
484 geomorphological standpoint, RGs constitute prominent sedimentary linkages within the alpine

485 environment, deeply contributing to alpine erosion [69]. Although they are chiefly considered one
486 of the main storage components of the coarse debris system [70], the amount of fine-grained
487 material in the RG interior can be relevant (Haeberli et al., 2006), as also observed in the frontal and
488 lateral scarps of the Col d'Olen Rock Glacier. Thus, since the RG offers a proportion of mobile
489 fine-grained material (cf. [71]) far higher than the surrounding vegetated soils, undergoing slower
490 rates of erosion (cf. [72]), we can expect to find an accumulation of such material on the bottom of
491 the pond in which the RG front is ending. As previously mentioned, the lithological characteristics
492 of the substrate are known to strongly influence microbial communities structure and composition
493 [73-75], thus the occurrence of RG sedimentary deposits in the pond may explain the analogies in
494 the dominant bacterial phyla/classes proportions and indicator OTUs between pond sediments and
495 RG samples.

496 Indicator analyses on the sampling point closest to the RG inflow (S3), where we expected
497 to detect a possible effect of RG hydrochemical contribution on the sediment communities, showed
498 a higher number of indicator OTUs shared with RG in comparison to the other sediment samples.
499 Nevertheless, the presence of OTUs closely related to phylotypes characterising the overall
500 sediment habitat or the combination sediments + RG did not allow to clearly confirm the link
501 between their occurrence and the presence of RG meltwater inputs.

502 In this study we also detected anomalies in the S3 samples collected in 2015, that could be
503 interpreted simply as a product of random interannual variability, but might also suggest that in
504 some circumstances the RG hydrochemical influence can be enhanced and become apparent.
505 Considering purely physical and chemical data, the waters in point S3 between mid-August and
506 mid-September 2015 were clearly differentiated from all the other sampling points across the pond
507 with colder water temperatures, higher electrical conductivity and higher solutes (including nitrate)
508 concentration. Colombo et al. [17, 20] attributed this evidence to a phase of high RG hydrological
509 contribution. Concerning the anomalies described in this study for samples S3_15, many of them
510 are in accordance with the physical and chemical observations. The higher nitrate concentrations in
511 sediments are coherent with those measured in waters. The increase in the relative abundance of
512 Thermoplasmata, with around 96% of sequences corresponding to a single unidentified OTU
513 present in lower proportions in all the other pond sediments samples, as well as the lower archaeal
514 diversity, are consistent with the possibility of a localised selection process, directly or indirectly
515 driven by RG influence. Finally, the higher relative abundance of Oxlobacteraceae, belonging to the
516 order Burkholderiales, that include many chemolithotrophic and autotrophic microorganisms

517 frequently associated with glacial and subglacial environments [57, 76], is in line with the
518 hypothesis of a localised, meltwater-driven microbial enrichment.

519 The absence of similar characteristics in the sediment samples S3 collected in 2016 could be
520 linked to the sampling, performed earlier in the snow-free season in comparison to 2015. Indeed,
521 Colombo et al. [20] showed that geochemically-enriched icemelt is likely to be exported from the
522 RG towards the late snow-free season (August/September), after the winter snowpack melting and
523 prolonged periods of atmospheric temperature above 0 °C. Unfortunately, the lack of data on water
524 temperature, electrical conductivity and solutes concentration for 2016 does not allow to verify if
525 the described anomalies represent actual markers of RG influence emerging as a response to
526 stronger input events, rather than products of random interannual variability. Due to the limited
527 amount of information on this subject, we believe these observations may represent useful hints for
528 future investigations.

529

530 In conclusion, our results showed that different habitats of the Col d'Olen RG-pond system
531 harboured different, well separated archaeal and bacterial communities. They also suggest that the
532 presence of meltwater inputs from an active RG has only a limited influence on the abundance and
533 diversity of microbial communities inhabiting sediments of an adjacent water body. Indeed, no clear
534 evidence of a stable modification of microbial ecology or sediments geochemistry in the area
535 closest to the predicted RG inflow has been found. Rather, factors involved in stably shaping
536 microbial diversity and distribution in the overall pond system seem to be more linked to the
537 potential of the RG to supply new sedimentary material or to other dynamics intrinsic to the pond,
538 such as differences in water column depth and exposition to freeze-thaw processes.

539

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777 Figure Captions

778 **Fig. 1** Location of the study area (a) and positioning of the sampling points (b, c) in the Col d'Olen
779 Rock Glacier Pond and neighbouring compartments. In S1, S2 and S3 sediments and porewater
780 were sampled and analysed. In RG and G fine material (0-10 cm depth) was sampled

781 **Fig. 2** Geochemical properties (a-h), and abundance of 16S rRNA genes, quantified by qPCR for
782 Archaea (i) and Bacteria (j) on RG, sediment (S1-S3) and soil (G) samples. In boxplots boxes
783 represent the interquartile range between the first and third quartiles, whiskers indicate the range of
784 measurements and bold bars show sample medians. In bar charts and dot plots bars and points
785 correspond to sample means and error bars represent the standard error of the mean. Different
786 colours correspond to different years (dark gray: 2015; light gray: 2016). Different letters indicate
787 significant differences ($P < 0.05$) among samples collected in the same year assessed with Tukey
788 HSD (a-g, i-j) or Dunn *post hoc* tests (h).

789 **Fig. 3** Alpha diversity estimators calculated for archaeal (a-e) and bacterial (f-j) communities in the
790 different sampling sites and times. Boxes represent the interquartile range between the first and
791 third quartiles, whiskers indicate the range of measurements and bold bars show sample medians.
792 Different letters indicate significant differences ($P < 0.05$) among samples collected in the same
793 year assessed with Dunn's *post hoc* tests.

794 **Fig. 4** NMDS ordination based on Bray-Curtis, Unweighted and Weighted UniFrac distance
795 matrixes calculated using total archaeal and bacterial OTUs

796 **Fig. 5** Relative abundance of the major archaeal (a) and bacterial phyla (b), with Proteobacteria split
797 in classes, in the different sampling points and times. Each bar represents the average of three
798 replicate samples. The group 'others' include all the taxonomic groups not explicitly reported in the
799 legend

800 **Fig. 6** Networks showing significant associations ($\text{IndVal} > 0.90$; $P_{\text{FDR}} < 0.05$) between different
801 sampling points and archaeal (a) or bacterial (b) indicator OTUs assessed by indicator species
802 analysis. For each group of associations, OTUs are represented as grouped in phyla/classes, and the
803 actual number of OTUs is reported beside. The dimension of circles size is proportional to the
804 number of representative OTUs included and the colour reflects the taxonomic affiliation. The
805 length and thickness of the edges connecting OTUs and sampling points is not proportional to any
806 evaluated parameter.

Figure 3

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Figure 5

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Figure 6

Table 1 Description of the sampling sites

Code	Environmental matrix	Dominant lithology	Depth (m)	n. of replicates
S1	Lacustrine sediment	Serpentinite	1	3
S2	Lacustrine sediment	Serpentinite	3	3
S3	Lacustrine sediment	Serpentinite	1	3
RG	RG fine grained surface material	Serpentinite	-	3
G	Soil	Calcschist	-	3

Table 2 Chemical characterisation of porewaters extracted from pond sediments. The maximum and minimum values measured for each year are reported

	2015	2016
pH	5.70 – 8.50	7.10 – 7.90
Si (mg L ⁻¹)	0.25 – 3.89	0.62 – 1.82
Na ⁺ (mg L ⁻¹)	0.36 – 1.72	0.37 – 0.75
K ⁺ (mg L ⁻¹)	0.96 – 4.60	1.01 – 4.52
Ca ²⁺ (mg L ⁻¹)	5.61 – 50.9	2.66 – 14.7
Mg ²⁺ (mg L ⁻¹)	2.50 – 22.4	1.02 – 4.02
Cl ⁻ (mg L ⁻¹)	0.68 – 4.98	0.11 – 1.52
NO ₂ ⁻ (mg L ⁻¹)	0.01 – 0.06	0.01 – 0.03
NO ₃ ⁻ (mg L ⁻¹)	0.01 – 4.46	< 0.01 – 0.05
SO ₄ ²⁻ (mg L ⁻¹)	1.67 – 11.4	< 0.01 – 17.6
PO ₄ ³⁻ (mg L ⁻¹)	0.01 – 0.02	0.01 – 0.04