

ORIGINAL ARTICLE

Possible interactions between bacterial diversity, microbial activity and supraglacial hydrology of cryoconite holes in Svalbard

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The diversity of highly active bacterial communities in cryoconite holes on three Arctic glaciers in Svalbard was investigated using terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA locus. Construction and sequencing of clone libraries allowed several members of these communities to be identified, with *Proteobacteria* being the dominant one, followed by *Cyanobacteria* and *Bacteroidetes*. T-RFLP data revealed significantly different communities in holes on the (cold) valley glacier Austre Brøggerbreen relative to two adjacent (polythermal) valley glaciers, Midtre Lovénbreen and Vestre Brøggerbreen. These population compositions correlate with differences in organic matter content, temperature and the metabolic activity of microbial communities concerned. No within-glacier spatial patterns were observed in the communities identified over the 2-year period and with the 1 km-spaced sampling. We infer that surface hydrology is an important factor in the development of cryoconite bacterial communities.

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Introduction

Glaciers cover ~10% of the earth's land area, and host diverse viral, prokaryotic and eukaryotic life-forms (Gunde-Cimerman *et al.*, 2003; Christner *et al.*, 2003a; Anesio *et al.*, 2007). Ironically, it is only as scientific interest has focused more closely on glaciers as a consequence of their vulnerability to climate change (Lemke *et al.*, 2007) that the perception of glaciers as inhospitable masses of ice is being displaced by the recognition of their biological diversity and an appreciation of the biogeochemical consequences of such activity (Hodson *et al.*, 2008).

Cryoconite holes are the most biologically active habitats within glacial ecosystems (Sävström *et al.*, 2002). High Arctic glaciers often contain significant amounts, up to 36 gm⁻² of cryoconite sediment (Hodson *et al.*, 2007), with cryoconite holes covering up to 6% of the glacier surface (Fountain *et al.*, 2004). These cylindrical holes are formed on the ice

surface by localized melting that is caused by the reduced albedo associated with the progressive accumulation of dark-coloured supraglacial material (Gerdel and Drouet, 1960; Takeuchi *et al.*, 2001; Takeuchi, 2002).

Arctic and Alpine holes are generally open to the atmosphere during each summer, permitting the exchange of gases, nutrients and biological propagules (Hodson *et al.*, 2007). In contrast, Antarctic cryoconite holes, most extensively studied on glaciers in the McMurdo dry valleys, commonly become lidded over by ice, insulating the contents from the influence of atmosphere and meltwater for as long as 10 years (Tranter *et al.*, 2004; Hodson *et al.*, 2008). In both cases, these cylindrical melt holes comprise pelagic (meltwater) and benthic (granular organic/mineral sediment) zones (Gerdel and Drouet, 1960).

On the basis of measurement of photosynthesis and respiration rates of cryoconite holes on glaciers in Svalbard, Greenland and the Alps, Anesio *et al.* (2009) have estimated that the net primary production of cryoconite habitats may be as much as 64 000 Tyr⁻¹ of carbon, challenging previous assumptions that glacial ecosystems are heterotrophic and dependent on an Aeolian flux of allochthonous organic matter (Stibal *et al.*, 2008).

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Most of this biological activity and biomass can be found in the cryoconite sediment. Photosynthesis rates of nearly $160 \mu\text{g Cl}^{-1} \text{h}^{-1}$ in the sediment (> 10 -fold greater than the meltwater above it) have been documented in High Arctic cryoconite holes (S awstr om *et al.*, 2002). Similarly, concentrations of bacteria, virus-like particles and nanoflagellates are higher in cryoconite sediment. Examinations of cryoconite organisms by S awstr om *et al.* (2002) and Porazinska *et al.* (2004) found that several bacterial taxa, in addition to ciliate, metazoan and algal species were present.

There is currently only one published culture-independent study of bacterial diversity in cryoconite sediment, in which Christner *et al.* (2003b) sequenced 16S rRNA genes taken from an Antarctic cryoconite hole. Despite the importance of this study, it raises further questions relating to the means whereby bacteria colonize cryoconite holes and the dynamics of bacterial communities, both temporally, between different seasons or years, and spatially, between different holes (within and between glaciers). During the ablation season, cryoconite material in open holes can be redistributed over the glacier surface by various hydrological processes, and also transported off the glacier to proglacial areas (Hodson *et al.*, 2008).

In this study, we have combined genetic fingerprinting by terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997) with sequencing of 16S rRNA gene clone libraries to examine the distributions of bacterial communities in cryoconite holes from three adjacent glaciers in the high Arctic. We were particularly interested

in detecting changes in community structure as a result of spatial and temporal variation, as well as in relation to the levels of microbial activity.

Methods

Field sites and sampling conditions

Sampling was conducted during July 2006 and 2007 on two polythermal valley glaciers (Midtre Lov enbreen (ML) and Vestre Br ggerbreen (VB)), and one cold valley glacier (Austre Br ggerbreen (AB)) located near the Ny  lesund research station at Spitsbergen in the high Arctic archipelago of Svalbard ($78^{\circ}55'N$ $11^{\circ}56'E$). In all, 36 samples were collected from these three glaciers during the sample-collection periods (six cryoconites were sampled at each glacier each year). Cryoconite samples were harvested by aspirating debris with a sterile 50 ml syringe into sterile tubes. The location of sampling sites (Figure 1) was recorded in relation to ablation stakes planted by the Norsk Polar Institutt's annual mass-balance monitoring campaigns on AB and ML, and by the global positioning system.

Sample handling and DNA extraction

Samples were transferred to the NERC Arctic Research Station at Ny  lesund before being frozen until transported by air, and frozen in an insulated container with freeze blocks, until archived at -80°C in Aberystwyth. DNA extractions were conducted aseptically using 250 mg (fresh weight) sub-samples of cryoconite sediment using the

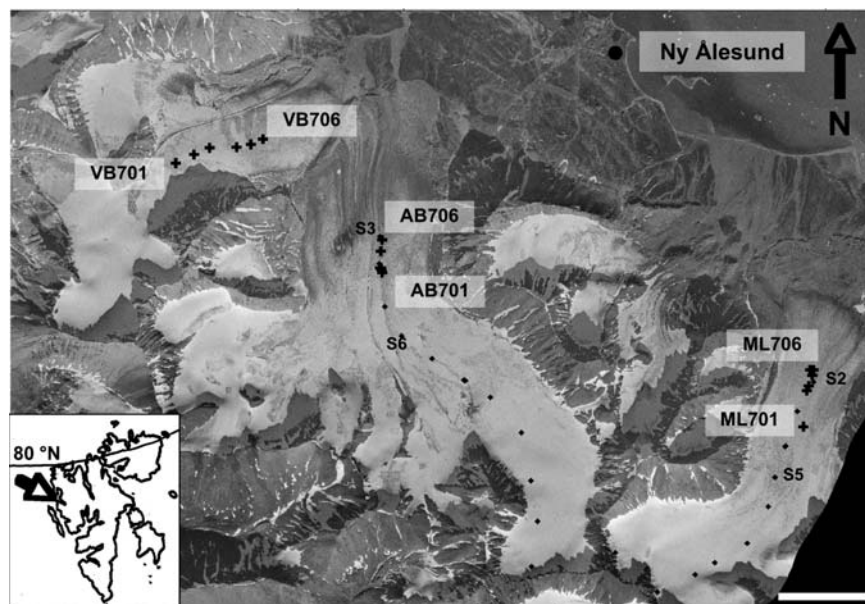


Figure 1 Ortho-rectified aerial photograph from 1977 (S77 0731, 0732, Norwegian Polar Institute) showing glaciated areas in the area of Ny  lesund (courtesy of Tim James, Swansea University, Wales, UK) with the location of the study area on Svalbard indicated (inset). The positions of the cryoconite holes sampled in 2007 are indicated by crosses (top and bottom holes are labeled). The positions of ablation stake lines on glaciers AB and ML are indicated by diamonds. The upper and lower extents of sampling in 2006 are indicated by stake labels (AB: S3–S6; ML: S2–S5). Scale bar indicates 1 km.

MoBio Laboratories (Solana, CA, USA) PowerSoil DNA extraction kit, as directed by the manufacturer, with DNA being eluted from the spin columns in a final volume of 100 µl elution buffer.

T-RFLP and clone libraries

For T-RFLP, PCR amplification of bacterial DNA extracted from all 36 of the sampled cryoconites was conducted with bacterial primers 27F (5'-tagged with fluorophore Cy5) and 1389R and analyzed by capillary electrophoresis, after restriction digestion with *Hae*III. Primers specific for archaea (Arch021F/Arch958R) and methanogenic archaea (mcrAF/mcrAR) were also tested. Detailed methodology for T-RFLP and PCR with archaea-specific primers is given in Supplementary Data S1.

For clone libraries, DNA extracted from the sediment of 12 cryoconites (a subset of two per glacier per sampling year, as indicated in Table 1) was used as template in real-time PCR reactions with the primers 27F and 1389R to crudely estimate differences in bacterial abundance (as described in Supplementary Data S1) prior to use as template DNA for clone libraries. For each glacier, one clone library was constructed after pooling of 16 PCR products (four replicates for four samples; two holes per year). For each clone library, 32 clones were sequenced. Further details of the clone library methodologies are given in Supplementary Data S1.

Measurement of microbial activity

Primary production (¹⁴C incorporation) and community respiration (oxygen consumption in dark incubations) were measured for several of the cryoconite holes sampled in 2006 for analyses of bacterial diversity. Six cryoconites from AB and three each from ML and VB were sampled. Depth and area measurements using photographs processed by Cell[^]Five software (Olympus Software Imaging Solutions) were also made.

For measurement of net primary production, 2 ml of cryoconite sediment was collected with a syringe into 80-ml Whirlpak bags (Nasco, Fort Atkinson, WI, USA). In total, 10 µl of NaH₂¹⁴CO₃ (370 KBq activity) was added to three 100 ml replicates, which were incubated *in situ* (unshaded on the glacier surface) for 24 h, with two parallel samples wrapped with aluminium foil serving as dark controls. Immediately after incubation, bags were transported to the laboratory and their contents filtered onto pre-weighed Whatman glass fiber filters (GF1; 47 mm diameter), acidified with HCl and dried overnight. Samples were counted with an LS6000IC (Beckman Coulter Inc., Brea, CA, USA) scintillation counter with internal quench curve. Dissolved inorganic carbon at the beginning of the incubations was calculated according to titration of alkalinity. Both net primary production and dissolved inorganic carbon for sediments were calculated on a

Table 1 Cryoconite holes sampled during this study during 2006 and 2007 on glaciers AB, ML and VB

Sample code	Physico-chemical data	Clone Library	T-RFLP No. of peaks	T-RFLP Shannon diversity index (H')	T-RFLP Gini coefficient (G _{corr})
AB601	+	+	ND	ND	ND
AB602	+		42	4.12	0.340
AB603	+		16	2.657	0.389
AB604	+		32	3.53	0.430
AB605	+		9	2.129	0.309
AB606	+	+	17	2.845	0.322
AB701		+	35	3.926	0.301
AB702			17	2.751	0.369
AB703			20	2.878	0.422
AB704			7	1.767	0.416
AB705			16	2.709	0.358
AB706		+	12	2.364	0.362
AB mean			20.3	2.88	0.365
ML601	+	+	33	3.53	0.426
ML602	+		47	4.261	0.469
ML603	+		37	3.635	0.500
ML604			12	2.283	0.448
ML605			37	3.679	0.484
ML606		+	39	3.721	0.488
ML701		+	24	3.15	0.406
ML702			15	2.55	0.439
ML703			14	2.537	0.391
ML704			8	1.886	0.419
ML705			13	2.458	0.375
ML706		+	12	2.307	0.407
ML mean			24.3	3.00	0.438
VB601	+	+	43	4.115	0.366
VB602	+		7	1.763	0.421
VB603	+		34	3.767	0.388
VB604			35	3.613	0.445
VB605			21	3.005	0.386
VB606		+	41	3.87	0.440
VB701		+	39	3.892	0.368
VB702			30	3.555	0.376
VB703			24	3.227	0.387
VB704			6	1.532	0.469
VB705			25	3.237	0.366
VB706		+	7	1.775	0.380
VB mean			26.0	3.11	0.399
<i>By glacier</i>					
ANOVA (P)			0.558	0.787	0.001
ANOVA (F)			0.59	0.24	9.24
<i>By year</i>					
ANOVA (P)			0.005	0.015	0.134
ANOVA (F)			8.91	6.56	2.35

ND indicates low quality T-RFLP data.

dry-weight basis. *p*CO₂ was calculated from dissolved inorganic carbon and pH was measured by using the method of Sturmm and Morgan (1970).

Community respiration was monitored by measurements of dissolved oxygen consumption using a dissolved oxygen meter (550A; YSI Hydrodata Ltd, Letchworth, Herts, UK) with a 0.01 mg l⁻¹ resolution. Cryoconite sediment (~10 g) was collected from the bottom of each cryoconite hole and

distributed into triplicate 100-ml Pyrex glass bottles with ground glass stoppers. Initial dissolved oxygen concentration was measured immediately and the bottles were incubated in the dark at *in situ* temperature. Oxygen consumption was converted to carbon, using a respiratory quotient of 1. The debris used in the incubations was also dried overnight at 70 °C and weighed for calculation of community respiration on dry-weight basis.

Inorganic matter content and mineral analyses

The inorganic matter content was analyzed by drying 1 g of cryoconite sediment in pre-dried and weighed crucibles at 105 °C for 48 h. Following cooling in a desiccator, samples were re-weighed (0.1 mg accuracy) and heated to 450 °C in a furnace for 24 h. The ashed material was cooled in a desiccator and re-weighed to determine the organic content.

Grain-size distributions of inorganic debris samples were determined, to a bin resolution of 1 ϕ , by dry sieving fractions coarser than 125 μm and by settling (Micromeritics SediGraph 5100, Norcross, GA, USA) for fractions finer than 125 μm . The finest bin analyzed was 'finer than 1 μm ' (>10 ϕ). Thin sections were prepared of cryoconite sediment, which were embedded in Araldite DBF resin (Huntsman Advanced Materials, Salt Lake City, UT, USA) and mounted on glass slides for examination using a Photomicroscope III polarizing microscope (Zeiss, Oberkochen, Germany) under illumination with both plane and cross-polarized light.

Statistical analyses

Data were initially organized in MS Excel. Analysis of variance was conducted using Minitab 1.13, PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2001) and canonical analysis of principal coordinates (CAP) (Anderson and Willis, 2003) on Bray–Curtis distances of fourth-root log or $\log(X+1)$ transforms of T-RFLP abundance data. PERMANOVA was conducted using default settings with 9999 unrestricted permutations, while CAP was conducted using default settings.

Pareto–Lorenz distribution curves were plotted from the cumulative relative abundance of each T-RF and its cumulative proportion of the number of T-RFs for each cryoconite hole. Gini coefficients (G_{corr}) were calculated geometrically (Dorfman, 1979) by estimating the area under the Lorenz curves by trapezoid approximation using the equation:

$$G = 1 - \left| \sum_{k=1}^n (Y_{k-1} + Y_k)(X_k - X_{k-1}) \right| \quad (1)$$

where Y is the cumulative relative abundance and X is the cumulative proportion of T-RFs. G_{corr} was

corrected to minimize the bias in estimating the underlying population, as it may result in skewed estimations of G_{corr} (Weiner and Solbrig, 1984; Deltas, 2003):

$$G_{\text{corr}} = G \left(\frac{n}{(n-1)} \right) \quad (2)$$

In order to explore spatial relationships between cryoconite holes on the same glacier, matrices of measures of similarity, richness and evenness determined from pair-wise comparison of T-RFLP profiles were generated. For distance–decay relationships of similarity, Bray–Curtis distances were generated using MVSP 3.1, (Kovach Computing Services, Anglesey, Wales, UK). For richness (S , number of T-RFs) and evenness, corrected G_{corr} was calculated for pair-wise means of relative abundances from T-RFLP profiles. These measurements of community structure were tested for correlation against a matrix of geographical distances between cryoconite holes obtained from geographic information system mapping (MapInfo Professional 7.8; <http://www.mapinfo.com>) by conducting Mantel tests using PAST 1.80 (Hammer *et al.*, 2001).

Canonical correspondence analysis (CCA; a constrained ordination method combining multiple regression with the underlying ordination) was conducted using MVSP 3.1, following the guidance of ter Braak (1986). Environmental variables were first standardized against the mean and s.d. of each variable in MS Excel and imported into MVSP for CCA by the Hill algorithm.

Results

T-RFLP analyses

T-RFLP analysis was conducted on 36 samples (six samples in each of the 2 years from each of the three glaciers) but one sample failed to produce an acceptable profile. The number of peaks for bacterial T-RFs (broadly equivalent to 'species richness') within the range of the size markers (60–600 bp) ranged from 6 to 47 per sample and Shannon Diversity Index ranged from 1.53 to 4.26 (Table 1). Both species richness and diversity were higher in 2006 than in 2007 (ANOVA: $P=0.005$, $F=8.91$; $P=0.015$, $F=6.56$, respectively), but there was no significant difference between the three glaciers.

The evenness of the bacterial communities in the holes was described using Pareto–Lorenz distribution curves (De Schryver *et al.*, 2008; Marzorati *et al.*, 2008) and the corrected Gini coefficients (G_{corr}) (Vainio *et al.*, 2005; Halet *et al.*, 2006; Wittebolle *et al.*, 2008) derived for each hole. The Pareto–Lorenz distribution curves obtained by plotting the cumulative relative abundance of each T-RF against its cumulative proportion of the number of T-RFs for each cryoconite hole are shown in Figure 2. G_{corr} derived from these Pareto–Lorenz distribution curves (Table 1) showed that the communities on

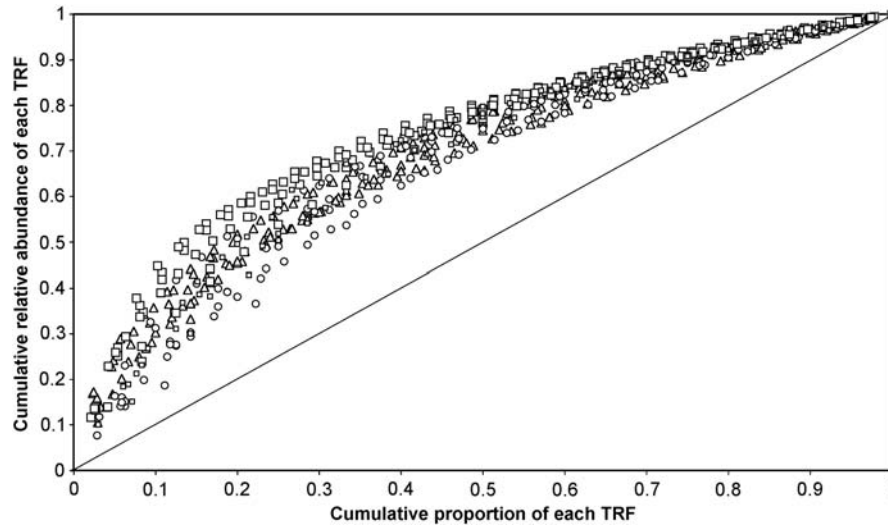


Figure 2 Pareto–Lorenz distribution curves for bacterial T-RF abundance patterns from the three glaciers. Samples from ML cryoconites (squares) show the most uneven distribution of taxa, with AB (circles) showing the most even distribution, and samples from VB (triangles) being intermediate. The diagonal gray line indicates a theoretical even distribution. Gini coefficients derived from Pareto–Lorenz curves for AB samples were significantly different (Tukey’s honest significant difference) from ML and VB samples.

ML were significantly more uneven than those on AB (ANOVA $P=0.001$, $F=9.24$). No significant difference between years was observed but comparison of each glacier–year combination showed that the communities sampled from ML in 2006 were significantly more uneven than those on AB (both years) and VB (2007 only).

To test the hypotheses that: (i) cryoconites on different glaciers harbor distinct bacterial communities and (ii) these communities vary from year to year, we used PERMANOVA, a powerful statistical tool for the hypothesis testing of multivariate datasets (McArdle and Anderson, 2001; Anderson, 2005), along with CAP. CAP, widely used for analysis of T-RFLP data (Nikolcheva and Barlocher, 2005; Gleeson *et al.*, 2008), is a constrained ordination method, which also cross-validates the model it derives.

PERMANOVA analysis of T-RFLP data showed a significant difference in cryoconite bacterial community structures between glaciers ($P=0.0001$; Pseudo- $F=5.34$) and also between years ($P=0.016$; Pseudo- $F=2.74$) but no significant interaction between these factors. Pair-wise testing using PERMANOVA on the basis of T-RFLP data revealed highly significant differences in bacterial community between glaciers, with communities on AB differing from both ML and VB ($P=0.0005$ and $P=0.0001$, respectively) but there being no significant difference between communities on ML and VB ($P=0.0795$). This is consistent with both the clustering patterns observed following CAP ordination (Figure 3) and also detrended correspondence analysis (Supplementary Data S2). The derived model correctly assigns 83.3% (VB), 72.7% (AB) and 83.3% (ML) of samples to the correct glacier (overall 80% correct).

The changes in bacterial communities between 2006 and 2007 were further explored using PERMANOVA

to examine each glacier separately. There was a clear difference between years for ML ($P=0.004$, Pseudo- $F=6.24$), but not for AB and VB ($P=0.233$ and 0.380 , respectively). The mean linear correlation coefficient of log-transformed T-RFLP abundance data for all pair-wise comparisons of all the holes on each glacier was lowest for AB (Supplementary Data S3), suggesting, as indicated by the spread of sites in Figure 3, that the bacterial communities on AB were the most heterogeneous, whereas those on ML and VB were more homogeneous in terms of community structure. Only one cryoconite was unequivocally sampled in both years (the uppermost hole on VB –VB601/VB701). Pair-wise comparisons of T-RF abundance data using Pearson’s product–moment correlation showed that the bacterial communities in these holes were more similar to each other ($r^2=0.834$) than to any other holes sampled.

Spatial variation of cryoconite communities

Global positioning system location of cryoconite holes in 2007 permitted the creation of distance matrices for the holes sampled on each glacier using the geographic information system (Supplementary Data S4). As it was clear that bacterial communities on AB were distinct, spatial relationships between the bacterial communities in the cryoconite holes were investigated separately for each glacier. The mean relative abundance of T-RFLP peaks was calculated pair-wise for all the holes on each glacier. Matrices of mean species richness (number of T-RFs) and G_{corr} were then derived, and Mantel tests were conducted in PAST to assess the correlation between the spatial matrix and the other matrices. A taxon–distance relationship was found for holes on ML and VB but not AB at $\alpha=0.05$ (Table 2). Bray–Curtis distance relationships were also tested

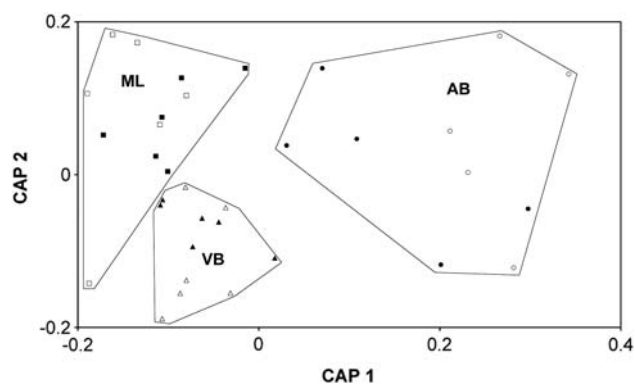


Figure 3 Canonical analysis of principal coordinates (CAP) ordination on the basis of Bray–Curtis similarity indices of fourth-root transformed T-RFLP profiles of bacterial communities from cryoconites on the three glaciers at Svalbard (AB, circles, ML, squares and VB, triangles; filled markers, 2006, empty markers, 2007.) Polygons have been added to define cryoconites from the same glacier.

Table 2 Mantel test correlation between physical distance and community structure

	ML		AB		VB	
	r	P	r	P	r	P
Bray–Curtis distance	0.001	0.007	0.000	0.392	0.002	0.169
Taxa (S)	0.838	0.041	0.044	0.491	0.407	0.047
Gini coefficient (G_{corr})	0.534	0.067	-0.497	0.944	0.233	0.178

r indicates the coefficient of correlation and *P* the significance of this correlation.

between pairs of holes, but no meaningful spatial correlation was found.

Correlation of bacterial diversity with cryoconite hole biogeochemistry

In 2006, measurements were conducted *in situ* and contemporaneously on 31 cryoconite holes, out of which 11 were also subjected to T-RFLP analysis (Supplementary Data S5). Significant differences between glaciers were observed in:

1. Temperature ($P=0.004$), with AB cryoconites showing a higher mean temperature (0.6 °C versus 0.1 °C and 0.2 °C for ML and VB, respectively);
2. percentage of organic content ($P<0.001$; AB significantly lower than ML and VB);
3. sediment primary production ($P<0.001$, AB significantly lower than ML and VB);
4. respiration rate ($P<0.001$; AB significantly lower than ML and VB).

Thus, cryoconite holes on different glaciers also show significant differences in their biogeochemical and metabolic activity, with AB differing from both ML and VB in a manner similar to the variation observed in T-RFLP profiles, and consistent with the findings of Anesio *et al.* (2009) regarding primary production and respiration.

The CCA was used to show whether any of these environmental variables accounted for changes in bacterial cryoconite community structure between holes. All environmental variables measured were initially included in the model but several were removed in a step-wise manner in order to develop a robust model, which explained as much variance as possible while avoiding multi-collinearity and high variance inflation. This model (Figure 4) explained 80.1% of the total variation within the T-RFLP profiles in relation to 9 of 11 environmental variables measured, with 66.5% of the explainable variance being explained within the first three axes (eigenvalues > 0.1: Supplementary Data S6).

Cryoconite temperature, pCO_2 and percentage inorganic content exert the strongest positive influences on environmental axis 1, with respiration and primary production acting most negatively. The impact of pCO_2 can be interpreted in the context of the highly autotrophic nature of cryoconite communities (Anesio *et al.*, 2009). Thus, low pCO_2 would correlate with active cryoconite phototrophs, and, as observed, high primary production. The relatively small temperature differences (<1 °C) are unlikely to exert a strong direct influence on community structure but may reflect differences in rates of meltwater flow into cryoconites, as discussed below.

Respiration rate, temperature and inorganic content also exert contrasting influences on environmental axis 2, but, interestingly, cryoconite volume has a positive influence on axis 2. Volume is a composite of depth and surface area, and is itself largely filled with meltwater, so this could reflect inputs into the cryoconites, in terms of either solar radiation or nutrients within meltwater. In all CCA plots, AB holes are resolved from both ML and VB, reflecting the differences shown earlier for bacterial communities. The dominant environmental influences on AB holes are also different (Supplementary Data S6), with the vectors of temperature, pCO_2 and inorganic matter being localized to the AB sites, while markers of microbial activity are strong influences on ML and VB sites.

As the high inorganic content (84–99% of dry-weight) of cryoconites sampled suggests the dominance of inorganic minerals in the structure of cryoconite sediment, representative samples from each glacier were analyzed to determine the mineral composition and size. Thin sections of cryoconite sediment viewed using polarized light microscopy consisted of a broad range of mineral types (Supplementary Data S7). Cryoconite sediment from hole ML701 contained quartz, white and biotite mica, feldspar and appeared consistent with metamorphic origins, as did a representative sample from hole AB703. Cryoconite sediment from VB701 contained clasts of sedimentary rocks, as well as carbonate, magnetite and biotite mica. In contrast to other samples, the biotite mica appeared to be more degraded in VB701, perhaps as a result of weathering; carbonate grains in the sample also appeared to

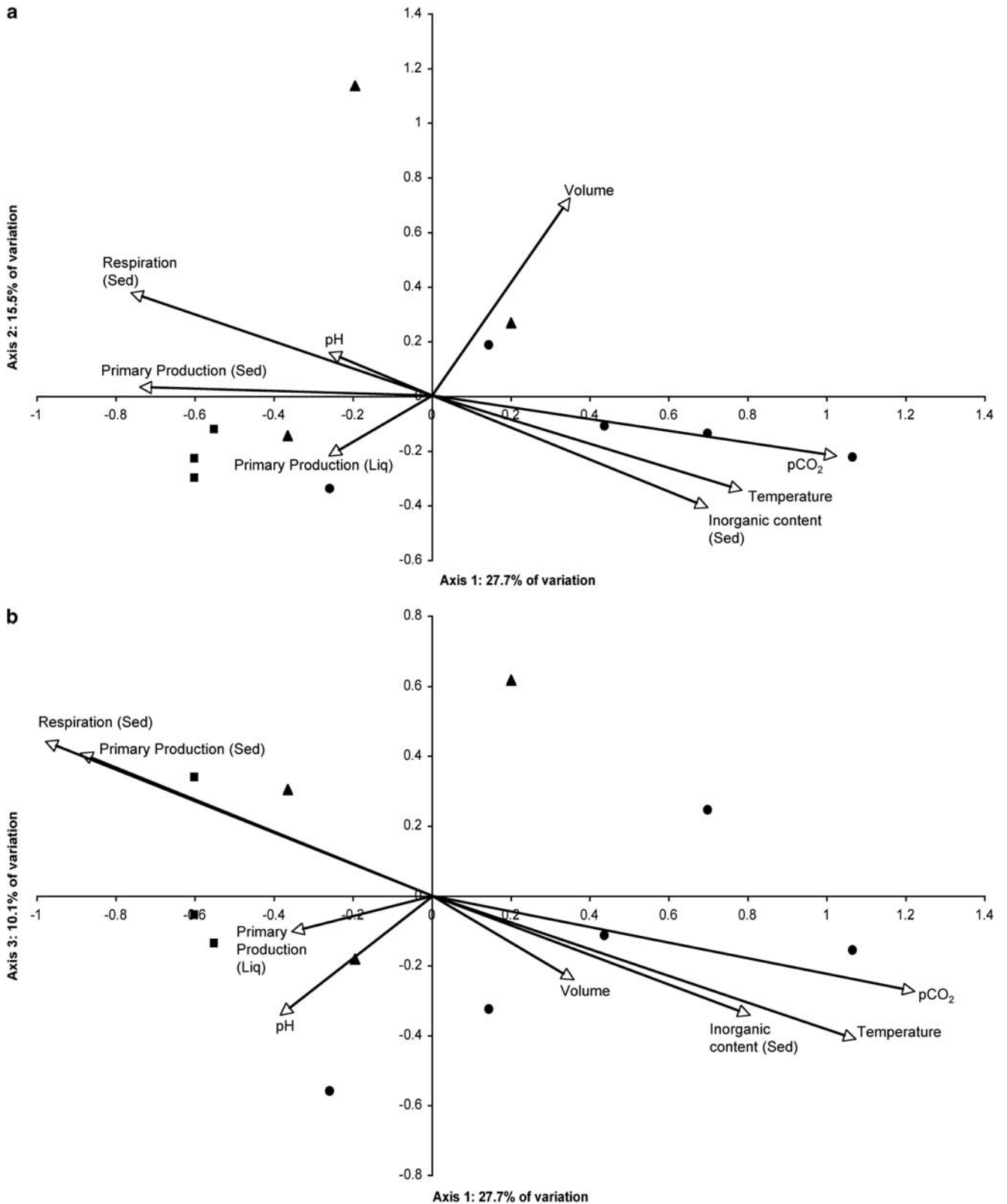


Figure 4 Canonical correspondence analysis of the effect of environmental variables on bacterial TRFs from cryoconite holes sampled in 2006 on AB (circles), ML (squares) and VB (triangles), using a model described by (a) axes 1 and 2 and (b) axes 1 and 3. Axes 1, 2 and 3 explain 27.7, 15.5 and 10.1% of the total variance, respectively.

contain structures resembling biological material. However, in general, the mineral particles in the cryoconite sediments examined had angular edges, suggesting local origin and recent deposition.

Particle size analysis conducted on pooled cryoconite ashes from holes sampled in 2006 showed that the particles from AB were finer than those taken from the other glaciers (Supplementary data S7). Calculation of overall surface area:volume ratio for the particle size profile found that this was $944 \text{ mm}^2 \text{ mg}^{-1}$ for AB, $715 \text{ mm}^2 \text{ mg}^{-1}$ for VB and $563 \text{ mm}^2 \text{ mg}^{-1}$ for ML. Thus, the expected correlation between finer particles and increased biological activity (that is, greater surface area for biofilm formation and weathering; see above and Supplementary Data S5) was not found.

Clone library diversity

Bacterial 16S sequence data were obtained for 90/96 clones, after removal of potential chimeric sequences using Bellerophon (Huber *et al.*, 2004) and Pintail (Ashelford *et al.*, 2005). The sequences have been deposited at the Genbank under accession numbers FN824532–FN824621. Sequence similarity (% identity) was determined using SeqMatch and alignment of the clones identified 54 OTUs at the 97% identity cutoff threshold (Supplementary Data S8). Nearly all OTUs (47/54) were matched to existing RDP-II sequences in the RDP taxonomic hierarchy (using the RDP Bayesian hierarchy classifier) and included 22 groups, which were previously found in Antarctic and alpine environments. Of the seven major bacterial lineages present, *Proteobacteria* were dominant (46% of clones), followed by *Cyanobacteria* (12%), *Bacteroidetes* (11%) and *Actinobacteria* (10%), with members of the phyla *Acidobacteria*, *Chloroflexus*, *Planctomycetes* and candidate phylum OP10 also being present (Figure 5).

As these clone libraries were generated from four pooled samples for each glacier, it was possible to compare the abundance of OTUs and phyla between glaciers by χ^2 -analysis (Supplementary Data S9). Five OTUs showed significantly different distributions between glaciers, with, for instance, the OTU52 (*Sphingobacteriales*) being found only on ML ($P=0.003$). As can be seen in Figure 5, proteobacteria were more dominant on AB ($P=0.014$), mainly because of the greater abundance of alpha-proteobacteria ($P=0.035$) and the absence of beta-proteobacteria (mostly *Burkholderiales*) on ML. Among the alphaproteobacteria, members of the order *Sphingomonadaceae* were dominant (18/29). *Sphingomonas* species comprised about 33% of the culturable bacteria isolated by Margesin *et al.* (2002) from Alpine cryoconites, but here they were mostly found on AB (8/9 clones; $P=0.002$). In contrast, *Sphingobacteriales* (*Bacteroidetes*) were mostly found on ML (7/9 clones; $P=0.018$).

In silico digestion of trimmed clone sequences with *Hae*III identified 42 distinct T-RFs among the 94 clones (Supplementary Data S8). Only 1 OTU

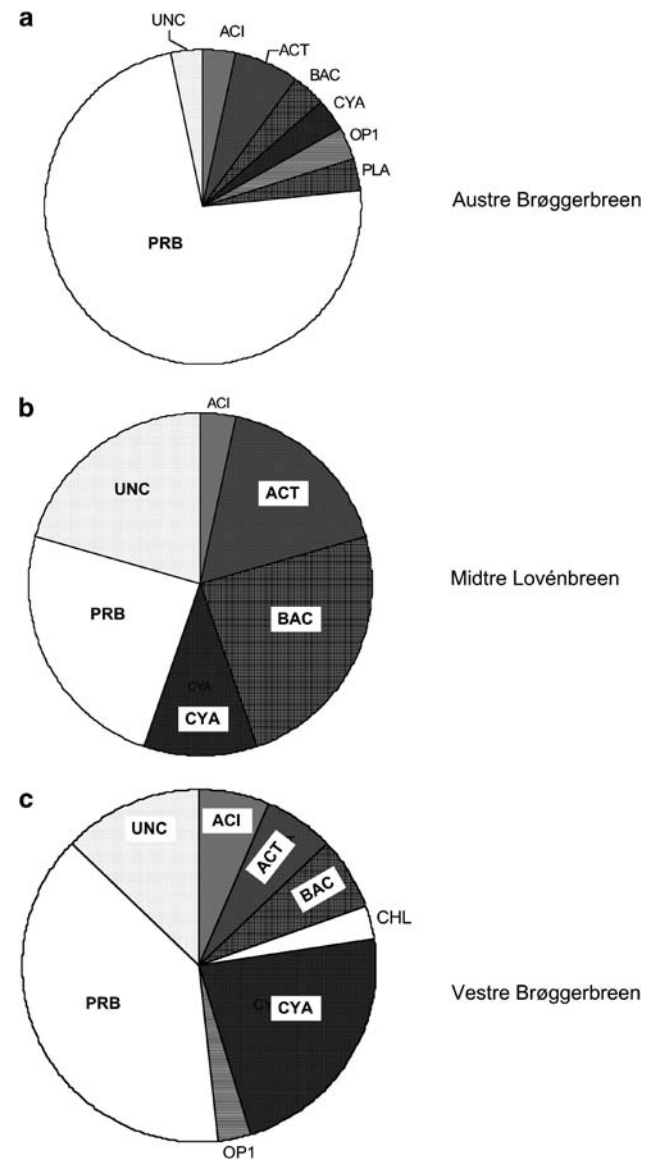


Figure 5 Phylum level classification of clones by glacier ($n=32$ per glacier; pooled from two holes per glacier per year); (UNC: Unclassified bacteria; ACI: *Acidobacteria*; ACT: *Actinobacteria*; BAC: *Bacteroidetes*; CHL: *Chloroflexus*; CYA: *Cyanobacteria*; OP1: OP10; PLA: *Planctomycetes*; PRB: *Proteobacteria*).

was predicted to have a T-RF outside the 60–600 bp range of the analysis, and of the 15 OTUs represented by multiple (≤ 7) clones, 7 were predicted to give multiple T-RF peaks (± 2 bp); with only one of these having more than two T-RFs. The three most abundant T-RFs across all T-RFLP samples at 294, 227 and 187 bp (± 2 bp), respectively occurred seven, four and three times among the 90 clones.

Discussion

To our knowledge, this is the first detailed comparison of bacterial diversity in cryoconite holes on neighboring glaciers. Previous microbiological analyses have predominantly focused on culture- and

microscopy-based approaches (Margesin *et al.*, 2002; Mueller and Pollard, 2004). Morphological examination of phototrophic microbes has been useful in comparison of Arctic and Antarctic cryoconite holes (Porazinska *et al.*, 2004; Stibal *et al.*, 2006), but the limited range of observable morphology hinders the application of this approach to most non-photosynthetic bacteria. Although archaea have been reported from several peri-glacial habitats (Sjöling and Cowan, 2003; Nicol *et al.*, 2006), other PCR-based investigations have also failed to detect their presence (Pointing *et al.*, 2009); (Supplementary Data S1). Further study using PCR-independent approaches (for example, phospholipid ether lipid analysis; Gattinger *et al.*, 2003) could usefully be applied to provide a definitive determination of the occurrence of archaea within cryoconites.

Our 16S rRNA gene clone library analysis confirms the presence of at least seven bacterial phyla within cryoconite sediments. The dominance of the alpha- and betaproteobacteria is consistent with the earlier culture-based studies of Margesin *et al.* (2002) of Alpine cryoconite bacteria and the 16S rRNA clones sequenced by Christner *et al.* (2003a) from an Antarctic cryoconite. The paucity of cyanobacterial sequences (12% of clones) implies that cyanobacteria are not the sole contributors to the high rates of primary production within cryoconite holes. Although non-cyanobacterial photosynthetic bacteria such as *Rhodobacter*, *Rhodospila* and *Chloroflexi* are present in the clone library, it is likely that eukaryotic photosynthetic organisms such as algae and diatoms are significant contributors to primary productivity in cryoconite holes (M Yallop and A Anesio, unpublished data). SeqMatch classification of bacterial clones also indicates that several of the taxa identified in this study are closely related to sequences identified at other cold, but mostly freshwater, environments. For instance, *Phormidium pristleyi* (OTU8) and other mat-forming *Oscillatoriales* have been found to be the most abundant cyanobacteria in cryoconite holes globally (Wharton *et al.*, 1981; Takeuchi *et al.*, 2000; Mueller *et al.*, 2001). However, the majority of OTUs identified in this study have similar sequences to those identified in temperate habitats, indicating that the cryoconite bacterial community also contains OTUs from broadly distributed taxa.

Several bacterial groups are characterized by an uneven spatial distribution between the three glaciers sampled, consistent with the differences observed in T-RFLP profiles between glaciers. The highly significant difference resolved by PERMANOVA and other multivariate analyses of T-RFLP profiles between AB and ML/VB is intriguing.

The annual mass-balance monitoring conducted by the Norsk Polar Institutt (Dowdeswell *et al.*, 1997) includes two of the glaciers studied here, with both AB and ML experiencing similar surface melt-rates of up to 1–2 m per year (Hagen *et al.*, 2003), and it is likely that surface melt rates would be similar

on VB. The upper surfaces of all three glaciers therefore experience ubiquitous melting in their lower reaches during the months of summer.

Although there is no reason to believe that the surface melt rate varies significantly between these three glaciers, supraglacial meltwater drainage may well differ at the sites investigated for at least two reasons. First, each of the glaciers included in the study has a unique shape and basal boundary. These differences manifest themselves as correspondingly unique flow fields and structural configurations (Hambrey *et al.*, 2005). As detailed patterns of surface meltwater drainage are to some degree controlled by glacier structure, it follows that at least the detail of each glacier's supraglacial meltwater network will be different. Second, supraglacial drainage networks generally evolve downglacier, with the proportion of smaller feeder channels gradually reducing relative to that of larger trunk channels, as the former merge into the latter. Thus, other things being equal, the greater the upglacier drainage 'fetch' above a sample site, the more discrete and less widespread the local drainage will be (Hodgkins, 1997; Nienow and Hubbard, 2005). As AB has a considerably longer fetch than ML or VB (Figure 1), it follows that the supraglacial drainage system is likely to be more discretely organized into fewer, larger channels in the area concerned. Although field observations on the three glaciers involved tentatively support this inference, it remains to be tested rigorously and is considered a research priority.

The low degree of spatial scaling that we have found on individual glaciers (Mantel test) and the greater variability between glaciers may be because of the downhill redistribution of both microbial propagules and larger particles by supraglacial meltwater over the glacier surface. Most of the open cryoconite holes are to some extent bathed with meltwater, and may be connected by supraglacial melt streams. Mueller and Pollard (2004) suggested that physico-chemical conditions and taxa in cryoconites on White Glacier in the Canadian High Arctic can be 'reset' by these interconnections.

However, such connections within glaciers do not exist between glaciers. In this study, the three glaciers were separated by de-glaciated ridgelines and 240 m or more apart at the closest. However, the sampled holes on ML and VB were 2–7 km apart, yet showed no significant difference in community structure. Thus, considerations of spatial proximity do not adequately explain the different T-RFLP profiles of bacterial communities on different glaciers. Equally, the CCA resolves different clusters of bacterial community structures in relation to the environmental variables included in the model. Cryoconites from ML and VB cluster closely suggesting similar environmental pressures on bacterial community structures, whereas AB cryoconites are well resolved during ordination, even from each other, and show differing influences from environmental variables on AB cryoconite holes.

Therefore, these variations in the route and rate of surface meltwater drainage potentially influence the availability of nutrients, debris and propagules to cryoconite holes on the glacier surface. In particular, this would restrict their availability to the cryoconite holes on the surfaces of longer and predominantly cold glaciers resulting in more heterogeneous environmental conditions than on glaciers with less well-developed surface drainage.

This is consistent with the observations that AB cryoconite sediments have considerably lower primary production and respiration rates and occupy a greater environmental gradient, as resolved by CCA, than both ML and VB. The relative abundance of T-RFs was more even on AB than on ML/VB, possibly reflecting a lower degree of functional organization, with unstable conditions constraining the emergence of dominant taxa (Marzorati *et al.*, 2008). This may be because of a lower and more sporadic influx of nutrients as a result of the more isolated nature of the cryoconite holes on AB. Correspondingly, the ordination space occupied by the bacterial T-RFLP profiles of cryoconite sites on AB is considerably greater than that of ML and VB using both detrended correspondence analysis and CAP.

Though dust particles on AB were finer, we did not identify any clear effect of inorganic particle size or composition from representative samples of cryoconite sediment that could correlate with the observed patterns of microbial diversity and activity. Although a complex and subtle set of influences and interactions between the biotic constituents of cryoconite and the inorganic substrates on which they reside no doubt exist, at the scale of this study, none could be identified as having a clear role in shaping microbial community structure.

In contrast, factors that have a clear biotic feedback (for example, humic and fulvic acid content and organic matter) correlate closely with levels of microbial activity and the patterns of bacterial community structure observed, both corroborating an earlier study showing the autochthonous origins of cryoconite carbon (Anesio *et al.*, 2009) and highlighting the importance of biotic processes to the structure and function of cryoconite holes.

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