The prokaryotic community in an extreme Antarctic environment: the brines of Boulder Clay lakes (Northern Victoria Land)

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Abstract During summer 2014, three hypersaline brines were discovered in two frozen lakes of Boulder Clay (Northern Victoria Valley, Antarctica). Ongoing research seeks to gain novel insights on the microbial ecology of such environments, in order to further the understanding of life adaptation to extreme conditions. To this aim, the abundance of prokaryotic cells (including cell morphologies and size for biomass conversion), the amount of viable cells (in terms of membrane-intact cells and respiring cells), the viral count, the physiological profiles at community level and the main microbial enzymatic activities were described. The brines differed each other in terms of prokaryotic cells' abundance, size, and viability as well as viral abundance. Cell morphotypes and metabolic responses also varied among the brine samples. Underground interconnections were likely to occur, with the microbial community becoming more abundant and structured to better exploit the limited resource availability. Overall, complex interactions among multiple environmental factors, including marine water origin, depth horizon, isolation time of the brines, and climatic variations, reflected on the microbial community distribution patterns and highlighted the need to preserve these niches of extreme life.

Keywords Prokaryotes · Viruses · Metabolic potentials · Brines · Antarctica

Introduction

Bacteria and Archaea (all together indicated as prokaryotes) represent the most abundant forms of life on the Earth (Watson, 2019), and are known to play a crucial role in trophic webs and biogeochemical cycles (Bellard et al., 2012). Conversely, viruses do not find a place on the universal tree of life, although a very close link with the living world exists (Brüssow, 2009). In polar areas, and even more in extreme environments, the ecological importance of prokaryotes and viruses is enhanced (Verde et al., 2016). Polar brines represent extreme environments where unfrozen conditions are kept at several degrees below 0°C due to their high salt content. At poles, brines have been found in perennial glacial lakes, sea-ice as well as permafrost (cryopegs) (Gilichinsky et al., 2003; Malone et al., 2010; Forte et al., 2016). These cryo-environments are considered relicts of ancestral seas, as observed in the cryopegs of Alaska (Colangelo-Lillis et al., 2016) and Siberia (Gilichinsky et al., 2003). Similarly, the geochemical analysis of the outflow from the Taylor glacier (McMurdo Dry Valleys, Southern Victoria Land) suggests its resemblance to a remnant sea, following the Pliocene intrusion of marine waters combined with weathering processes (Mikucki et al., 2015).

Despite their harsh conditions for most organisms, polar brines are able to host extreme forms of life, including specialized microbes that can represent a potential source of novel biotechnologically exploitable molecules (Murray et al., 2012; Michaud et al., 2014; Rizzo et al., 2020). In particular, polar brines host a variety of halophilic microorganisms, including prokaryotes, green algae, cyanobacteria and viruses (Madigan et al., 2003; Murray et al., 2012; Rampelotto 2014; Papale et al., 2019; Heinz et al., 2020). The discovery of a viable prokaryotic assemblage in the encapsulated brines of the Lake Vida dates back to 2002 (Murray et al., 2012); the survival for millennia of these microorganisms was assumed to depend on their ability of using the available organic carbon resources. The very high concentrations of dissolved organic carbon (range: 48.3–64.7 mM C, of which the 7% made by carbohydrates) and small amounts of low molecular weight organic compounds supported the potential biological carbon processing. Also the fluorescence index values (1.73–1.79) showed that the dissolved organic matter was microbially derived (Murray et al., 2012). Nevertheless, inconsistencies regarding the metabolic capacities of microbes within brines were observed: while low metabolism at a temperature below +4°C was documented (Murray et al., 2012; Pearce, 2012), enhanced survival was detected in subzero eutectic brines compared with their warmer analogs (Heinz et al., 2018). Anyway, microbial survival in polar brines involves adaptive modifications of their structural and physiological organization (La Ferla et al., 2017; Papale et al., 2019), including the production of antifreeze substances and the maintenance of turgor pressure and membrane fluidity (Fillinger et al., 2001). However, the mechanisms regulating the degradation of dissolved organic matter as well as the origin of the utilizable organic matter remain unclear (Mann et al., 2014). All the microorganisms of the perennially frozen ground and lakes are greatly susceptible to environmental changes and warming. In this regard, the microbial ecology of permafrost, and of its associated environments, was reviewed by (Jansson & Taş, 2014). Changes in microbial biogeochemistry and their potential impacts on trophic webs and greenhouse-gas emissions were debated by (Schuur et al., 2015).

Within the brine systems the presence of viruses represents another knowledge gap. Everywhere, viruses have been recognized to be important agents that influence microbial dynamics and biogeochemical cycles (Parikka et al., 2016). At the Poles, characterized by peculiar seasonality, the importance of viruses in the microbial ecosystem functioning is likely even greater than in temperate areas (Yau & Seth-Pasricha, 2019), since virus-host interactions not only control remineralization and community composition, but also influence the productivity cycles (Cavicchioli, 2015). Viruses have been discovered in the Antarctic sea, sea-ice, lakes and brines (Colangelo-Lillis et al., 2016; Luhtanene et al., 2018; Papale et al., 2019), but the knowledge of viruses and their functions as well as the interplay and intraspecificity between viruses and prokaryotes has so far been fairly limited in these aquatic polar environments (Yau & Seth-Pasricha, 2019).

In October–November 2014, a screening of several frozen lakes in the Northern Victoria Land through Ground Penetrating Radar (GPR) was carried out, in the framework of the Italian National Antarctic Research Program (PNRA). In two adjacent lakes of

Boulder Clay (BC), further indicated as L-A and L-B, three lenses of liquid brines at different depths were detected by GPR. Exploiting the opportunity to study these so far unknown brines, three samples (two in L-A and one in L-B) were drilled in order to find new extreme refugia for microbial life. In addition, a brine sample was collected from a borehole already existing (cored in 2005) on a side of a frost mound occurring in L-A. Our study describes the microbiological properties of newly discovered brines of Antarctic perennially-frozen lakes in the Northern Victoria Land. The working hypothesis of our research was that these brines, located at different depths in two adjacent lakes and with their diverse abiotic features and history, could host different microbial assemblages. To this end, several methodological approaches were adopted: Image analysis was applied to estimate the prokaryotic cells abundance, their phenotypic traits and the percentage of membrane intact cells and actively respiring cells; flow cytometry to enumerate the viruses (in terms of virus-like particles). Moreover, the metabolic responses in terms of potential utilization of carbon sources by Biolog-Ecoplates and the potential enzymatic activity rates on proteinaceous and glucidic organic polymers as well as on organic phosphates by fluorimetry were assessed.

The aims of this study were: (i) to explore the microbial ecology of the newly discovered BC brines, (ii) to relate the microbial patterns of each brine to its environmental and geomorphological properties, and (iii) to formulate new hypotheses on the historical evolution of these cryo-environments.

Methods

Site description and samples' collection

Boulder Clay (BC; 74°44′45″S, 164°01′17″E, 205 m a.s.l.) is an ice-free area located in the Northern Victoria Valley (Antarctica) about 6 km south of Mario Zucchelli Station (MZS). The soils here are mainly glacic haplorthels. The chemical and physical parameters of the soil show relatively high values of Al and Fe and scattered mosses and epilithic lichens constitute the vegetation types (Cannone et al., 2008; Guglielmin et al., 2014). The mean annual air temperature—based on observations over the last 20 years—is around −14°C. Permafrost is ice cemented, and the mean annual permafrost temperature (at the permafrost table at around 30 cm of depth) is around −16.5°C, but it appears to be increasing over time (Guglielmin & Cannone, 2012; Guglielmin et al., 2014). The thickness of the active layer, monitored in the adjacent circumarctic active layer monitoring (CALM) grid, ranges between 0 and about 90 cm but it appears to be increasing over time (Guglielmin et al., 2014).

GPR data were collected on November 2014 with a ProEx (Malå Geoscience) instrument equipped with 250 or 500 shielded antennas, both in common offset configuration. These antennas were selected in order to obtain the best trade-off between achievable resolution and investigation depth. GPR was triggered by an electro-mechanical odometer every 10 cm, while a DGPS device was used for measurement (trace) positioning. Drift removal (zero-time correction), spectral analysis, band-pass filtering, geometrical spreading, static corrections, depth correction, as well as Kirchhoff migration were performed by using a constant electro-magnetic velocity typical of pure ice and equal to 0.17 m/ns. Profiles were interpreted considering lateral phase continuity, amplitude and other specifically calculated attributes (frequency related and cosine of instantaneous phase attributes).

After their discovery, four pockets of liquid brines were reached and aseptically sampled through a borehole. Their sampling dates were different, and namely BC1 was sampled on November 29, whereas BC2, BCM and BC3 on December 1. The samples were collected at different depths by a peristaltic pump and a sterilized tubing. In L-A (Guglielmin et al., 2009), the brine samples BC1, BC2, BCM were collected at 2.5, 0.9 and 0.5 m depths, respectively; in L-B, the sample BC3 was picked up at a 2 m depth (Fig. 1). At these depths in the surrounding ground the temperatures ranged between − 31.4°C and − 0.9°C (Table 1). It should be noted that BCM was collected within the piezometer in a borehole drilled in 2003 where a brine pocket was intercepted between 350 and 400 cm depths. The level of collection in 2014 was only 50 cm, suggesting that the brine within the piezometer was mixed with surface melting water or made only by the latter.

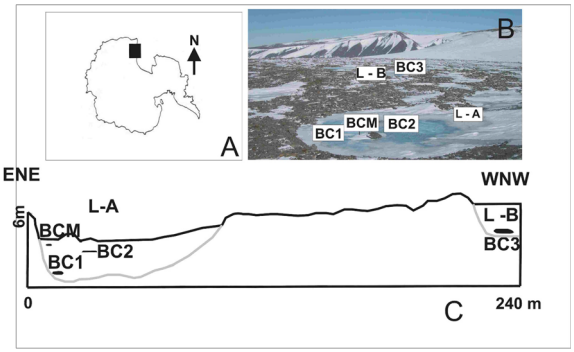

Fig. 1 Location of the study area: **A** Location of Boulder Clay area within Antarctica; **B** color picture of the lake A (L-A) and the other perennial frozen lakes (among which lake B, L-B) in Boulder Clay area taken from the helicopter southward; **C** Sketch of the area (grey line = lake bottom surface; black areas = brine pockets, Grey area = brine pocket within the borehole from which partially comes up BCM). Please note that the horizontal and vertical scales are different

Microbial abundance

Prokaryotic cells' abundance, volumes, morphotypes and biomass by image analysis

After collection, three sub-samples per each brine were collected in sterile 50 ml-polyethylene tubes, immediately fixed with filter-sterilized formaldehyde (2%, final concentration) and stored at 4°C in the dark until their transfer and lab processing in Italy. Three replicates per brine were filtered through polycarbonate black membranes (porosity 0.22 μm; GE Water & Process Technologies) and stained for 10–20 min with DAPI (4′6-diamidino-2-phenylindole, final concentration 10 μg ml^{-1} by Sigma-Aldrich) according to (Porter & Feig, 1980). The prokaryotic cells were quantified through an AXIOPLAN 2 Imaging microscope (Zeiss), provided with the specific DAPI filter set (G365; FT395; LP420) and equipped with an AXIOCAMHR digital camera (Zeiss) and an AXIOVISION 3.1 software. Cell counts were performed on a minimum of 20 randomly selected fields on duplicate slides. When cell abundance was low, 30-40 random microscope fields were counted (La Ferla et al., 2012).

The prokaryotic individual cells' volume (VOL) was derived from two manually obtained linear dimensions (width, W, and length, L). Details of the technical features and calibration are reported in (La Ferla et al., 2015) and references herein. The VOL of each single cell was calculated according to the geometrical formula:

$$\mathrm{VOL}\ \left(\mu m^3\right) = (\pi/4) \times W^2 \times (L - W/3),$$

where $W = L$ for coccal forms, assuming that the cells were cylindrical straight rods with hemispherical or, in the case of coccoid forms, spherical caps. The mean cell VOL was converted into Cell Carbon Content (CCC, expressed in fg C $cell^{-1}$) using the allometric relation proposed by Loferer-Krőbacher et al. (1998). The prokaryotic biomass (PB, μg C l^{-1}) was calculated multiplying PA by CCC determined per each sample.

Six morphotypes were recognizable: cocci (when their length and width differed by less than 0.10 μm), coccobacilli (length and width differed by more than 0.10 μm), rods (having a length at least double of their

Table 1 Ground temperature (T, ° C) and salinity (mg l^{-1}) of the brines

	BC1	BC2	BCM	BC3
Annual mean T[a]	16.6	16.4	16.4	16.5
Max	6.7	1.4	0.9	5
Min	24.9	30.4	31.4	26.2
Current mean T[b]	14.8	9.5	7.7	13.1
Max	11.6	4.4	2.3	9.3
Min	18.0	15.1	14.1	17.1
Salinity	6333 ± 15	37.6 ± 2.1	97.8 ± 10.4	1583 ± 6.81

[a]Annual mean, maximum and minimum ground temperature at the depths of brines' collection reconstructed for the period 1996–2012 using the data of the Boulder Clay station borehole that is located less than 100 m from the lake. Note that these ground temperature is indicative of the ground surrounding the frozen lake. [b]Current mean, maximum and minimum brine temperature and salinity at the moment of sampling

width), vibrios (V-shaped cells), curved rods (C-shaped cells) and filamentous bacteria (cells exceeding 4 μm in length).

The morphological index (MINDEX) was calculated (M = %rods/%cocci), which allows to determine the extent of pollution and self-purification in the brines, because the increase in the number of rod-shaped cells is an indicator of increased organic content in the water (Pernthaler, 2005).

Viable prokaryotic cells

The cells having intact membranes were quantified by the Live/Dead Bac Light Bacterial Viability Kit™ (Molecular Probes) (*L/D*). Using this staining kit that includes two separate solutions of SYTO® 9 (a green-fluorescing nucleic acid stain) and propidium iodide (a red-fluorescing nucleic acid stain,a marker of membrane-damaged cells), stock solutions were prepared per each dye using 5 ml of Milli-Q ultrapure water and the final concentration was 6 μM for SYTO 9 and 30 μM for propidium iodide. The two solutions were thoroughly mixed. After collection, triplicate subsamples (1 ml) per each brine were immediately treated. Briefly, 1 ml of sample was stained with 1 ml of *L/D* stain mixture, incubated at 4°C in the dark for 1 h and kept at − 80°C until its analysis in Italy. After filtration of 1 ml through a 0.22 μm pore size Nuclepore polycarbonate black membrane, the above-referred procedure for DAPI staining was performed on all the replicates. Cells were counted using the same Imaging microscope (AXIOPLAN 2, Zeiss), equipped with the specific fluoresceine (BP450-490; FT510; LP520) and rhodamine filter sets (BP546/12; FT580; LP590) under aerobic conditions.

The actively respiring cells were quantified by the Bac Light Redox Sensor CTC Vitality Kit™ (Molecular Probes). After collection, 1 ml of the sample, collected in three replicates from each brine,was treated with 0.1 ml of 5-cyano-2,3-ditolyltetrazolium chloride (CTC; final concentration 5 mM). The solutions were incubated at 4°C in the dark for 5 h that proved to be the best incubation time among trials of 5, 10 and 18 h. Cells with a highly active respiratory chain (CTC+) reduced CTC into intracellular red-fluorescing formazan granules. Prefiltered (through a 0.22 μm pore size filter) formalin (final concentration 2%) was used to stop the reaction. For each replicate, a killed control was prepared by injecting formalin before CTC addition. The pretreated samples were stored at − 20°C until their analysis in Italy by image analysis. Slides were prepared in three replicates according to the DAPI procedure earlier described, and observed under the epifluorescence microscope using the rhodamine filter set (BP546/12; FT580; LP590). All the counts were performed under aerobic conditions.

Virus and prokaryotic cells abundance by flow cytometry

For the determination of viral abundance (VA as virus-like particles), immediately after collection, 1.5 ml of each brine were collected in triplicate in cryogenic tubes and fixed with glutaraldehyde (0.05%) for 30 min at 4°C, followed by freezing and storage in liquid nitrogen (N_2; − 196°C) until their analysis in the lab. For viral enumeration, aliquots were stained with SYBR Green I (Molecular Probes, at a final concentration 5×10^{-4} of the commercial stock solution) according to (Brussaard, 2004a) and analyzed using a FACSCalibur flow cytometer (BD Biosciences) equipped with a 488 nm argon laser. To relate prokaryotic abundance (PAC) with viral counts in the virus to prokaryote ratio (VPR), aliquots of the samples were fixed with paraformaldehyde (1%) and glutaraldehyde (0.05%) and assessed using flow cytometry (Gasol & del Giorgio, 2000).

Microbial metabolic responses

Physiological profiles

Following the standard procedure (Garland & Mills, 1991; Garland, 1996), Biolog-Ecoplates™ were used to determine the potential physiological profiles of microbial assemblages hosted in the brines BC1, BC2 and BC3 and their differences. The experiments started at the dates of samplings, i.e. on November 29 for BC1 and on December 1 for BC2 and BC3. Ninety-six-well microtiter plates, containing 31 carbon sources and a control in triplicate together with the redox dye tetrazolium violet, were inoculated with 150 μl of sample. Each plate was incubated at 4°C in the dark under aerobic conditions (La Ferla et al., 2017). At MZS, the oxidation of the carbon sources into formazan was quantified—as absorbance—at 590 nm using a microplate-reader spectrophotometer

(MICROTITER ELX-808, Bio Whittaker, Inc.) equipped with an Automatic Microplate Reader and the specific software (WIN KQCL) for data processing. The optical density (OD) of the reaction product was measured at 4°C immediately after inoculation (at time T0), and thereafter every 2 days (from T1 until T6 for BC1, and until T5 for BC2 and BC3 due to differences in the timing of the analytical procedure).

According to Sala et al. (2005), the color development for each plate was expressed as the average substrate color development (ASCD) using the following equation:

$$\mathrm{ASCD} = \Sigma\left((R - C)/31\right),$$

where R was the average absorbance of the three wells with substrate and C was the average absorbance of the control wells (without substrate). The absorbance percentages of each substrate were determined according to (Sala et al., 2006), and a value of 2% of the total absorbance measured per plate was used as the threshold for substrate utilization.

The carbon substrates were grouped into six guilds: complex carbon sources (polymers), carbohydrates, phosphate carbon sources, carboxylic and acetic acids, amino acids, and amines.

Potential enzymatic activities

Three enzymes, such as leucine aminopeptidase (LAP), ß-glucosidase (ß-GLU) and alkaline-phosphatase (AP) involved in the degradation of proteinaceous and polysaccharidic substrates, and organic phosphates, respectively—were measured in terms of whole (both particulate and dissolved) activity rates potentially exhibited by the microbial community inhabiting the examined brine samples.

The enzymatic assays were carried out according to the Hoppe's method (1993), based on the addition to the sample of fluorogenic substrates, that are methylcoumarine (MCA) or methylumbelliferone (MUF) derivatives, analogs to natural compounds. Such substrates were each specific to the enzyme to be determined; in particular, L-leucine-7-amido-4-methylcoumarin hydrochloride, 4-methylumbelliferyl B-D-glucopyranoside and 4-methylumbelliferyl phosphate (Sigma) were used to measure the potential LAP, ß-GLU and AP activity rates, respectively. Triplicate 5-ml sub-volumes of each brine sample (previously diluted 1:100 in sterile physiological saline) were incubated with increasing amounts (from 40 to 320 nmol l^{-1}) of each substrate. The increase in fluorescence between the initial time (T0, immediately after the substrate addition) and 3 h after incubation at 4°C was recorded with a fluorimeter (Turner TD-700 model) at 365 nm (excitation wavelenghts) and 440–455 nm (emission wavelenghts for MCA and MUF substrates, respectively). After calibration with known amounts of the standards 7-amino-4-methylcoumarin (MCA) or 4-methylumbelliferone (MUF), the maximum reaction velocity (V_{max}) of hydrolysis of the substrates was calculated, in terms of nanomoles of leucine, glucoside and PO_4 potentially released per gram and per hour, by LAP, ß-GLU and AP, respectively, taking into account the initial dilution of each sample.

Statistical analyses

The logarithmic transformation of the data was performed in order to standardize those data whose original values were expressed with different measurement units. All the statistical analyses were performed in the R statistical environment package v3.2.3. To identify relationships linking the microbial parameters with the environmental factors, redundancy analyses (RDA) were performed using the function rda in the vegan package (v2.3-3) (Oksanen et al., 2018). Furthermore, preliminary detrended correspondence analysis (DCA) showed that the gradients in functional structure were rather short (DCA axis 1: 0.559; DCA axis 2: 0.115), implying linear responses and supporting the use of RDA. The inclusion of explanatory variables in the RDA model was based on forward stepwise selection ($\alpha = 0.05$), being the significance of the variables assessed by Monte Carlo randomizations (999 runs).

Heterogeneity of variance precluded the use of parametric statistics. Therefore, the Kruskall-Wallis test was used to compare data (PB, VOL, VA, CTC, *L/D*, AP, LAP, GLU and MINDEX) among the four brine pockets, followed by a posteriori multiple comparison with the Nemenyi test (Zar 1996).

To study the association between the parameters, correlations between all pairs of data variables were estimated using a Spearman correlation coefficient followed by a significance test (P value < 0.01) based on t test. These correlations were analyzed and plotted using 'corrplot' (Wei, 2013), a graphical correlation

matrix plotting package in the R statistical environment package (Team, 2013).

Results

GPR brines identification

Among several perennially frozen lakes investigated through GPR in order to find saline brines, L-A and L-B were the two lakes in which the greatest saline pocket brines were detected. It is interesting to note that, from the geophysical point of view, brines are peculiar habitats with characteristics strictly dependent on their salinity, density and pressure. Our study showed that brine detection on GPR data was not always trivial. In fact, while liquid high-conductive brines produced peculiar spectral and amplitude effects on GPR data and were well highlighted by different attributes, the signature of frozen brines was much more elusive.

The brines were clearly identified using GPR data due to their peculiar characteristics responsible for their high electrical conductivity, causing in turn an extremely high intrinsic attenuation and remarkable bright spots (Fig. 2).

In L-A, the brines were identified at 0.9–1.5 m of depth in the middle of transparent ice (BC2) and close to the bottom within a depression of the lake (BC1), below a frost mound; in L-B, they were found only at the bottom of the lake between 1.9 and 2.5 m of depth (BC3) below a frost mound (Fig. 2), similarly to what found by some of the authors at Tarn Flat (Forte et al., 2016).

Geochemical characteristics of BC1, BC2 and BC3 have been reported by Sannino et al. (2020).

Microbial abundances

Prokaryotic cells' abundance, volumes, morphotypes and biomass by image analysis

In Fig. 3a, a scatterplot of prokaryotic cell abundance (PA) versus prokaryotic biomass (PB) together with the prokaryotic cells' volume (VOL) is reported. The highest PA values (in the order of 10^6 cells ml^{-1}) occurred in BC1 while in the other brines PA was in the order of 10^5 cells ml^{-1}; significant differences among the brines (p value < 0.016) were found. Great variability was also observed for VOL whose values ranged, on the whole, from 0.01 to 3.170 μm^3 (mean value 0.204 ± 0.373 μm^3) and wide differences among the studied brines (P value < 0.001) were detected. The largest cell volumes were observed in BC1 again, the smallest ones in BCM and the intermediate in BC3 and BC2. CCC values, dependent on cell volume, varied widely between 6.6 and 588 fg C $cell^{-1}$, with a mean value of 50.4 ± 74.1 fg C $cell^{-1}$ and significant differences were detected among the brines ($P < 0.001$). PB, modulated by both PA and CCC, significantly differed among the brine samples (P value < 0.016), being surprisingly high in BC1 in relation to the very great VOL and, consequently, high CCC of the filamentous forms that occurred here only. In BC2 and BC3, similar PB values were detected, while the lowest one was found in BCM (Fig. 4).

The length and width of cells measured in each sample are reported in Table 1 Suppl. Total cell lengths and widths showed significant differences (P value < 0.001) among the examined samples. The greatest and the smallest values were observed in BC1 and BCM, respectively.

The morphotypes' abundance and their relative percentage found in each brine are shown in Fig. 4. Significant differences in the prokaryotic types among the brines were observed (P-value < 0.001) even if, on the whole, the most abundant cell groups were rods and coccobacilli (accounting, on average, for the 50 and the 27% of the total morphotypes, respectively). In detail, in BC1, five morphotypes were observed, and namely, rods, cocci, filamentous forms, curved rods and vibrios; coccobacilli were lacking. Rods, cocci and filamentous forms prevailed. Cocci and rods showed sizes smaller than 0.1 μm^3, while filamentous bacteria were the largest morphotype (mean value 0.892 μm^3). It is noteworthy that filamentous forms consisted of long or short filaments, formed by small to medium-sized rods, curved filaments with septae visible; curved and s-shaped filaments. Vibrios and curved rods, fairly negligible, were larger than 0.1 μm^3. In the other samples, less morphotypes were detected: three in BC2, where coccobacilli dominated the prokaryotic assemblages followed by rods and cocci. Large volumes (> 0.1 μm^3) characterized rods and coccobacilli while cocci were the smallest ones. In BCM, rods and coccobacilli were the most abundant morphotypes with volumes < 0.1 μm^3 and smaller cocci were in negligible numbers. Finally, in BC3,

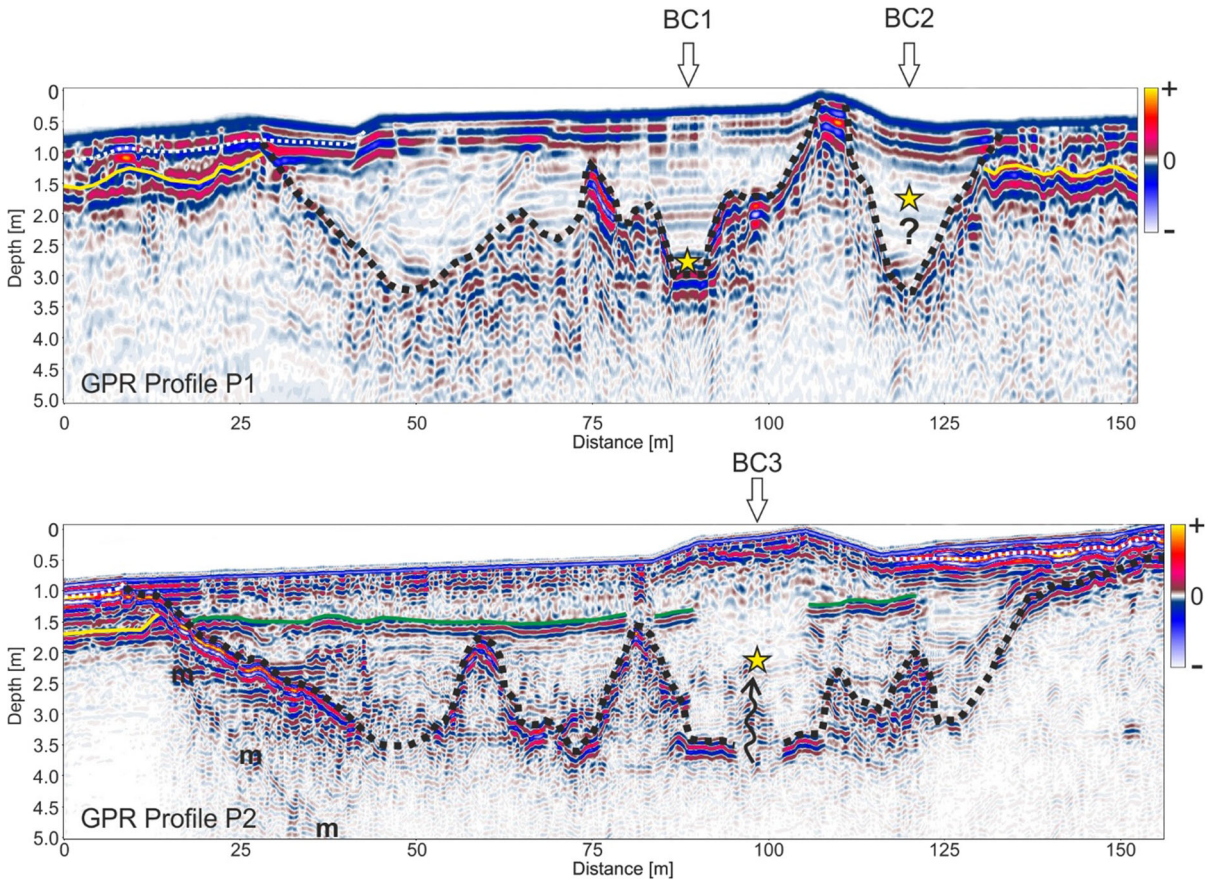

Fig. 2 Processed and interpreted amplitude GPR profile on lake A (L-A, top) acquired at 250 MHz and on lake B (L-B) acquired at 500 MHz. Dotted black line marks the lake bottom; yellow line the top of LGM glacial ice; white dotted line the base of snow and firn, while the green line highlights a bright reflector between the lake ice. BC1, BC2 and BC3 labels point out the location of the three boreholes from which brines were sampled (yellow stars). Letter m depicts multiple reflections, while the question mark is located in a zone characterized by low amplitude sub-horizontal reflectors just below the sample brine. Vertical exaggeration 10x

only rods and coccobacilli occurred with volumes > 0.1 and < 0.1 μm^3, respectively.

MINDEX reached the maximum value in BCM ($M = 32.1$) while in BC1 and BC2 its values were 1.1 and 1.2, respectively.

Viable prokaryotic cells

The obtained *L/D* and CTC counts are shown in Fig. 3b. The dead cells were always more abundant than the live ones. The highest percentages of membrane intact cells were once again found in BC1, followed by BCM, while in BC3 and BC2 low percentages were observed. Consequently, the *L/D* ratio was < 1 in all the samples. Actively respiring cells (CTC+) showed the highest abundances in BC1 and BC3, intermediate values in BCM and the lowest ones in BC2.

Virus and prokaryotic cells abundance by flow cytometry

VA was in the order of 10^6–10^8 particles ml^{-1} and the highest counts were observed in BC1 followed by BC3 (Fig. 3c). The lowest abundances were found in BCM. Three different sub-populations (V1, V2 and V3) were distinguished according to their fluorescence signals. V1 group was the most abundant sub-group everywhere and mainly in BC2 and BC3, where it accounted for the 97% and the 91% of the total VA, respectively. In BC1 and BCM, V1 group accounted for the 56% and the 69%, respectively, while V2 accounted for the

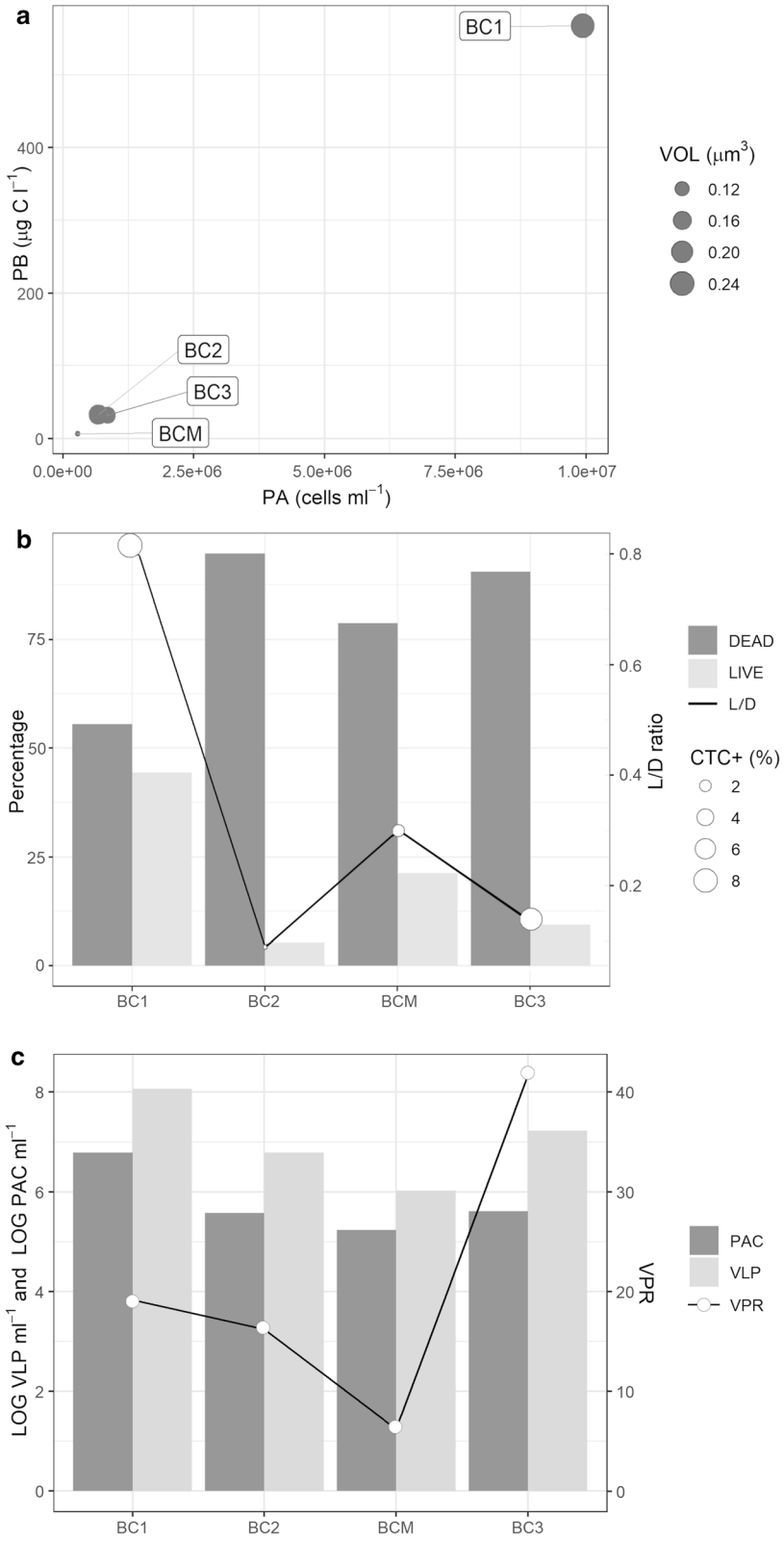

Fig. 3 Distribution of abundance in BC1, BC2, BCM and BC3 brines: **a** scatterplot of prokaryotic abundance (PA) versus prokaryotic biomass (PB) by image analysis. The areas of the circles are proportional to the cells' volume (VOL); **b** percentage of membrane intact cells (live, L, and dead, D) on total cells, *L/D* ratio and percentage of actively respiring prokaryotes (CTC +) on the total cells; **c** prokaryotic abundance (PAC), virus-like particle (VA) by flow cytometry and virus-to-prokaryote ratio (VPR)

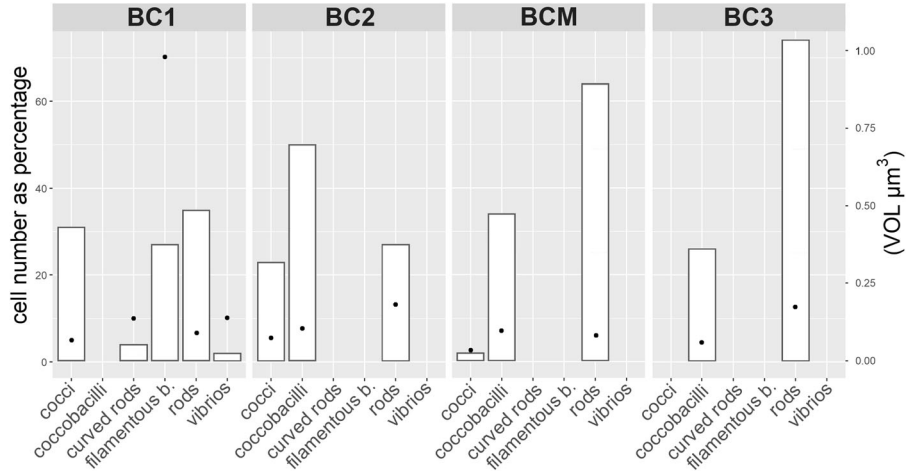

Fig. 4 Abundance as percentage of the total (white column) and volume of the morphotypes (filled circle) in BC1, BC2, BCM and BC3 samples

40% and the 27%, respectively. V3 group was negligible in all the samples.

PAC, whose determination in relation to viral counts allows to calculate the VPR, was in the order of 10^5–10^6 cell ml^{-1} with the highest and lowest values in BC1 and BCM, respectively. VPR fell between 6.2 (BCM) and 41.7 (BC3), while intermediate and similar ratios were calculated in BC1 and BC2.

Microbial metabolic responses

Physiological profiles

The average substrate color development (ASCD) over time, expressed as the absorbance values higher than 0.10 OD, and the number of positive wells (S) per each plate is reported in Table 2 Suppl. A slightly increasing trend over time in BC1 only was detected; here the mean ASCD value was high (0.121 ± 0.07) and a high percentage of positive results was detected (79% of the total wells per each plate). In both BC2 and BC3, the metabolism was constantly low (mean ASCD values: 0.012 ± 0.002 and 0.015 ± 0.002, respectively), and the percentages of positive wells on the total plate were the 51% and 36%, respectively. The microbial utilization of the assayed substrate guilds differed among the three analyzed brines. Kruskal–Wallis One Way Analysis of Variance on Ranks revealed the occurrence of significant differences (P-value < 0.001) among the samples. All pairwise comparisons using Nemenji-test highlighted that BC1 differed significantly (P-value < 0.05) from the other brines but no significant diversity occurred between BC2 and BC3.

In Table 2, the positive and negative responses to each substrate per each brine are reported. BC1 hosted a microbial community able to oxidize all the substrates, with the exception of 2-hydroxy benzoic acid and L-phenylalanine, both not used by the microbial community in any brine. Carboxylic acids and aminoacids were poorly oxidized in both BC2 and BC3. Anyhow, the microbial assemblage showed to have the capability to metabolize all the substrate guilds (Fig. 5a) even if at different levels. The complex carbon sources were the most utilized compounds, accounting for the 51% of the total carbon sources, with the highest utilization efficiency in BC3. Thereafter, the carbohydrates, and phosphate carbon sources (at least in BC1 and BC2) and amines (in BC2 and BC3) were utilized at discrete levels. Carboxylic acids (except for BC1) and aminoacids were poorly used.

Potential enzymatic activities

The potential enzymatic activities measured in the four brine samples exhibited different patterns. Generally, they showed higher mean AP activity compared to ß-GLU and LAP (Fig. 5b). Moreover, the relative importance of the three enzymes was different among the brine samples. Both LAP and AP values, indicating proteolytic and phosphatasic activities, depicted a

Table 2 Pattern of utilization of the 31 carbon sources for BC1, BC2 and BC3 samples. The box color is indicative of positive and negative response (gray and white, respectively)

		BC1	BC2	BC3
Complex carbon souce	*Tween 40*			
	Tween 80			
	α-cyclodextrin			
	Glycogen			
Carbohydrates	*D-cellobiose*			
	α-D-lactose			
	β-methyl-D-glucoside			
	D-xylose			
	i-erythritol			
	D-mannitol			
	N-acetyl-D-glucosamine			
Phosphate-carbon	*Glucose-l-phosphate*			
	D,l-α-glycerol phosphate			
Carboxylic acid	*Pyruvic acid mèthyl ester*			
	D-glucosamic acid			
	D-galactonic acid γ-lactone			
	D-galactonic acid			
	2-hydroxy benzoic acid			
	4-hydroxy benzoic acid			
	γ-hydroxy butyric acid			
	Itaconic acid			
	α-ketobutyric acid			
	D-malic Acid			
Amino acids	*L-arginine*			
	L-asparagine			
	l-phenylalanine			
	L-serine			
	L-threonine			
	Glycyl-L-glutamic acid			
Amines	*Phenylethyl-amine*			
	Putrescine			

decreasing gradient from BC1 to BCM, that was inversely related to the depth horizon where these samples were collected from. Conversely, BC3 showed intermediate levels for both these enzymes. ß-GLU, an enzyme active on polysaccharidic substrates, prevailed in the brines BC2 and BC3, while BC1 and BCM were characterized by similarly low glycolytic activities.

Statistical analyses

The correlation analysis among the studied parameters yielded the outputs shown in Fig. 6a. Analyzing all data, several correlations among the microbial and physical parameters were observed. Most of highly significant correlations computed among the abundance data were positive, with the exception of coccobacilli whose correlations were negative.

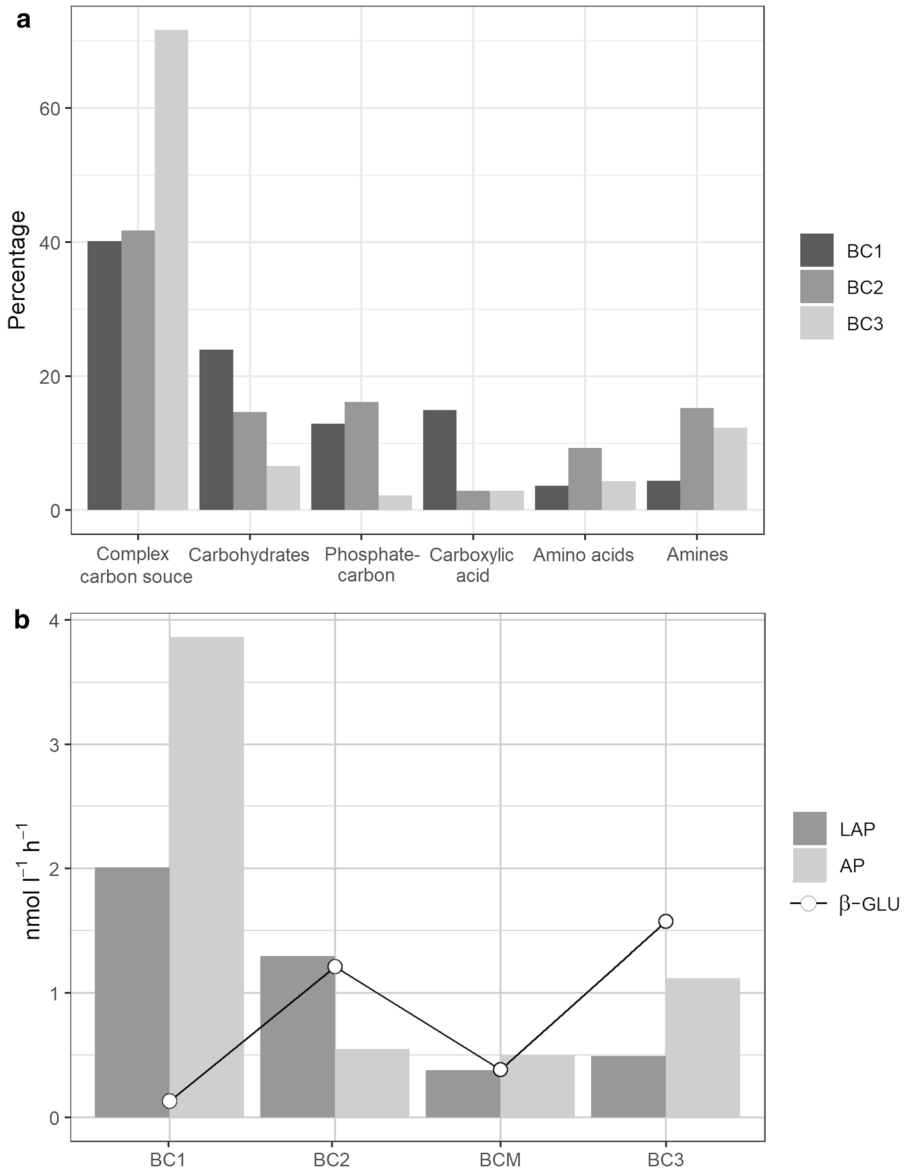

Fig. 5 Microbial metabolic responses: **a** carbon substrate utilization measured as percentage by Biolog ecoplates in BC1, BC2 and BC3 brines; **b** enzymatic activity rates (*LAP* Leucine Amino-Peptidase, *AP* Alkaline Phosphatase, *ß-GLU* ß-Glucosidase) measured in BC1, BC2, BCM and BC3 samples

Viability counts correlated significantly with the other parameters, with a higher significance level for *L/D* than for CTC +. Regarding the abundance of morphotypes, coccobacilli showed highly significant positive correlations with all the parameters considered, with the only exception of S, AP and ASCD for which the correlations were negative. Rods were not influenced by physical and microbiological parameters, while the coccal abundance was negatively affected by microbiological variables, excepting LAP. The enzymatic activities were not related significantly to CTC +, but the fraction of membrane-intact cells (*L/D*) was positively related to LAP and AP and negatively to GLU.

Forward selection in the RDA showed that temperature (P-value < 0.05, VIF value: 2.5), depth (P-value < 0.05, VIF value: 2.1) and salinity (P-value $<$ 0.05, VIF value: 1.8) were responsible for the

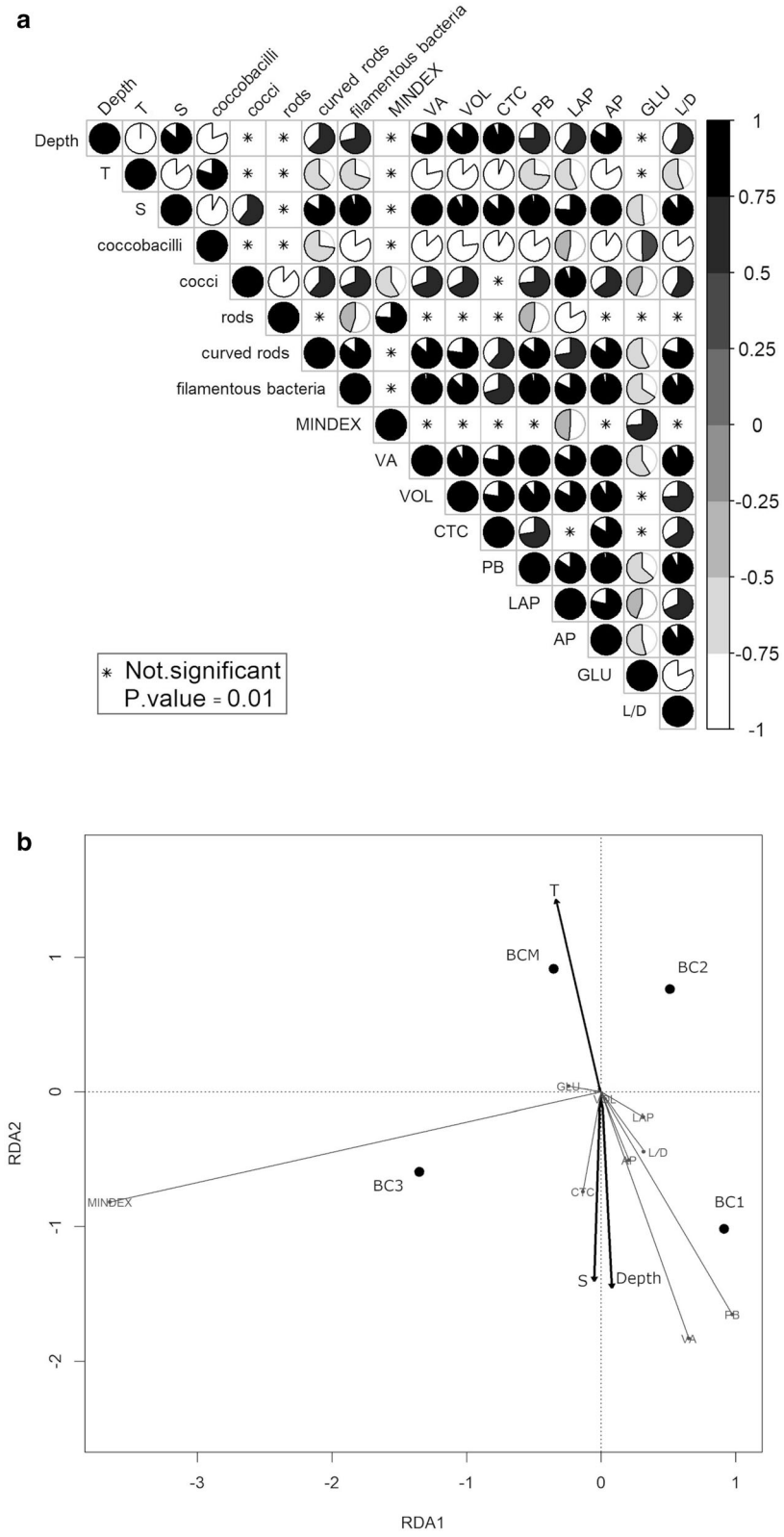

Fig. 6 Statistical analyses: **a** Pearson correlation coefficient matrix comparing paired environmental data. The color key for the correlation values is shown on the right of the figure: negative correlations are shaded white; positive correlations are shaded black. The strength of the correlation is indicated by the size of the pie. The definition of each covariate (*y*-axis) and its coded counterpart (upper *x*-axis) are defined per comparison. Correlations with a *P*-value > 0.01 are considered as not significant and indicated with an asterisc "*"; **b** Triplot of correlations based on Redundancy Analysis (RDA) computed between prokaryotic parameters (VA, VOL, PB, LAP, AP, GLU, *L/D*, MINDEX) and three environmental factors (depth, temperature, salinity) for all the brines. Each vector represents the direction of maximum variation and its length indicates the correlation strength. Vectors showing the same direction indicate correlation between variables

observed variations in the microbial functional structure.

The prokaryotic variables were included in RDA as dependent variables, while the environmental factors as independent variables. The statistical elaborations were performed using a level of significance *P* (probability) with 5% risk of error (*P*-value < 0.05). RDA explained 90% of the prokaryotic–environmental relationship through the first two axes (global model significance test *P*-value < 0.001, two first axes significance test *P*-value < 0.01).

According to Spearman correlation coefficients, the prokaryotic biomass showed a strongly positive correlation with LAP and AP, while NTOT a slightly negative correlation with LIVE but a positive one with GLU. Unlike salinity, temperature was negatively correlated with VA, VOL, PB and AP. VA was strongly positively correlated with VOL, PB, AP (Fig. 6b). VOL correlated positively with depth and salinity, negatively with temperature.

According to Kruskal–Wallis ANOVA (Table 3), PB, VOL, VA, CTC, *L/D*, AP, LAP, GLU and MINDEX differed significantly among the four sites (*P*-value < 0.001).

Discussion

Microbial abundances, morphotypes and viability

This study describes the microbial ecology of newly discovered brines in Boulder Clay. It underlines the need to protect and preserve these biological niches of extreme life also in the light of the construction of a runway in this area (Urbini et al., 2019). To date, the Boulder Clay lake brine pockets have not extensively been explored, with the exception of two contemporary studies on fungal and prokaryotic diversity by Sannino et al. (2020) and Lo Giudice et al. (2021).

In BC brines, our PA was in a range roughly comparable to that found in the Antarctic Dry Valleys permafrost (Gilichinsky et al., 2007), while it was higher than the values reported in the Lake Vostok (Karl et al., 1999) and lower than those detected in the Lake Vida (McMurdo Dry Valleys, Mosier et al., 2007) and in the Siberian cryopegs (Gilichinsky et al., 2005). Comparing BC brines with Tarn Flat ones (Papale et al., 2019), PA displayed significant quantitative differences (10^5cells ml^{-1} vs. 10^6 cells ml^{-1}, respectively). The observed discrepancy could depend on several interacting factors such as the possible competition among organisms inhabiting the same environment (i.e. grazers) or the availability of trophic resources. Our data revealed, at first glance, differences among the BC brines, as PA was one order of magnitude higher in BC1 compared to the other brines. Conversely, similar PA values were found in BC2 and BC3.

As concerns the prokaryotic VOL, it was on average > 0.1 μm^3 and cells having the large volumes dominated in BC1. Compared to the previous research carried out in the Northern Victoria Land, our mean VOL was roughly greater than that of the prokaryotic cells detected in the BC permafrost (La Ferla et al., 2017). Anyway, cell volumes smaller than 0.1 μm^3 were observed in the active layer of permafrost at Edmonson Point and in Tarn Flat brines, and these prokaryotic cells were supposed to be stressed, starved, or dormant (Papale et al., 2018, 2019). In the Lake Vida, very small prokaryotic cells (VOL range 0.028–0.083 μm^3) were detected also in ice cores collected at 15.9 and 14 m depth (Mosier et al., 2007). Using confocal, scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDS) analysis, small prokaryotic cells were found (Kuhn et al., 2014). These cells had the capability to reduce volume and body or change their life cycle in

Table 3 Statistical results of Nemenyi tests (* indicate *P*-values < 0.05, ** indicate *P*-values < 0.01 and *** indicate *P*-values < 0.001)

	PB	VOL	VA	CTC	*L/D*	AP	LAP	GLU	MINDEX
BCM–BC1	***	***	***	**	–	***	***	–	*
BCM–BC2	–	–	–	–	**	–	**	–	–
BCM–BC3	*	*	**	–	–	**	–	**	–
BC1–BC2	*	**	**	***	***	***	–	**	–
BC1–BC3	–	–	–	–	**	–	**	***	***
BC2–BC3	–	–	–	**	–	–	–	–	***

response to unfavorable environmental factors. Laboratory experiments on *Oleispira antarctica* RB8^T strain showed its transition from spiral to rod and coccoid shapes in relation to the different substrates (oil mixtures) and/or growth temperatures (Gentile et al., 2020). These findings suggest that in BC1, the cells were presumably larger than those capable of altering their phenotype to face environmental changes, as presumably happened in the other brines. Consequently, the study of microbial morphotypes assumes an ecological relevance because it allows to compare different communities (La Ferla et al., 2014); indeed, the environmental forcings act in concert, causing combined responses that can influence cell body. As a matter of fact, microorganisms are subject to such rapid and frequent environmental changes, therefore they can respond more quickly than other biological components in terms of phenotypic properties (Young, 2006). Cocci, that are the most abundant morphotype present in natural samples, did not assume a particular relevance in the BC brines conversely to what observed in the Antarctic permafrost active layer and in Turn Flat brines (La Ferla et al., 2017; Papale et al., 2018, 2019). Differently, the prevalence of rods in BC3 and BCM brine samples could indicate an increased organic content in the water (Pernthaler, 2005), and could be linked with inputs of organic substances more recalcitrant to decomposition (Kalcheva et al., 2008). In fact, nutrient limitation influences the in situ composition of morphotypes and their activities (Thingstad et al., 2005) as well as the enzymatic activities and substrate uptake, growth properties, temperature adaptation and phage infection. The presence of filamentous cells with VOL > 0.4 μm^3 was observed in BC1 only, underlining once again the peculiarity of this brine. The absence or limitation in the availability of one or more nutrients was found to inhibit cell division and produce filamentation in bacterial isolates (Young, 2006). In this context, studies of the genomic diversity could help to understand the intriguing properties of these cryo-ecosystems. Lo Giudice et al. (2021) observed in BC1 brine the massive presence of *Flavobacterum,* a bacterial strain meanly sized 0.4 μm^3. Anyhow, all these findings, together with the presence of five different morphotypes, let us suppose that in BC1 a more complex community was hosted than that of the other brines. The absence of vibrios, curved rods and filamentous cells, observed in the BC2, BCM and BC3, could be due to a more simplex community but the effect of random local factors should not be excluded. Finally, the morphological index, which gives an indication of easily degradable organic matter, was greater than 1, mainly in BCM, suggesting an efficient cell growth strategy (Kalcheva et al., 2014).

As concerns the prokaryotic viability in BC brines, our results evidenced low percentages of living cells in terms of both actively respiring and membrane intact cells. However, the CTC + cells were always lower than the LIVE ones detected by *L/D*, with the exception of BC3 only, where their percentages were reciprocally similar. This discrepancy between *L/D* and CTC + counts could be due to the different approaches used to detect the living cells based on the cell membrane integrity (*L/D*) and the active respiring capacity (CTC +), respectively. Accordingly, the low percentages of CTC + cells detected in an oligotrophic high-mountain lake (Posch et al., 1997) were ascribed to alive but dormant cells or with respiration rates below the detection limit of the method. The comparison among BC permafrost, Turn Flat brines and Edmonson Point active layer (La Ferla et al., 2017; Papale et al., 2018, 2019) showed that the BC brines hosted an even less viable and respiring prokaryotic community. In any case, the microbial community composition revealed a higher number of membrane intact and actively respiring cells in BC1 than in the other examined brines, confirming the distinctiveness of such a brine compared to the others. An intriguing property of the brines could be that more limited the resources are, more complex the community is. In this context, controversial opinions exist about the metabolic capacities of microbes within brines. Actually, compared with their warmer analogues, in subzero eutectic brines enhanced microbial survival was detected (Heinz et al., 2018), as a result of the increase in the stability of hydration shells around ions at lower temperatures that reduces osmotic and chaotropic stress for microorganisms. Nevertheless, the potential survival ability of isolates (i.e. *Planococcus halocryophilus*), and not natural populations, increased, in spite of low temperatures, in chloride-containing samples. According to (Murray et al., 2012), microbial natural population survival in brines was not limited to the maintenance of life in a steady state but implied also the capability to metabolize in situ thermogenic or biogenic sources and to play an important role in

biogeochemical cycles. In the Lake Vida, the encapsulated brines, at − 13°C and a salinity of 200, contained organic carbon resources and microbial populations survived for millennia. Indeed, metabolic studies of several isolates suggested that, at a temperature decreasing below +4°C, much more energy is needed for cell maintenance (Pearce, 2012).

The viral abundance was relatively high, as often happens at cold temperature and hypersaline systems (Reese et al., 2014) and was similar to that observed in Arctic cryopeg brines (Zhong et al. 2020), Alaska permafrost brines (Colangelo-Lillis et al., 2016), Arctic sea-ice, Antarctic Ross Sea pack ice (Le Romancer et al., 2007) and Tarn Flat brines (Papale et al., 2019). According to (Reese et al., 2014), a decreasing structural complexity increased the physiological accessibility to a large range of environmental conditions, like those considered as extreme. Among the environmental forcings, temperature seemed to affect viral community since the highest values were observed in the coldest brine (BC1), as happened in the sea-ice horizons in Arctic brines (Wells & Deming, 2006). Moreover, in relation to salinity increase, switching from a lytic to lysogenic modality of virus infection was observed by (Reese et al., 2014), suggesting that lysogeny enabled viruses to evade the harsh environments. In our samples, excepting BCM, the predominance of V1 group was found. Laboratory tests with isolated phages showed that only viral particles as small as bacteriophages were available in the V1 position (Brussaard, 2004b). Insights into virus–host relationships in a variety of aquatic ecosystems showed that in every habitat VPR fluctuates due to different reasons, as it is linked to a multitude of factors related to virus–host dynamics and host speciation (Parrika et al., 2016). Usually, high VPR are attributed to high and ongoing viral production; low ratios have often been considered the result of diminished viral activity or high viral decay rates (Parrika et al., 2016). The VPR ratios in our samples were similar to those usually reported in warm and cold brines as well as in seawater (Shapira et al., 2009; Jacquet et al., 2010; Papale et al., 2019) where an active microbial community existed (Wommack & Colwell, 2000). However, the true explanatory power of a linear relationship and its robustness across diverse ocean environments is unclear (Parrika et al., 2016).

Metabolic responses

Low physiological responses were observed in most of our samples. In agreement with the low metabolism found in stressing habitats, the potential metabolic functions detected in the Tarn Flat brines (Papale et al., 2019) were in a range near the minimum detectable, with mean ASCD values of 0.057 ± 0.018 for the samples collected at 4 m depth and 0.126 ± 0.154 for the one taken at 5 m depth. These findings were probably ascribable to the environmental constraints. In BC brines, the ASCD differed among the studied samples: in both BC2 and BC3 low metabolism (by physiological profiles) was detected and the lack of lag phase during incubations suggested that the prokaryotic community was metabolically viable as stated by (Meola et al., 2015). The low metabolism observed, near the maintenance level, probably allowed cells to preserve their energetic reserves. Differently, in BC1, ASCD was relatively high, and a high percentage of positive responses to the available organic substrates was recorded (80%), highlighting the peculiar ecosystem functioning. According to Cavicchioli (2015), niche adaptation could be linked to distinctions among the main genera and strain-level variation within populations, which result in differences in resource utilization. A recent research (Lo Giudice et al., 2021), carried out on the metabolically active fraction of the bacterial community hosted by three of the same brines of the present study, depicted a massive occurrence of *Bacteroidetes*, chemo-organotrophic bacteria involved in the detritus food web and carbon cycle. Nevertheless, in BC1 the active fraction included many sequences referable to *Flavobacterum,* suggesting the presence of complex dissolved and particulate organic matter, among which biopolymers. Differently, *Betaproteobacteria*—that need copious amounts of organic nutrients—dominated in BC2 and BC3 brines. Anyway, the DNA/RNA sequencing of these communities could further elucidate the complexity of these brine ecosystems.

Doubtless, the metabolic profiles of microbial community in our study revealed different utilization patterns of the carbon substrates in each brine, with the exception of the complex carbon sources that were efficiently utilized everywhere. An efficient utilization of polyols, acting as emulsifiers, surfactants and solubilizers (i.e. Tween 40, Tween 80 and α-

ciclodextrin) or as energy reserve (glycogen), probably influenced the adaptability of the prokaryotic community to freezing conditions. Similarly, carbohydrates, together with the phosphate carbon sources, known as antifreeze substances as well as energy reserves, may have affected the microbial ecophysiological patterns. In Siberian permafrost (Wagner et al., 2009) carbohydrates were found to be actively metabolized by the prokaryotic community as well as in Antarctic permafrost (La Ferla et al., 2017), where D-cellobiose, being a product of cellulose hydrolysis, could indicate the occurrence of allochthonous organic carbon of algal origin that could derive from sea ice remnants. On the contrary, carboxylic acids, constituents of hydrophobic biomolecules, were intensively used in BC1 only. Among these substrates, the pyruvic acid could be utilized in the methanogenesis and this feature corroborated the hypothesis that the methane cycling could be a current microbial metabolic pathway in the brine of Northern Victoria Land (Papale et al., 2019). Anyway, microbial survival in polar environments involves modifications of the structural and physiological organization to adapt to extreme temperature and salinity conditions (La Ferla et al., 2017; Papale et al., 2019). For example, in order to keep turgor pressure and membrane fluidity, adaptation to extreme environments involves physiological modifications for the utilization of low molecular weight solutes (Fillinger et al., 2001).

Regarding the enzymatic activities in sea ice, microorganisms seem to adapt their metabolism to low temperatures by showing a high specific extracellular activity in the decomposition of dissolved and particulate organic carbon under these conditions (Mock & Thomas, 2005). The strategies of adaptation would also imply the capability to change the metabolic state from dormant to active, perhaps stimulating the production of cold-adapted enzymes (Gilichinsky et al., 2005). New insights on the amount of organic carbon and phosphorus potentially available to sustain microbial metabolism were provided by the enzymatic activities measured in the BC brines. Knowledge on this topic is almost scarce. In our samples, the microbial community was found to be still active to degrade organic polymers, particularly organic phosphates. In spite of the close proximity of the two BC lakes examined in this study, however, differences were observed in the enzymatic activities of the microorganisms hosted within the brine samples. In BC1, the presence of cells with high proteolytic and phosphatasic activities was suggested by the detection of high LAP and AP levels. The increasing trend of LAP activity with depth, reaching a maximum at a 2.5 m depth, indicated the progressive decomposition of labile organic matter. The different magnitude order of the enzymatic expression detected in the brine samples was likely ascribable to the different environmental variables that characterized the examined cryo-environments.

A complex picture of Boulder Clay brines

A complex microbial picture was obtained as a result of multiple factors. First of all, the discovery of prokaryotes in brines stimulates further questions about their survival and adaptive mechanisms in both extreme temperature and osmolarity conditions, made possible by changes in membrane fluidity (Los & Murata, 2004). In our study, the utilization of substrates acting as emulsifier, surfactant and antifreeze compounds or energy reserves allowed the adaptability of the prokaryotic community to freezing conditions.

The local physical and chemical conditions partially explained the microbial differences in abundance, viability and metabolism among the studied brines. In this regard, differences in ion composition, total organic carbon, conductivity and pH have been observed among BC1 and the other brines (Sannino et al., 2020). These findings suggested that a more complex community was hosted in BC1 than that inhabiting the other brines. Moreover, similarities in terms of abundance and metabolism between the other brines were observed. All these features opened the question about the specific historical origin of these brines. In Antarctica, and in particular in Terra Nova Bay (Northern Victoria Land), the widespread presence of subfossils of marine fauna suggested that the edge of Ross's platform incorporated marine sediments deposited on the coast (Orombelli et al., 1990). The geochemistry of lakes in Victoria Land also suggested that the chemical composition of lakes varies either as a direct inheritance of seawater or as the result of salt accumulation due to repeated cycles of water evaporation and freezing over time (Porcino et al., 2020). Several geophysical prospecting studies (e.g. Cowan et al., 2002; Guglielmin et al., 1997) related the location of the study sites at

elevations < 300 m a.s.l. within massive ice remnants of the Last Glacial Maximum Ice Shelf, covered by a layer of frozen sublimation or ablation till of variable thickness from 30 to 40 cm to more than 3 m in some spots. The glacimarine sediments were also testified by the findings of many marine shells, pelecypods, serpulids, sponge spicules, echinoderms and foraminifera (Orombelli et al., 1990). The icing blisters of the permafrost in BC site were supposed to reflect the occurrence of brines at the base of perennially frozen lakes with high salt contents in a hydrostatic extremely cold and arid environment (Guglielmin et al., 2009). It is likely that microorganisms may have been trapped at the same age as the brines, when the dynamics of polar ocean and geological conditions favoured the formation of these cryo-environments as affirmed by (Stan-Lotter, 2012). In BC brines, the order of magnitude of PA corroborated the hypothesis of a marine origin (Porcino et al., 2020). The distribution pattern of microorganisms likely depended on the time in which the brines remained at a frozen state (in relation to their salinity) as well as on the connection with the atmosphere that was relevant for BCM and negligible for BC1. The marine origin of the studied brines was also suggested by the VPR values > 10 registered in BC1, BC2 and BC3.

These findings, together with the different levels of viability and metabolic activities in the diverse brines, led us to suppose that BC1 possessed intrinsic peculiarities different from the other ones likely related to the vicinity of the frost mound that could be related to other hypersaline brines flowing within the underlying permafrost or glacier ice.

Finally, the extensive occurrence of subglacial waters in Antarctica has been postulated in the last decade, but little is known about groundwater, mainly in ice-free zones. The presumable interconnectivity among underground aqueous systems within a glacier or a lake is not yet known. Adopting an airborne transient electromagnetic (AEM) sensor to produce extensive imagery of resistivity, in Taylor Valley (Antarctic Dry Valley) a deep briny groundwater system appeared to exist, beneath glaciers, lakes and permafrost (Mikucki et al., 2004). Similarities between BC2 and BC3 corroborated the hypothesis that icing blisters of the permafrost in BC site were due to a dry-based perennially frozen lake. Coherently, liquid veins of salty water presumably interconnected the single brine pockets, also contributing to resources exchanges (Guglielmin et al., 2009). Recently, Sannino et al. (2020) guessed the interconnection among the brines of the L-A and their difference with respect to the frost mound occurring in the same lake, as revealed by the fungal diversity. Also the interactions between rock and brine by groundwater advection in Arctic cryopegs (Colangelo-Lillis et al., 2016) as well as the interactions between brine and iron-rich sediment (Murray et al., 2012) could provide energy sources for the microbial community inhabiting the brine pockets.

Conclusions

This study has contributed to deepen the current knowledge on microbial ecology of hypersaline brines found at the Boulder Clay (Northern Victoria Valley, Antarctica). In spite of their spatial proximity, the brines were inhabited by diversified prokaryotic assemblages, which make these extreme ecosystems even more attractive from a scientific point of view. In particular, BC1 (the deepest, the saltiest, the coldest brine and the least exposed to heating then the other brines) was inhabited by a more abundant and complex microbial community than the other brines, able to exploit the limited resources available. BC2 and BC3 showed analogies in their community patterns supporting the existence of underground interconnections. The interplay of a lot of factors determined the microbiological peculiarities of each brine, i.e. abiotic factors, marine origin, depth horizon, isolation time.

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References

Bellard, C., C. Bertelsmeier, P. Leadley, W. Thuiller & F. Courchamp, 2012. Impacts of climate change on the future of biodiversity. Ecology Letters 15: 365–377.

Brussaard, C., 2004a. Viral control of phytoplankton populations – a review. Journal of Eukaryotic Microbiology 51(2): 125–138.

Brussaard, C., 2004b. Optimization of procedures for counting viruses by flow cytometry. Applied and Environmental Microbiology 70: 1506–1513.

Brüssow, H., 2009. The not so universal tree of life or the place of viruses in the living world. Philosophical Transactions Royal Society B Biological Sciences 364: 2263–2274.

Cannone, N., D. Wagner, H. W. Hubberten & M. Guglielmin, 2008. Biotic and abiotic factors influencing soil properties across a latitudinal gradient in Victoria Land, Antarctica. Geoderma 144: 50–65.

Cavicchioli, R., 2015. Microbial ecology of Antarctic aquatic systems. Nature Reviews Microbiology 13: 691–706.

Colangelo-Lillis, J., H. Eicken, S. D. Carpenter & J. W. Deming, 2016. Evidence for marine origin and microbial-viral habitability of sub-zero hypersaline aqueous inclusions within permafrost near Barrow, Alaska. FEMS Microbiology Ecology 92: fiw053.

Cowan, D. A., N. J. Russell, A. Mamais & D. M. Sheppard, 2002. Antarctic Dry Valley mineral soils contain unexpectedly high levels of microbial biomass. Extremophiles 6: 431–436.

Fillinger, S., G. Ruijter, M. J. Tamás, J. Visser, J. M. Yhevelein & C. d'Enfert, 2001. Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus *Aspergillus nidulans*. Molecular Microbiology 39: 145–157.

Forte, E., M. Dalle Fratte, M. Azzaro & M. Guglielmin, 2016. Pressurized brines in continental Antarctica as a possible analogue of Mars. Scientific Reports 6: 33158.

Garland, J. R., 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biology Biochemistry 28(2): 213–221.

Garland, J. R. & L. Mills, 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied Environmental Microbiology 57(8): 2351–2359.

Gasol, J. M. & A. Del Giorgio, 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Scientia Marina 64: 197–224.

Gentile, G., G. Maimone, R. La Ferla, M. Azzaro, M. Catalfamo, M. Genovese, S. Santisi, M. Maldani, A. Macrì & S. Cappello, 2020. Phenotypic variations of *Oleispira antarctica* RB8^{T}in different growth conditions. Current Microbiology. https://doi.org/10.1007/s00284-020-02143-8.

Gilichinsky, D., E. Rivkina, V. Shcherbakova, K. Laurinavichuis & J. Tiedje, 2003. Super cooled water brines within permafrost – an unknown ecological niche for microorganism: a model for astrobiology. Astrobiology 3(2): 331–341.

Gilichinsky, D., E. Rivkina, C. Bakermans, V. Shcherbakova, L. Petrovskaya, S. Ozerskaya, N. Ivanushkina, G. Kochkina, K. Laurinavichuis, S. Pecheritsina, R. Fattakhova & J. M. Tiedje, 2005. Biodiversity of cryopegs in permafrost. FEMS Microbial Ecology 53: 117–128.

Gilichinsky, D. A., G. S. Wilson, E. I. Friedmann, C. P. McKay, R. S. Sletten, E. M. Rivkina, T. A. Vishnivetskaya, L. G. Erokhina, N. E. Ivanushkina, G. A. Kochkina, V. A. Shcherbakova, V. S. Soina, E. V. Spirina, E. A. Vorobyova, D. G. Fyodorov-Davydov, B. Hallet, S. M. Ozerskaya, V. A. Sorokovikov, K. S. Laurinavichyus, A. V. Shatilovich, J. P. Chanton, V. E. Ostroumov & J. M. Tiedje, 2007. Microbial populations in Antarctic permafrost: biodiversity, state, age, and implication for astrobiology. Astrobiology 7(2): 275–311.

Gugliemin, M. & N. Cannone, 2012. A permafrost warming in a cooling Antarctica? Climatic Change 111: 177–195.

Guglielmin, M., A. Biasini & C. Smiraglia, 1997. The contribution of geoelectrical investigations in the analysis of periglacial and glacial landforms in ice free areas of the northern foothills (Northern Victoria Land, Antarctica). Geografiska Annaler: Series A, Physical Geography 79(1–2): 17–24.

Guglielmin, M., A. G. Lewkowicz, H. M. French & A. Strini, 2009. Lake-ice blisters, Terra Nova Bay area, Northern Victoria Land, Antarctica. Geografiska Annale Series A, Physical Geography 91(2): 99–111.

Guglielmin, M., M. Dalle Fratte & N. Cannone, 2014. Permafrost warming and vegetation changes in continental Antarctica. Environmental Research Letters 9: 45001.

Heinz, J., J. Schirmack, A. Airo, S. P. Kounaves & D. Schulze-Makuch, 2018. Enhanced microbial survivability in subzero brines. Astrobiology 18(9): 1171–1180.

Heinz, J., A. C. Waajen, A. Airo, A. Alibrandi, J. Schirmack & D. Schulze-Makuch, 2020. Bacterial growth in chloride and perchlorate brines: halotolerances and salt stress responses of *Planococcus halocryophilus*. Astrobiology 20(2): 1377–1387.

Hoppe, H. G., 1993. Use of fluorogenic model substrates for extracellular enzyme activity (EEA) measurement of bacteria. In Kemp, P. F., B. F. Sherr, E. B. Sherr & J. J. Cole (eds), Handbook of methods in aquatic microbial ecology. FL Lewis Publisher, Boca Raton: 423–432.

Jacquet, S., T. Miki, R. Noble, P. Peduzzi & S. Wilhelm, 2010. Viruses in aquatic ecosystems: important advancements of the last 20 years and prospects for the future in the field of microbial oceanography and limnology. Advances in Oceanography and Limnology 1(1): 97–141.

Jansson, J. K. & N. Taş, 2014. The microbial ecology of permafrost. Nature Reviews Microbiology 12(6): 414–425.

Kalcheva, H., M. Beshkova, L. Pehlivanov & R. Kalcev, 2008. Bacterioplankton dynamics and the influence of environmental factors on it in the SrebarnaLake. The third international scientific conference BALWOIS, Ohrid, Republic of Macedonia 27, 31 May 2008.

Kalcheva, H., R. Kalchev & M. Beshkova, 2014. Bacterioplankton of Wetlands along the lower Danube (Bulgaria) and its relation to environmental factors. Acta Zoologica Bulgarica 7: 83–89.

Karl, D. M., D. F. Bird, K. Björkman, T. Houlihan, R. Shackelford & L. Tupas, 1999. Microorganisms in the accreted ice of LakeVostok, Antarctica. Science 286: 144–2147.

Kuhn, E., A. S. Ichimura, V. Peng, C. H. Fritsen, G. Trubl, P. T. Doran & A. E. Murray, 2014. Brine assemblages of ultrasmall microbial cells within the ice cover of Lake Vida, Antarctica. Applied Environmental Microbiology 80(12): 687–3698.

La Ferla, R., G. Maimone, M. Azzaro, F. Conversano, C. Brunet, A. S. Cabral & R. Paranhos, 2012. Vertical distribution of the prokaryotic cell size in the Mediterranean Sea. Helgoland Marine Research 66: 635–650.

La Ferla, R., G. Maimone, G. Caruso, F. Azzaro, M. Azzaro, F. Decembrini, A. Cosenza, M. Leonardi & R. Paranhos, 2014. Are prokaryotic cell shape and size suitable to ecosystem characterization? Hydrobiologia 726: 65–80.

La Ferla, R., G. Maimone, A. Lo Giudice, F. Azzaro, A. Cosenza & M. Azzaro, 2015. Cell size and other phenotypic traits of prokaryotic cells in pelagic areas of the Ross Sea (Antarctica). Hydrobiologia 761: 181–194.

La Ferla, R., M. Azzaro, L. Michaud, G. Caruso, A. Lo Giudice, R. Paranhos, A. S. Cabral, A. Conte, A. Cosenza, G. Maimone, M. Papale, A. C. Rappazzo & M. Guglielmin, 2017. Prokaryotic abundance and activity in permafrost of the northern Victoria Land and upper Victoria Valley (Antarctica). Microbial Ecology 74(2): 402–415.

Le Romancer, M., M. Gaillard, C. Geslin & D. Prieur, 2007. Viruses in extreme environments. Reviews Environmental Science and Bio-Tecnology 6: 17–31.

Lo Giudice, A., A. Conte, M. Papale, C. Rizzo, M. Azzaro & M. Guglielmin, 2021. Prokaryotic diversity and metabolically active communities in brines from two perennially ice-covered Antarctic lakes. Astrobiology. https://doi.org/10.1089/ast.2020.2238.

Loferer-Krössbacher, M., J. Klima & R. Psenner, 1998. Determination of bacterial cell dry mass by transmission electron microscopy and densitometric image analysis. Applied Environmental Microbiology 64: 688–694.

Los, D. A. & N. Murata, 2004. Membrane fluidity and its roles in the perception of environmental signals. BiochimicaBiophysicaActa 1666: 142–157.

Luhtanene, M. A., E. Eronen-Rasimus, H. M. Oksanen, J. L. Tison, B. Delille, G. S. Dieckmann, J. M. Rintala & D. H. Bamford, 2018. The first known virus isolates from Antarctic sea ice have complex infection patterns. FEMS Microbiology Ecology 94: fiy028.

Madigan, M. T., J. M. Martinko & J. Parker, 2003. Extremely halophilic Archaea. In Carlson, G., S. L. Snavely, D. Wechsler & K. Schiaparelli (eds), Brock Biology of Microorganisms. Prentice Hall, Upper Saddle River: 448–452.

Malone, J. L., M. C. Castro, C. M. Hall, P. T. Doran, F. Kenig & C. P. McKay, 2010. New insights into the origin and evolution of lake Vida, McMurdo Dry Valleys, Antarctica – a noble gas study in ice and brines. Earth Planetary Science Letters 289: 112–122.

Mann, P. J., W. V. Sobczak, M. M. LaRue, E. Bulygina, J. E. Vonk, J. Schade, S. Davydov, N. Zimov, R. M. Holmes & R. G. M. Spencer, 2014. Evidence for key enzymatic controls on metabolism of Arctic river organic matter. Global Change Biology 20: 1089–1100.

Meola, M., A. Lazzaro & J. Zeyer, 2015. Bacterial composition and survival on Sahara Dust particles transported to the European Alps. Frontiers Microbiology 6: 1454.

Michaud, L., A. Lo Giudice, M. Mysara, P. Monsieurs, C. Raffa, N. Leys, S. Amalfitano & R. Van Houdt, 2014. Snow surface microbiome on the high Antarctic Plateau (DOME C). PLoS ONE 9: e104505.

Mikucki, J. A., C. M. Foreman, B. Sattler, W. B. Lyons & J. C. Priscu, 2004. Geomicrobiology of blood falls: an iron-rich saline discharge at the terminus of the Taylor glacier, Antarctica. Aquatic Geochemistry 10: 199–220.

Mikucki, J. A., E. Auken, S. Tulaczyk, R. A. Virginia, C. Schamper, K. I. Sørensen, P. T. Doran, H. Dugan & N. Foley, 2015. Deep groundwater and potential subsurface habitats beneath an Antarctic dry valley. Nature Communications 6: 6831.

Mock, T. & D. N. Thomas, 2005. Recent advances in sea-ice microbiology. Environmental Microbiology 7: 605–619.

Mosier, A. C., A. E. Murray & C. H. Fritsen, 2007. Microbiota within the perennial ice cover of Lake Vida, Antarctica. FEMS Microbiology Ecology 59: 274–288.

Murray, A. E., F. Kenig, C. H. Fritsen, C. P. McKay, K. M. Cawley, R. Edwards, E. Kuhn, D. M. McKnight, N. E. Ostrom, V. Peng, A. Ponce, J. C. Priscu, V. Samarkin, A. T. Townsend, P. Wagh, S. A. Young, P. T. Yung & P. T. Doran, 2012. Microbial life at -13 C in the brine of an ice-sealed Antarctic lake. Proceedings National Academy Sciences USA 109(50): 20626–20631.

Oksanen J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens, E. Szoecs, H. Wagner, 2018. vegan: Community Ecology Package. R package version 2.3-3. https://CRAN.R-project.org/packagevegan.

Orombelli, G., C. Baroni & G. H. Denton, 1990. Late Cenozoic glacial history of the Terra Nova Bay region, Northern Victoria Land, Antarctica. Geografia Fisica Dinamica Quaternaria 13: 139–163.

Papale, M., A. Conte, A. Mikkonen, L. Michaud, R. La Ferla, M. Azzaro, G. Caruso, R. Paranhos, A. S. Cabral, G. Maimone, A. C. Rappazzo, C. Rizzo, N. Spanò, A. Lo Giudice & M. Guglielmin, 2018. Prokaryotic assemblages within permafrost active layer at Edmonson Point (Northern Victoria Land, Antarctica). Soil Biology & Biochemistry 123: 165–179.

Papale, M., A. Lo Giudice, A. Conte, C. Rizzo, A. C. Rappazzo, G. Maimone, G. Caruso, R. La Ferla, M. Azzaro, C. Gugliandolo, R. Paranhos, A. S. Cabral, V. R. Spica & M. Guglielmin, 2019. Microbial assemblages in pressurized Antarctic brine pockets (Tarn Flat, Northern Victoria Land): a hotspot of biodiversity and activity. Microorganisms 7: 333.

Parikka, K. J., M. Le Romancer, N. Wauters & S. Jacquet, 2016. Deciphering the virus-to-prokaryote ratio (VPR): insights into virus–host relationships in a variety of ecosystems. Biological Reviews 92(2): 1081–1100.

Pearce, D. A., 2012. Extremophiles in Antarctica: Life at Low Temperatures. In Stan-Lotter, H. & S. Fendrihan (eds), Adaption of Microbial Life to Environmental Extremes. Springer, Vienna: 87–218.

Pernthaler, J., 2005. Predation on prokaryotes in the water column and its ecological implications. Nature Reviews Microbiology 3: 537–546.

Porcino, N., A. Cosenza & M. Azzaro, 2020. A review on the geochemistry of lakes in Victoria Land (Antarctica). Chemosphere 251: 126229.

Porter, K. G. & Y. S. Feig, 1980. The use of DAPI for identifying and counting aquatic microflora. Limnology Oceanography 25: 943–948.

Posch, T., J. Pernthaler, A. Alfreider & R. Psenner, 1997. Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. Applied Environmental Microbiology 63(3): 867–873.

Rampelotto, P. H., 2014. Polar microbiology: recent advances and future perspectives. Biology 3(1): 81–84.

Reese, B.K., J.A. Koester, J. Kirkpatrick, T. Konotchick, L. Zeigler Allen & C. Dziallas, 2014. How extreme is extreme? In P.F. Kemp (ed), Eco-DAS X Symposium Proceedings ASLO pp. 66–87

Rizzo, C., A. Conte, M. Azzaro, M. Papale, A. C. Rappazzo, D. Battistel, M. Roman, A. Lo Giudice & M. Guglielmin, 2020. Cultivable bacterial communities in brines from perennially ice-covered and Pristine AntarcticLakes: ecological and biotechnological implications. Microorganisms 8: 819.

Sala, M. M., L. Arin, V. Balagué, J. Felipe, Ò. Guadayol & D. Vaqué, 2005. Functional diversity of bacterioplankton assemblages in Western Antarctic seawaters during late spring. Marine Ecology Progress Series 292: 13–21.

Sala, M. M., M. Estrada & J. M. Gasol, 2006. Seasonal changes in the functional diversity of bacterioplankton in contrasting coastal environments of the NW Mediterranean. Aquatic Microbial Ecology 44: 1–9.

Sannino, C., L. Borruso, A. Mezzasoma, D. Battistel, L. Zucconi, L. Selbmann, M. Azzaro, S. Onofri, B. Turchetti, P. Buzzini & M. Guglielmin, 2020. Intra- and inter-cores fungal diversity suggests interconnection of different habitats in an Antarctic frozen lake (Boulder Clay, Northern Victoria Land). Environmental Microbiology. 22(8): 3463–3477.

Shapira, M., M. J. Buscot, S. C. Leterme, T. Pollet, C. Chapperon & L. Seuront, 2009. Distribution of heterotrophic bacteria and virus-like particles along a salinity gradient in a hypersaline coastal lagoon. Aquatic Microbial Ecology 54: 171–183.

Schuur, E. A. G., A. D. McGuire, C. Schädel, G. Grosse, J. W. Harden, D. J. Hayes, G. Hugelius, C. D. Koven, P. Kuhry, D. M. Lawrence, S. M. Natali, D. Olefeldt, V. E. Romanovsky, K. Schaefer, M. R. Turetsky, C. C. Treat & J. E. Vonk, 2015. Climate change and the permafrost carbon feedback. Nature 520: 171–179.

Stan-Lotter, H., 2012. Physico-chemical boundaries of life. In Stan-Lotter, H. & S. Fendrihan (eds), Adaptation of Microbial Life to Environmental Extremes. Novel Research Results and Application. Springer, Wien New York: 7–12. ISBN 978-3-211-99690-4.

Team, R.C., 2013. R: a Language and Environment for Statistical Computing. Vienna, Austria. http://www.R-project.org. Accessed 2014 April 15.

Thingstad, T. F., L. Øvreås, J. K. Egge, T. Løvadal & M. Heldal, 2005. Use of non-limiting substrates to increase size: a generic strategy to simultaneously optimize uptake and minimize predation in pelagic osmotrophs? Ecology Letters 8: 675–682.

Urbini, S., G. Bianchi-Fasani, P. Mozzanti, A. Rocca, L. Vittuari, A. Zanutta, V. A. Girelli, M. Serafini, A. Zirizzotti & M. Frezzotti, 2019. Multi-temporal investigation of the Boulder Clay Glacier and Northern Foothills (Victoria Land, Antarctica) by integrated surveying techniques. Remote Sensing 11(12): 1501.

Verde, C., D. Giordano, C. M. Bellas, G. di Prisco & A. M. Anesio, 2016. Polar marine microorganisms and climate change. In Poole, R. K. (ed.), Advances in Microbial Physiology, Vol. 69. Academic Press, Oxford.

Wagner, D., S. Kobabe & S. Liebner, 2009. Bacterial community structure and carbon turnover in permafrost-affected soils of the Lena Delta, north eastern Siberia. Canadian Journal Microbiology 55: 73–83.

Watson, T., 2019. The trickster microbes shaking up the tree of life. Nature 569: 322–324.

Wei, T., 2013. Corrplot: visualization of a correlation matrix. R package version 0.60.

Wells, L. & J. W. Deming, 2006. Modelled and measured dynamics of viruses in Arctic winter sea-ice brines. Environmental Microbiology 8(6): 1115–1121.

Wommack, K. E. & R. R. Colwell, 2000. Virioplankton: Viruses in aquatic ecosystems. Microbiology Molecular Biology Reviews 64: 69–114.

Yau, S. & M. Seth-Pasricha, 2019. Viruses of polar aquatic environments. Viruses 11: 189.

Young, K. D., 2006. The selective value of bacterial shape. Microbiology Molecular Biology Reviews 70: 660–703.

Zar, J. H., 1996. Biostatistical Analysis, 3rd ed. Prentice Hall, New Jersey.

Zhong, Z. P., J. Z. Rapp, J. M. Wainaina, N. E. Solonenko, H. Maughan, S. D. Carpenter, Z. S. Cooper, H. B. Jang, B. Bolduc, J. W. Deming & M. B. Sullivan, 2020. Viral ecogenomics of Arctic Cryopeg Brine and Sea Ice. Ecological and Evolutionary Science. https://doi.org/10.1128/mSystems.00246-20.

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