The archaeal community in sediments of freshwater lakes of north-east Antarctic Peninsula: Structure and diversity

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Abstract

This research represents the first attempt to study the structure and diversity of the archaeal and methanogenic archaeal community in selected lakes around the Czech polar station J.G. Mendel on James Ross Island (JRI), Antarctica. Sediment samples from a total of 19 of JRI and the nearby Vega Island and Long Island, were analyzed using 16S rRNA and mcrA genes sequencing and real-time qPCR. Contrary to the sequences retrieved by 16S rRNA analysis, many more reads belonging to methanogens were found with *mcrA* gene sequencing. Generally, archaea represented only a small proportion (0–8.8%) of the total prokaryotic community. With the exception of lakes in the Solorina Valley and Lagoons Mesa area and Lake Esmeralda on Vega Island, methanogenic archaea made up a small proportion of the archaea present in most lakes. The genera *Methanothrix* and *Methanosarcina* were identified as the predominant methanogenic representatives in the lake samples. Sequences of representatives belonging to *Methanothermobacter* sp. and Methanomassiliicoccales and a high proportion of sequences belonging to Methanoperedens-like archaea, methanotrophs that associate anaerobic methane oxidation with denitrification, were recorded for the first time in Antarctica. It is also the first time that the presence of the genus *Methanobacterium* has been detected to such a large extent. Generally, sequences of the methanogens which might be involved in all three pathways of methane production were found in our samples, indicating the broad metabolic potential of the methanogens present. Individual lakes from one area shared much higher similarity in their methanogenic diversity with the lakes from another area rather than with the lakes within the same area, suggesting that a lake location is probably not the main factor influencing the diversity of the methanogens. Indeed, archaeal and methanogenic community structure and *mcrA* gene copy numbers varied even within a single lake, suggesting that more sampling within a single lake, preferably at different times of the year, will be necessary in the future for more comprehensive information. Although this is an initial study, our research unambiguously provides evidence that the lakes of the JRI and surrounding islands may be potential sources of new archaeal species or metabolic pathways.

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Introduction

Low temperature and temperature extremes are considered dominating driving factors affecting antarctic life. Antarctica is considered to be the most hostile continent of the World with temperatures ranging from -5° to -70°C during the polar summer and winter, respectively (Siddiqui et al. 2013). Besides a low temperature, Antarctic life is restricted also by the low availability of nutrients and water, along with whole daylight availability during austral summer and its absence during the winter. Due to continental ice sheet, Antarctica plays an important role in climate change and ocean functioning. On the other hand, this continent is very sensitive to any changes related to ongoing climate change (Cavicchioli 2015). All these factors make Antarctica a unique place study to microbial life and understanding of local ecosystems' functioning.

Microbial biodiversity in polar aquatic environments depends on topography of terrestrial ecosystems, local/regional climatic factors, and represents a basis for the studies of endemism and genetic diversity (Pearce and Galand 2024). Antarctic habitats include freshwater lakes and ponds located in the deglaciated parts of the continent (that forms only about 0.4% of the total area of Antarctica), primarily in the regions on the coastal fringe such as the Vestfold Hills and McMurdo Dry Valley (Wilkins et al. 2013, Cavicchioli 2015). Within this ice-free area, the diversity of aquatic system types is considerable, ranging from fresh to hypersaline, permanently ice-covered to perenially ice-free, and mixed to stratified (Wilkins et al. 2013). Subglacial lakes provide additional, potentially important reservoirs of microbial life, as do cryoconite holes (Cavicchioli 2015). In the Antarctica, liquid water is usually oligotrophic to ultra-oligotrophic with a minimum of nutrients. It can be occasionally enriched with the bird or seal feces or organic material derived from their carcasses (Ruiz-Fernández et al. 2019). Generally, coastal lakes are more enriched with nutrients, allowing a vast density of planktonic animals like fairy shrimp *Branchinecta gaini* or copepod *Boeckella poppei* (Pociecha and Dumont 2008, Nedbalová et al. 2017).

Microbial diversity studies in antarctic lakes focused mainly on bacteria as they usually represent the largest part of the microbial community. They are present in water column, lake bottom and lake sediments where they show vertical distribution along the depth of the sediment due to the prevalence of anoxic conditions (Shivaji et al. 2011). Previous studies using molecular approaches have also revealed significant bacterial diversity (Bowman et al. 2000, Kurosawa et al. 2010, Michaud et al. 2012). Recently, several studies focused on biodiversity of bacteria in antarctic terrestrial ecosystems including archaeal communities (*e.g.* Doytchinov and Dimov 2022). Archaea are usually less important inhabitants of the lake sediments, they often represent no more than 5% of the microbial diversity (Mulyukin et al. 2014, Achberger et al. 2016, Gugliandolo et al. 2016), although rare exceptions to this rule do exist (Purdy et al. 2003). Hypersaline lakes where haloarchea might account for a majority of the microorganisms (Bowman et al. 2000), however, represent different niche. The key aspect driving the diversity of the microorganisms in such lakes is their engagement and role in carbon, nitrogen, and sulfur cycling. Carbon

entering lakes comes from thawing or non-thawing soils surrounding lakes, from lake primary production, and dead organisms.

Generally, chemoheterotrophic and chemolithotrophic microorganisms are involved in the remineralization of organic matter while chemolithotrophic activity, is increased during winter months with low light availability in lake sediments (Vick-Majors et al. 2014). Members of Delta-Proteobacteria are involved in dissimilative sulfate reduction as well as Firmicutes which contribute to the metabolism of nitrates, sulfates, and metals occuring also within the lake sediments. Deeper layers of lake sediment are usually dominated by methanogenic archaea, namely those where other electron acceptors like nitrates and sulfates were already exhausted.

Members of genera *Methanosarcina*, *Methanothrix*, and *Methanoculleus* are the most common methanogenic representatives (Bowman et al. 2003, Karr et al. 2006, Chaya et al. 2019). Worthy notice is the isolation of two important methanogens, *Methanococcoides burtonii* and *Methanogenium frigidum* from meromictic Ace Lake in Vestfold Hills (Cavicchioli 2006). Owing to their capability to grow at low temperatures, both species are used as model organisms in research of methanogenic psychrophiles (Franzmann et al. 1997, Williams et al. 2011).

Ice-free area of the James Ross Island (JRI), the Ulu Peninsula in particular, is reported the largest ice-free area in the Antarctic Peninsula region (Hrbáček et al. 2017). Moreover, neighbouring Vega Island and Long Island are partly deglaciated and possess ice-free areas as well. Such ice-free areas offer suitable conditions for scientific investigations of lakes. This area represents one of the largest ice-free areas in the northern part of the Antarctic Peninsula. The lakes are found at altitudes ranging from < 20 m above sea level (a.s.l.) near the coast to 400 m a.s.l. in the mountain areas. The lakes are relatively young and were formed by the deglaciation processes of the ice sheet, retreat of the JRI ice cap, and postglacial isostatic uplift (Nedbalová et al. 2013). It was showm for the JRI freshwater ponds and lakes that bacterial biodiversity of such relatively young water bodies is lower than in older ones located at JRI, *i.e.* older than 2000 years (Kollár et al. 2023). Compared to brackish and saline lakes experiencing marine incursions and successive periods of meromixis like Ace Lake in the Vestfold Hills (Laybourn-Parry and Pearce 2007), all of the lakes on JRI are freshwater, but some of them may be influenced by sea spray due to their geomorphological position (Lachman Lakes, Phormidium and Muddy Lake, Green 2 Lake, Long Island and Esmeralda Lake). Six different types of lakes were defined, while bedrock, lake age, and morphometry are the most important factors underlying the observed limnological variability (Nedbalová et al. 2013). Investigations of JRI lakes and lakes on Vega Island were concentrated mostly to paleolimnology (Björck et al. 1996, Píšková et al. 2019), chemicophysical limnology (Nedbalová et al. 2013, Roman et al. 2019), algology (Kopalová et al. 2013, 2019; Nedbalová et al. 2017, Bulínová et al. 2020) and crustacean biology (Nedbalová et al. 2017). Till now, however, only a very small research effort has been devoted to the study of the bacterial-cyanobacterial mats occurring on the bottom of freshwater lakes (Komárek et al. 2008, 2012; Kollár et al. 2023). Importance of studying the biodiversity of microbial communities covering the bottom of lakes is apparent, since such microhabitats represent hotspots of biodiversity and biological activity in an otherwise harsh and generally unproductive Antarctic terrestrial environments (Cavicchioli 2015).

Diversity, density of archaea, and methanogenic archaea in sediments of selected Antarctic freshwater lakes was explored in this paper. Samples from JRI and the nearby Vega Island and Long Island were col-

lected and analyzed, using 16S rRNA and mcrA genes sequencing. The 16S rRNA gene sequencing provided a general view of the diversity of total archaeal members in the samples and had allowed also a comparison of archaeal diversity with bacterial one. The mcrA gene sequencing has offered deeper insight into the taxonomical diversity of methanogenic archaea and archaea capable of anaerobic methane oxidation (AOM). Such approach was used in order toto identify as many microbes as possible.

Material and Methods

S*ampling sites*

Lake sediment sampling was performed during the Australian summer in 2019 from January to February, as a research part of the Czech Antarctic expedition to the J. G. Mendel Polar Station on 2019. Sediment samples for further processing in the laboratory were taken from 19 different lakes in the deglaciated areas of the northern part of James Ross Island (Ulu Peninsula), as well as lakes in the southwestern deglaciated part of Vega Island and the lake on Long Island (Fig. 1). The main criterion for the selection of these lakes, apart from their different locations and limited research so far, was mainly the accessibility from the J. G. Mendel Polar Station.

Brief characteristics together with the identification codes are summarised in Table 1. Location and classification of lakes into the types according to their origin, geomorphological position, hydrological stability, and the presence of the given subsoil can be found in a paper by Nedbalová et al. (2013).

Sampled localities were divided into six geographical parts: Lachman lakes area, Sollorina Valley area, Lagoon Mesa area, Brandy Bay area, and two close islands – Vega and Long Island (*see* Fig. 1).

Lachman lakes area

One of the studied localities lies at the northeastern promontory of Ullu Peninsula close to Cape Lachman. We can find here Lachman lakes (LA1, LA2) also known as Big and Small Lachman Lake. There are also small shallow lakes and bodies of water in the area, which act as a connection between these larger lakes. They can be supplied from melting snow plates and in case of a lack of snow, they are endangered by drying out (Váczi and Hájek 2013).

Lagoons Mesa (JRI)

The Lagoons Mesa area is characterized by an irregular elevated surface from the rocks of the James Ross volcanic group formed by glacial erosion, which is covered by glacial sediments with basalt fragments (Lecomte et al. 2016). Numerous lakes are perched at an altitude of over 160 m a. s. l. (ASL) and the type belongs to the stable shallow lakes. Lakes BLA, CYA, and LM1 (Lake Cecil according to Lecomte et al. 2016), were sampled as well as lakes LM2 and LM3, which lie west of lakes Vondra, and have not yet been described in detail, so only values from temperature, pH and conductivity measurements were recorded.

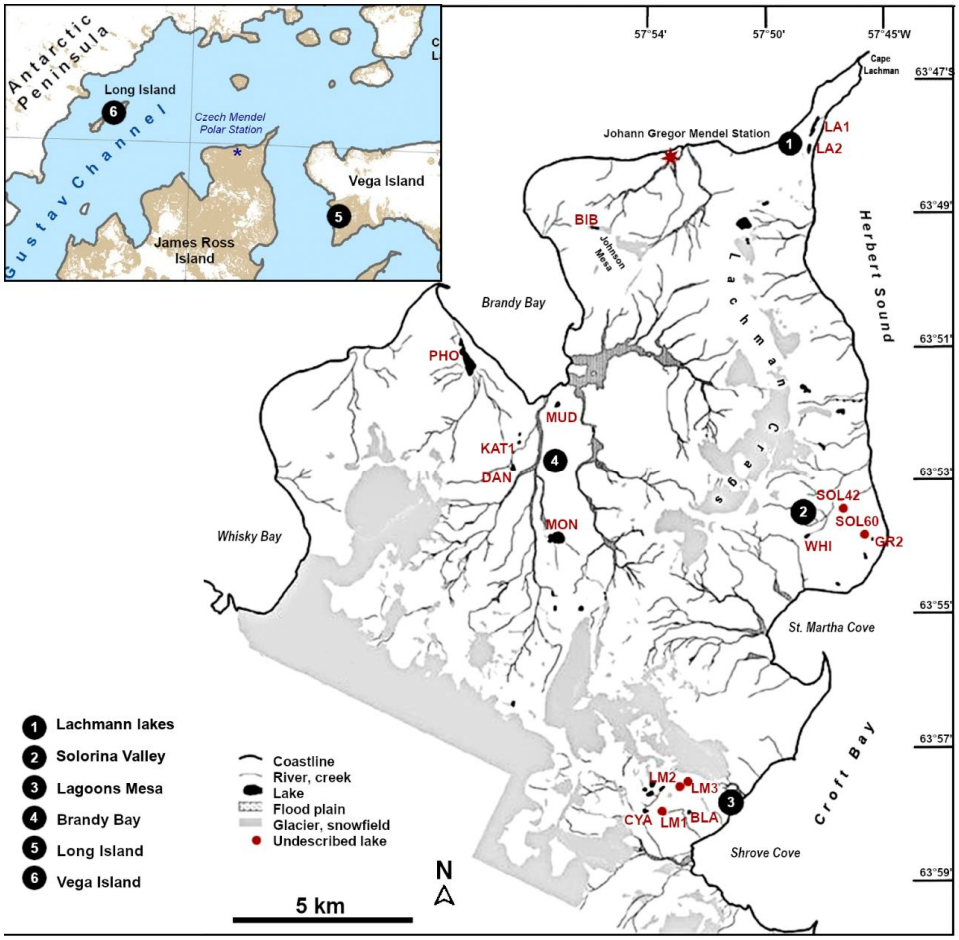

Fig. 1. Location of the sampled lakes areas. Lakes marked with an asterisk may be affected by sea spray. a) Rectangular marked sampled areas on JRI, Vega, and Long Island, b) North part of JMI with marked sampled lakes. Adapted according to the Czech Geological Survey (2009)[2].

Solorina Valley area (JRI)

Solorina Valley is located about ten kilometers from the polar station on the northeast side of the JRI. With a length of three kilometers, it is one of the longest in the area. The climate is colder here than in the other areas studied because of constant winds, humidity, and the transfer of sea aerosol (Zvěřina et al. 2017).

The lakes here are characterized as stable shallow lakes, similarly to lakes in the Lagoons Mesa area. They are one of the oldest created after the deglaciation of the area in the early Holocene and occur not far from the coast at an altitude of 65 ASL. Only two of the previously described lakes were sampled, namely White lake (WHI) and Green 2 lake (GR2), and other two sampled Solorina lakes (SOL42, SOL60) were not described yet.

Brandy Bay area

Lakes Monolith, Dan and Katia1 at JRI

The second largest lake in the deglaciated part of the Ulu Peninsula is Lake Monolith (MON) at an altitude of 67 m ASL, about six kilometers from Brandy Bay. It is a stable lake formed after the retreat of the Whiskey Glacier during the Middle Holocene (Nedbalová et al. 2013). Numerous tributaries lead to the lake and are rich in freshwater communities of algae and cyanobacteria. The area around the tributaries is abundantly covered with mosses, lichens, and microbial growths (Skácelová et al. 2013).

The small lakes Dan (DAN) and Katia1 (KAT1) occur northwest of Lake Monolith and southwest of Lake Muddy. Although they are shallow lakes, their location is at higher altitudes in the area of the old moraine ranks among the stable lakes several thousand years old, as well as Lake Monolith and Phormidium (Nedbalová et al. 2013).

Phormidium and Muddy Lakes at JRI

Lake Phormidium (PHO) is the largest lake in the deglaciated part of UP. With Muddy Lake (MUD), which is relatively smaller, PHO is representative of coastal shallow lakes. PHO and MUD are located on the shores of Brandy Bay. Like Lake Lachman, it lies on a terrace made up of a mixture of Upper Cretaceous marine sediments and Miocene to Holocene glacial sediments. Lake Phormidium is considered more stable because it is probably several thousand years old moraine lake.

Bibby lake

Lake Bibby (BIB) is several decades to hundreds of years old glacial lake, formed after the melting of a glacier in the karst area of Johnson Mesa. It lies at an altitude of 250 m ASL. During the summer months, the lake partially freezes and is supplied by snow cover on the slopes of Johnson Mesa (Skácelová et al. 2013, Coufalík et al. 2016). The lake is surrounded by a rock wall in the southwest and dammed by a moraine in the northeast.

Vega Island

Vega Island is characterized by a similar climate as JRI. Dry and cold winds from the south and southwest with the presence of rain shadows from the Antarctic Peninsula are reported by Engel et al. (2012). The vast majority of the island's area is covered by glaciers. Deglaciated area, however, is found on the western side of the island around Cape Lamb. There are numerous lakes, which have been studied already (Moreno et al. 2012, Lecomte et al. 2016, Píšková et al. 2019).

Lake Esmeralda is located on the southwestern tip of the island of Vega, in the Cape area. It lies at an altitude of 68 m ASL. The depth is greater than in most of the studied lakes, and reaches 6 m. At present, the lake is supplied mainly by the thawing of the active layer of permafrost and from occasional precipitation, as the tributaries are currently diverted. The bottom is formed mainly by sandstone and claystone formations of Snow Hill Island, so the bottom is muddy in nature (Píšková et al. 2019).

Long Island area

The lake located on the northwestern part of Long Island is of glacial origin with a markedly rocky bottom, but it has not yet been sufficiently described and there are only data from sampling.

Lake	Lake Code	Latitude	Longitude	Type	Altitude ASL (m)	Max. depth (m)	Distance from the shore (km)
Bibby	BIB	63.8197	57.9302	4	250	2.7	1.25
Black	BLA	63.9654	57.8824	1	166	0.5	0.84
Cyanobacteria	CYA	63.9655	57.9063	1	185	1.5	1.56
Dan	DAN	63.8788	57.9789	3	41	0.2	1.82
Esmeralda	ESM	63.8739	57.6063	-	68	6.1	1.02
Green 2	GR2	63.8984	57.7765	1	40	0.9	0.38
Katia1	KAT1	63.8709	57.9755	3	36	0.5	0.9
Lagoons Mesa 1	LM1	63.9660	57.8971	1	169	-	1.2
Lagoons Mesa 2	LM2	63.9591	57.8834	-	188	-	1.52
Lagoons Mesa 3	LM3	63.9598	57.8864	-	193	-	1.44
Lachman 1	LA1	63.7942	57.8065	2	9	0.3	0.2
Lachman 2	LA2	63.7999	57.8087	2	13	0.4	0.13
Long Island	LI	63.7652	58.1836	-	22	-	0.24
Monolith	MON	63.8980	57.9571	3	67	2.2	3.69
Muddy	MUD	63.8635	57.9537	2	4	-	0.36
Phormidium	PHO	63.8521	58.0043	3	10	0.3	0.37
Solorina 42	SOL42	63.8886	57.7943	-	36	-	0.99
Solorina 60	SOL60	63.8977	57.7794	-	67	-	0.54
White	WHI	63.8975	57.8137	1	65	1.8	1.48

Table 1. Geomorphological characteristics of sampled Antarctic lakes.
Types of lakes: 1 – stable shallow lake, 2 – shallow coastal lake, 3 – lake in the area of the old moraine, 4 – small unstable lake in the area of the young moraine. Adapted according to Nedbalová et al. (2013) and supplemented by data (Moreno et al. 2012, Lecomte et al. 2016).

Sampling method

Sediment samples from the upper 10 cm layer were collected manually using nitrile gloves and a sterile sampler about 50 cm from the lake shore into sterile 2 ml cryovials (Brand GmbH and CO KG, Germany) in triplicate. The viability of the microbial cells in the samples was maintained by LifeGuard Soil Preservation (Qiagen, Germany). Upon return to the J. G. Mendel station, the samples were stored at -20°C and transported to the Czech Republic. The temperature, pH, and conductivity of the lake surface water were measured in situ during sampling using the Combo pH / EC / TDS / T tester HI 98130 (Hanna Instruments, Italy).

DNA isolation

Subsamples from all lakes were pooled before the genomic DNA isolation. Hence, 19 samples from 19 different lakes were used for subsequent molecular analyses. Prior to isolation, the cryovials were removed from -20°C and allowed to thaw by themselves. The sediments were then manually homogenized and centrifuged on a MiniSpin® plus microcentrifuge (Eppendorf AG, Germany) at maximum speed to remove excess liquid from the sediment sample. For isolation, approximately 0.4 g of sediment was weighed on an Explorer EX124 / AD analytical balance (OHAUS, USA). An aliquot of each cryovial was added to the pool of samples. The DNeasy PowerSoil Kit (Qiagen, Germany) was used for isolation and the manufacturer's recommended protocol was followed, except for the last step, where only 80 µl was used for DNA elution to concentrate the DNA, since a relatively low yield was expected. DNA concentration and purity were measured on a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Germany).

Real-time PCR

Real-time qPCR was used to detect and quantify methanogenic archaea from lake sediment samples. The *mcrA* gene encoding the alpha subunit of methyl-coenzyme M reductase was selected as the target sequence, which catalyzes the last reaction in methane formation by methanogens, and conversely the first reaction in archaea capable of anaerobic methane oxidation (AOM). This gene is generally used to detect methanogens in the environment (Steinberg and Regan 2009, Laskar et al. 2018).

For absolute quantification of methanogens, the qPCR method (quantitative polymerase chain reaction) was used. The reaction was performed on Light Cycler 480 II (Roche, Switzerland) in triplicates for every sample. The volume of the reaction was 14 µL, including 4 µL of template DNA and 9 µL of Luna Master Mix (BioLabs, New England) with two forward (0.25 µL per one) and one reverse primer (0.5 µL) with a final concentration of 250 nM each. The primer was targeted at the mcrA gene which is supposed to be a single copy gene (one gene per one methanogen cell – *e.g.* Vaksmaa et al. 2017). For this study, a combination of three primers was designed. Reverse mcrA primer 5´-CGTTCATBGCGTAGTTVGGRTAGT-3´ was mixed with an equal volume of two forward primers mcrAF1 5´-ACTTCGGTGGATCDCARAGRGC-3´ and mcrAF2 5´-ACTTCGGCGGTTCDCARAGRGC-3´ (Denman et al. 2007, Steinberg and Regan 2008). Reaction conditions included an initial denaturation step at 95°C for 3 min., followed by five cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s, with a ramp rate of 0.1°C/s from the annealing to the extension temperature. These initial five cycles were followed with 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s, followed by a final extension step at 72°C for 10 min. The expected length of amplicons was around 300 bp. A pure culture of *Methanobrevibacter smithii* DSM 861 (DSMZ) was used as a template for the standard and the melting curve analysis showed that the qPCR reaction is specific and only a single product was observed. DNA concentration was measured on a fluorometer Qubit 4 (Thermo Fisher Scientific, USA) and the sample was diluted to the required concentrations (103–106 copies per µL).

Next-Generation Sequencing-Illumina

Preparation of the library for sequencing the 16S rRNA gene and the *mcrA* gene was performed using single-step PCR. The 16S rRNA gene sequencing provides a general view of the diversity of total archaeal members in the samples and allows also the comparison of archaeal sequences diversity and frequency with bacterial ones. The *mcrA* gene sequencing offers deeper insight into the taxonomical diversity of methanogenic archaea and archaea capable of anaerobic methane oxidation (AOM). DNA extracted from lake sediments was used as a template in PCRs with specific primers flanking either a fragment of the *mcrA* gene or the V4 hypervariable region of the 16S rRNA gene sequence as described previously (Pichler et al. 2018). Amplification was done using Platinum™ II Hot-Start PCR Master Mix (ThermoFisher, USA) according to the Earth Microbiome Project with 0.8× final concentration of the polymerase and 200 nM concentration of primers in a final volume of 25 µl. Reaction conditions included an initial denaturation step at 95°C for 3 min., followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s, with a ramp rate of 60% for the annealing. After the PCR, the amplification products were purified using Agencourt® AMPure XP beads (Beckman Coulter, USA). Consequently, the purified PCR samples were quantified before and after pooling using the Qubit 4 fluorometer (ThermoFisher, USA) and characterized using the FragmentAnalyzer (Agilent, USA).

The final library was sequenced using an Illumina MiniSeq sequencer together with the MidOutput MiniSeq Reagent Kit (Illumina, USA) (2×150 paired-end sequencing) according to the manufacturer's instructions.

Raw fastq reads were processed using the DADA2 package (version 1.16.0), (Callahan et al. 2016) in R (version 4.0.0). The analysis was carried out according to the standard operating procedure. Reads were first filtered, trimmed, de-replicated, and de-noised. Afterward forward and reverse reads were merged, chimeras were removed, and the taxonomy was assigned by the RDP naive Bayesian classifier method (Wang et al. 2010) against either the 16S Silva v138 database or an improved version of the *mcrA* reference database (Yang et al. 2014). Multiple alignments were carried out using the DECIPHER package and a phylogenetic tree was built using the phangorn package (Schliep 2011). Subsequently, phylogenetic and statistical analyses were done in R using the phyloseq package (McMurdie and Holmes 2013). Datasets generated and analyzed during the current study are available in the SRA under project number BioProject ID: PRJNA813029.

Biodiversity analysis

Analysis of alpha and beta diversity was performed on results from *mcrA* gene sequencing based on the number of readings for individual amplicon sequence variants (ASVs) with assigned taxonomy. Alpha diversity refers to the diversity within a particular ecosystem and is usually expressed as a number of species (species richness respectively). To estimate the richness in the sample, the Chao1 index was used, which shows the assumed ASV in the sample. Shannon and inverse Simpson indexes were chosen to describe the biodiversity of microbial communities taking into account the richness (number of different units in the community) and the equability (relative frequency of individual units in the community) in the sample. Indexes were calculated using phyloseq paq (McMurdie and Holmes 2013). The index

values for each lake were shown in graphs created using the new ggplot2 software in R (version 4.0.4) (Wickham et al. 2021[1]).

Beta diversity describes the structural complexity of the environment, it is a measure of the difference (or, conversely, similarity) of species composition between communities along a particular gradient of the environment or between the community and its surroundings. The beta diversity is higher the less common species the communities contain.

The Bray-Curtis difference matrix method was chosen, which takes into account the presence and frequency of ASVs (Bray and Curtis 1957). Visualization of the sample similarities was shown using non-metric multidimensional scaling (NMDS), where objects are represented in two-dimensional space based on a matrix of differences. The processing was performed in R software using the phyloseq package (McMurdie and Holmes 2013).

Results

Physical-chemical parameters and water chemistry

Water temperature ranged between 0.6-16.5°C with an average temperature of 8.9°C ± 5.4. The lowest temperature was recorded in MUD, which, however, was almost frozen during the sampling. Lakes BIB, CYA, and PHO were also almost frozen. In contrast, the highest temperature was measured for BLA. The pH values were less variable compared to temperature and varied from neutral to basic spectrum 7.2-10.4 (KAT1, GR2) with an average value of 8.7 ± 0.8. The conductivity values ranged from 30 to 922 μS cm^{-1} with a mean value of 190.3 ± 233.8 μS cm^{-1}. The highest conductivity was found in the ESM and the lowest at BIB, respectively (Table 2).

Quantitative analysis of methanogens (qPCR)- archaeal mcrA gene abundance

The presence of the *mcrA* gene was found in the sediment samples from all 19 lakes (Fig. 2). The numbers of *mcrA* gene copies varied in the order of 10^1-10^5 with an average abundance of 6.36 x 10^4 per gram of wet sediment. The highest number of *mcrA* gene copies was recorded in the LM1 sample (5.74 x10^5 per gram of sediment), whereas the lowest yield was found in the sediment of the CYA, where the number of *mcrA* gene copies was at the method detection limit. Generally, higher values of the *mcrA* gene copies were recorded in the lakes from the Lagoons Mesa and Solorina valley (Fig. 2).

Sequencing analysis of 16S rRNA gene

Due to insufficient sample quality, only 16 samples with the highest DNA yield were sequenced on the Illumina MiniSeq platform. After sequence modification and error correction, 2 597 884 sequences were obtained which had been further purified from sequences to which no taxonomy had been assigned and which belonged to eukaryotes.

Lake (code)	Date	Temperature (°C)	pH	Conductivity (µS/cm)	DOC	NO3-N
BIB	11.02.2019	1.1	8.9	30	mg/l	(mg/l)
BLA	23.01.2019	16.5	8.7	53	0.29	0.05
CYA	23.01.2019	5.6	9.7	61	1.51	0.00
DAN	01.02.2019	15.4	7.4	154	x	0.00
ESM	31.01.2019	4.6	8.6	922	0.7	0.02
GR2	24.01.2019	11.0	10.4	108	2.7	0.07
KAT1	01.02.2019	11.0	7.2	75	?	0.00
LA1	17.01.2019	12.9	8.2	398	1.3	0.00
LA2	17.01.2019	10.9	8.4	114	2.46	0.03
LI	06.02.2019	2.5	8.2	132	1.69	0.00
LM1	23.01.2019	6.7	9.5	58	x	x
LM2	23.01.2019	15.0	9.3	69	x	x
LM3	23.01.2019	14.3	9.0	60	1.36	x
MON	01.02.2019	0.9	8.0	65	x	x
MUD	15.02.2019	0.6	7.4	754	0.79	0.00
PHO	15.02.2019	2.2	7.9	158	1.91	0.01
SOL42	24.01.2019	10.9	9.0	302	8.93	0.00
SOL60	24.01.2019	7.8	9.7	75	x	x
WHI (J)	24.01.2019	16.2	9.7	111	x	x
WHI (S)	24.01.2019	12.2	8.7	106	0.65	0.00

Table 2. Physico-chemical parameters of sampled antarctic lakes water (x – under the limit of detection).

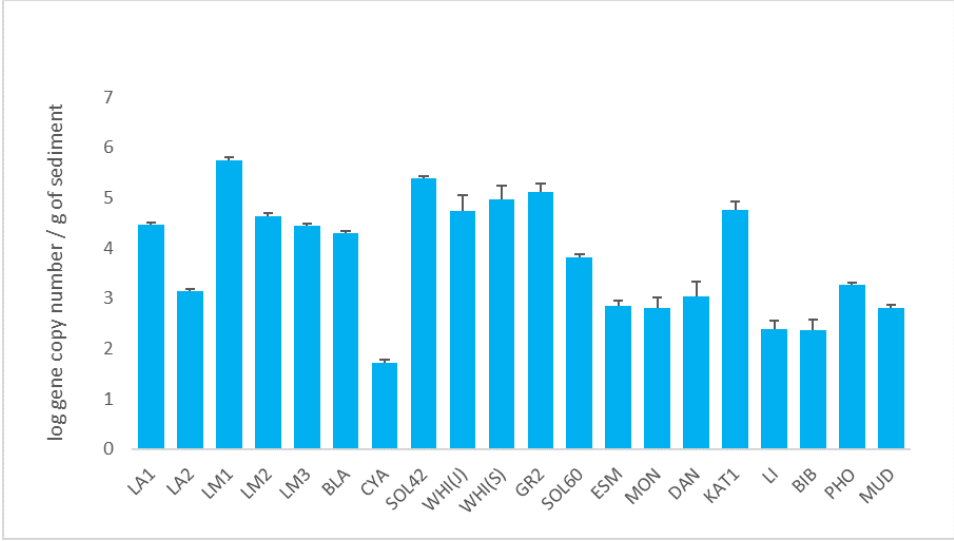

Fig. 2. Logarithm of *mcrA* gene copies number per gram wet sediment. Error bars represent standard deviation.

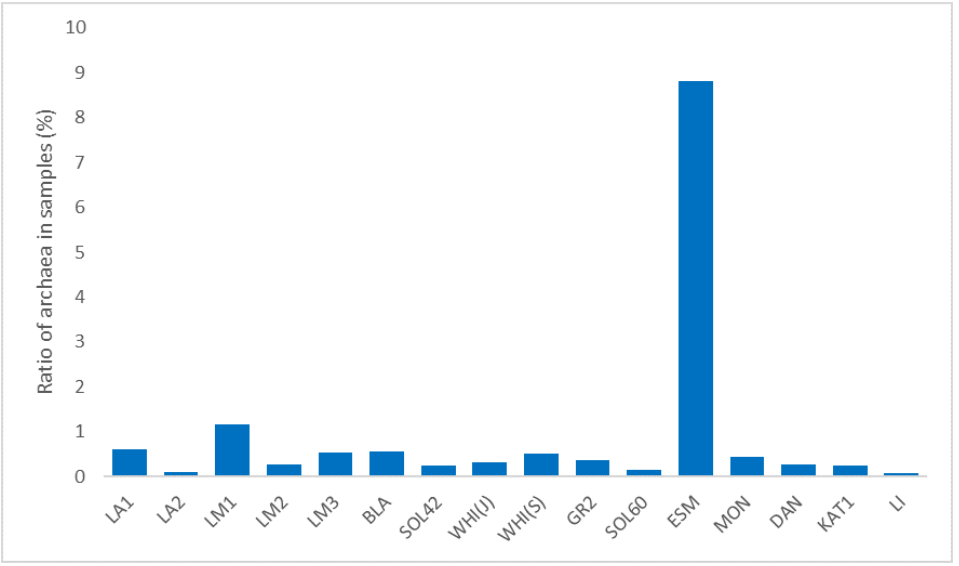

Fig. 3. The percentual proportion of archaea sequencing ASVs in the total prokaryote community based on 16S rRNA gene.

The sequences were classified as 25 617 ASVs, of which 23 620 ASVs belonged to bacteria and 2 217 ASVs were assigned to the archaeal sequence.

Generally, archaea represented only a small proportion (0–8.8%) of the total prokaryotic community. The highest archaeal ASV numbers were found in ESM (Fig. 3), while the average relative frequency of archaea within the prokaryotic community was 0.93%.

The majority of the archaeal sequences belonged to the Woesearchaeia class ("Woesearchaeota"). The sequences of this group accounted for up to 96.4% of the archaeal population (Fig. 4). Another numerous archaeal ASVs were affiliated with Nitrososphaeria ("Thaumarchaeota"); these ASVs contributed to 0.6–81.9% of the archaeal population and were absent only in two lakes LA1 and LM2. Potential representatives of methanogens belonged to Methanobacteria, Methanomicrobia, and Thermoplasmata (Euryarchaeota). No sequences related to any of these three classes were detected in just three lakes SOL60, MON, and LI, indicating the absence of methanogens or their low concentration below the detection threshold in these lakes. The highest number of possible methanogens occurred in samples LM1, LM2, LM3, and ESM where the sequences belonging to Methanobacteria and Methanomicrobia formed the vast majority of the total archaeal ASVs. In the ESM, Methanobacteria sequences accounted even for 95.6%. The lowest proportion of archaeal sequences was assigned to the classes Altiarchaeia and Bathyarchaeia (Fig. 4).

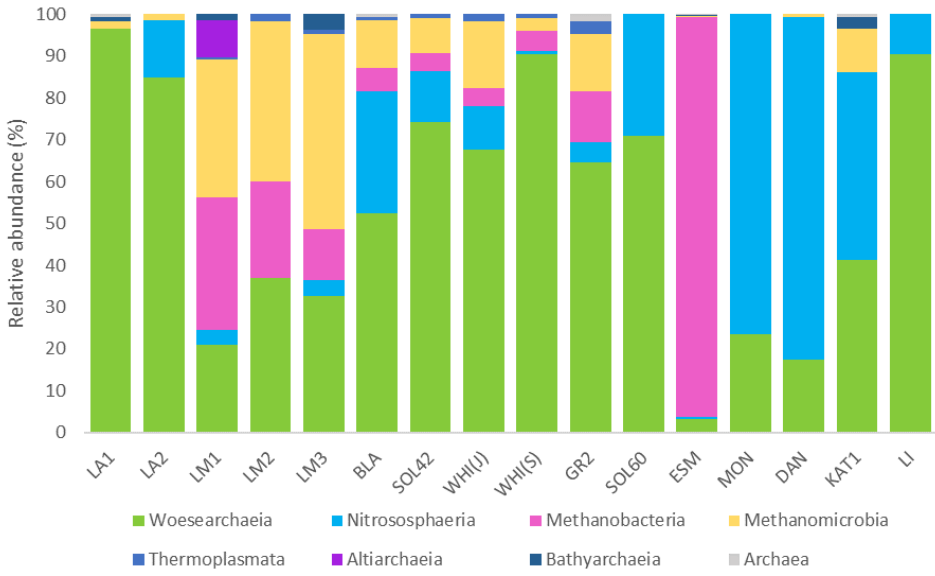

Fig. 4. Taxonomic composition of archaea in lake sediment samples.

Sequencing analysis of mcrA gene

Sequencing of the *mcrA* gene by NGS provided deeper insight into the composition of the methanogenic archaea and archaea capable of AOM within sediments of Antarctic lakes and revealed a wide diversity of methanogens. In the end, 8 sediment samples were selected for sequencing according to the location and DNA yield after amplification. After filtering out inappropriate sequences and removing chimeras, 1,977,126 sequences in 1 231 ASVs were obtained and classified into 19 taxonomic groups at the genus level. The archaeal community compositions of all samples (Fig. 5) and from individual sampling sites (Fig. 6) are shown. For greater clarity, only 10 taxa that reached a relative frequency of 5% in at least one sample are shown in these graphs.

Among methanogens, *Methanothrix* sp. (20.5 ± 22.7%) has the highest relative frequency, followed by *Methanobacterium* sp. (17.2 ± 13.5%) and *Methanoperedens* sp. (14.7 ± 25.5%), which is capable of anaerobic methane oxidation. A smaller part of archaeal diversity consists of unclassifiable archaea of the class Thermoplasmata related to sequences of the order Methanomassiliicoccales (11 ± 12.3%), *Methanosarcina* sp. (8.7 ± 10.4%), archaea of the class *Methanomicrobia* (7.8 ± 19%), *Methanothermobacter* sp. (3.6 ± 5.2%), *Methanoculleus* sp. (3 ± 3.9%) and archaea belonging to the family Methanomicrobiaceae (1.5 ± 2.1) were detected (Fig. 5).

A high proportion of the methanogens with the *mcrA* gene in a total archaeal population was found in BLA (67.7%) and SOL42 (48.4%) lakes. Archaea were also recorded in all samples, which could not be classified up to the genus level and were included only in the taxa Euryarchaeota, Methanomicrobia, Thermoplasmata, or Methanomicrobiaceae (Fig. 6).

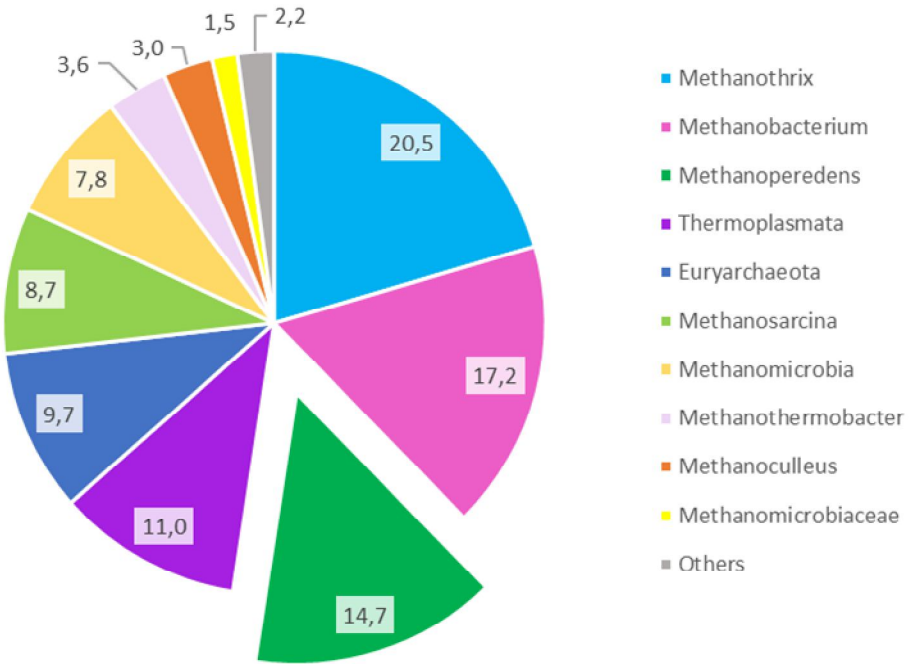

Fig. 5. Taxonomical diversity of archaea capable of methanogenesis and AOM found in all samples according to the *mcrA* gene sequencing. The separated sector of the circle belongs to the genus *Methanoperedens* with the capability to carry out AOM. Mentioned taxa have reached at least one sample frequency ≥5%.

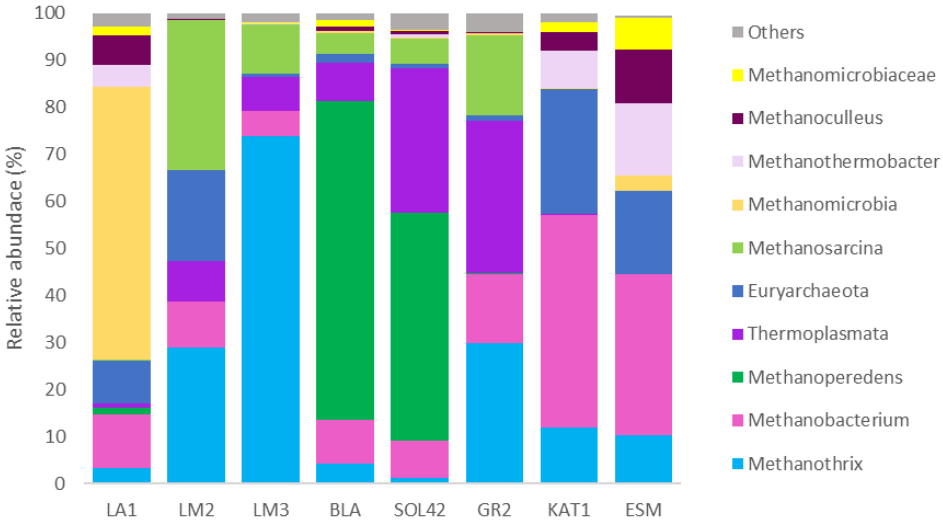

Fig. 6. Proportional representation of various archaeal genera according to the *mcrA* gene sequencing. Mentioned taxa have reached at least one sample frequency ≥5%.

In the LA1 sample, sequences classified only in the Methanomicrobia class accounted for 58%. The presence of the genera *Methanothrix* and *Methanobacterium*, which form the majority of the methanogen population found in the sampled Antarctic lakes, was recorded in all samples. The highest relative frequency of the genus *Methanothrix* was found in LM3 (73.9%), LM2 (28.9%), and GR2 (29.9%) samples. Representatives of the genus *Methanobacterium* were found with the highest proportion in samples from lakes KAT1 (45%) and ESM (34.3%). Sequences belonging to the genus *Methanoperedens* were also recorded in all sampled sites except KAT1 and ESM. Genus *Methanoculleus* was also recorded in all samples, was the highest proportion in the sample from Lake ESM (11.5%). The genus *Methanosarcina* has been recorded in seven sequenced lakes, but higher relative frequencies have been recorded in lakes from the Lagoons Mesa area and the Solorina valley. It reached the highest frequencies in samples LM2 (31.8%) and GR2 (17.1%).

A surprisingly high frequency of sequences belonging to the thermoplasmata was detected in our samples. In SOL42 (30.7%) and GR2 (32.3%) samples, thermoplasmata represented up to one-third of the archaeal assemblage. Higher frequencies of the genus *Methanothermobacter* were also recorded in samples (LA1, KAT1, ESM), where, on the contrary, thermoplasmata members were found only in very low numbers.

The unclassifiable archaea belonging to the Euryarchaeota represented 9.7 ± 9.6%.

Diversity of methanogenic archaea based on mcrA gene

The diversity of archaea containing the *mcrA* gene in Antarctic lake sediment samples was evaluated as diversity within one sample (alpha diversity) or diversity among different samples (beta diversity). First, the alpha diversity of the sample was compared using the three indexes Chao1, Shannon, and inverse Simpson (Fig. 7). The Chao1 index showing the estimated number of ASVs in the sample ranged from 75 (LM3) to 351 (ESM). Shannon and inverse Simpson index values were also highest for ESM (3.77, 22.99) and KAT1 (3.33, 9.54) and conversely, the lowest for LM3 (1.59, 2.29).

Beta diversity was described using Nonmetric Multidimensional Scaling (NMDS) (R software) where the stress value represents the difference between distance in the reduced dimension compared to the complete multidimensional space (Fig. 8). A stress value of 0.07 indicates an excellent agreement of the model with the observed data, although the resulting distribution of points from only eight places must be taken with a reserve. Two "groups" of lakes were separated in which methanogens and archaea capable of AOM are mutually more similar than others. The most similar samples were those from GR2 with LM3 and BLA with SOL42 (right side of a panel). On the other hand, the samples from lakes LA1 and ESM were the most different from samples GR2 and LM3. The resulting placement of samples in the diagram correlates well with the visualization of shared ASVs (Fig. 9). For more information, the recorded environmental gradients were tested using the envfit function. At the 5% level of significance, none of the tested parameters was statistically significantly correlated with ordination.

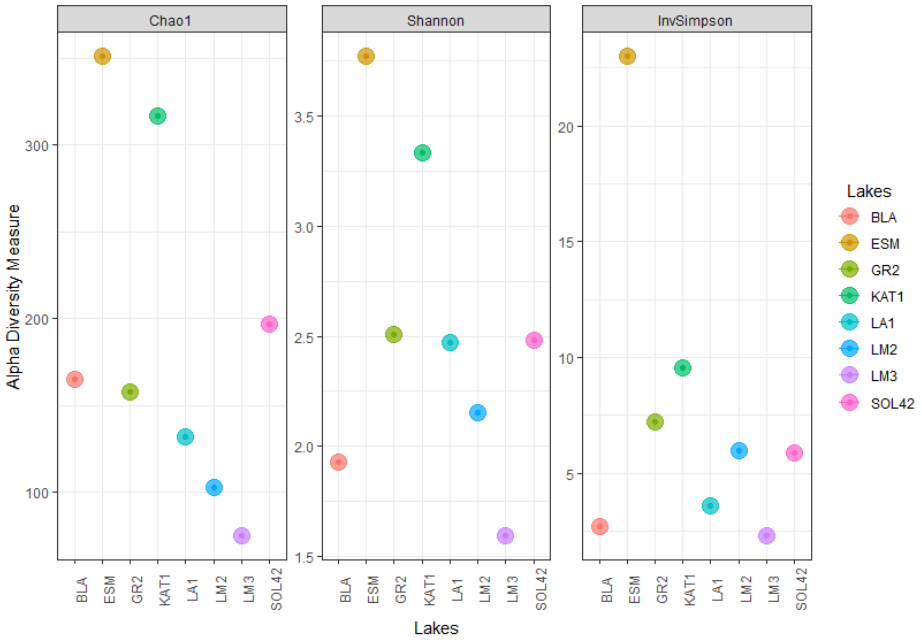

Fig. 7. Alpha diversity of *mcrA* gene. Each dot represents an individual lake.

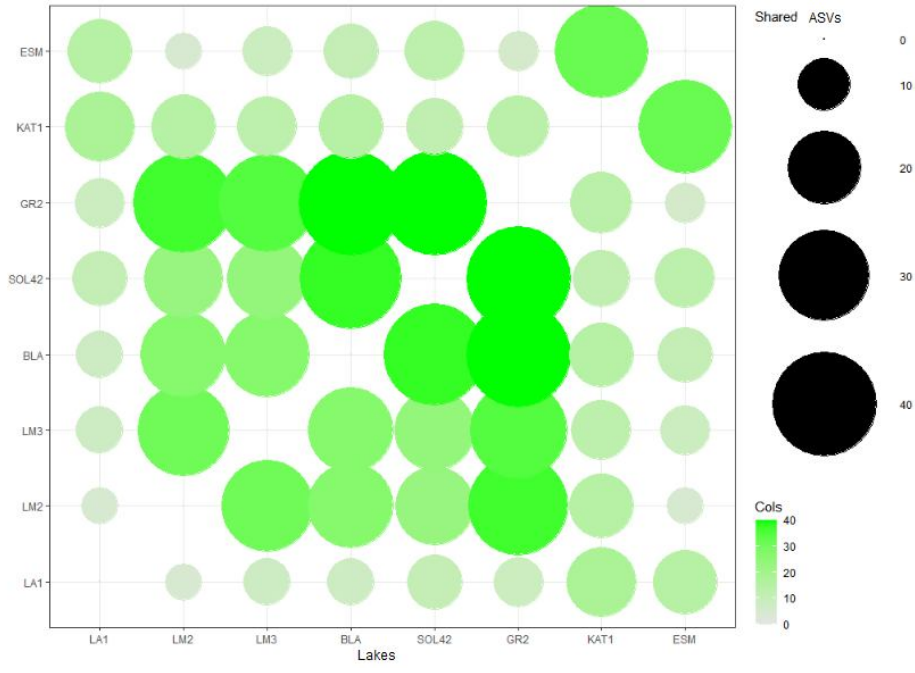

Fig. 8. Beta-diversity analysis based on the *mcrA* gene using non-metric multidimensional scaling (NMDS). Each dot represents an individual lake. A stress value is 0.07.

Beta diversity or dissimilarity in microbial composition among the samples is described by visualization of hypothetical sharing of ASVs among various lake samples (Fig. 9). Lakes LA1, ESM, and KAT1 shared the least ASVs with other lake samples, in addition, the ESM and KAT1 samples share mutually the higher number of ASVs. Lakes BLA, LM2, LM3, SOL42, and GR2, which belong to the Lagoons Mesa and Solorina valley, also showed a larger number of shared ASVs.

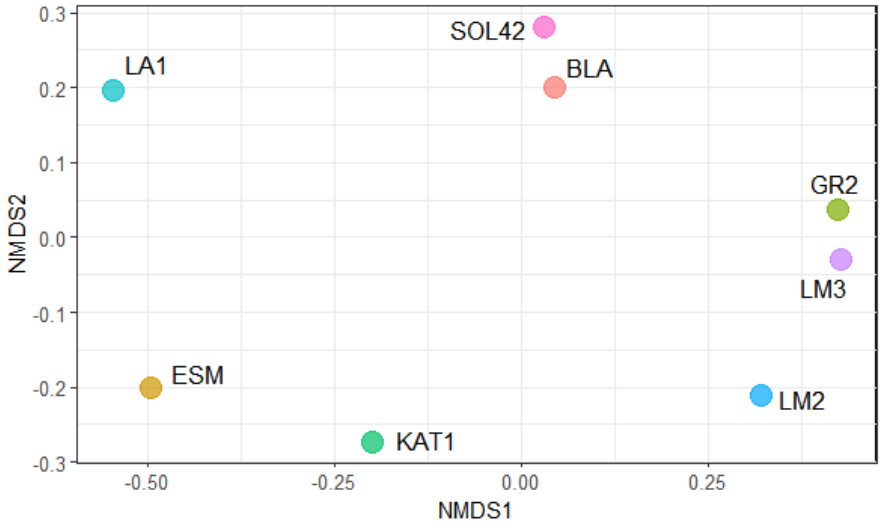

Fig. 9. Shared ASVs among lake sediment samples. The number of the shared ASVs is determined both by the circle size and coloration intensity. The greater circle and more intensive green color, the more shared ASVs.

Discussion

To our best knowledge, this is the first study focusing on methanogenic archaea in samples collected from lakes nearby Czech polar station J. G. Mendel on James Ross Island. Moreover, the lake Long Island from the Long Island was sampled for the first time. Limnological data available for some other lakes like Lagoons Mesa 2 and 3 or Solorina 42 and 60 were obtained during this research in the 2019 expedition.

Low temperature and ice cover of the lakes during the sampling led to reduced light availability. These factors could probably affect the final DNA yield from sediments of lakes BIB, CYA, MUD, and PHO. Finally, samples from all these lakes were not used for subsequent 16S rRNA analysis. Both the low intensity of irradiation and low temperature hinders the development of primary production which is also supported by cyanobacteria and microscopic algae here. Restriction of the primary producers also limits the presence of other microorganisms depending on them as has been reported for some Antarctic lakes (Laybourn-Parry and Pearce 2007, Nedbalová et al. 2013).

Bacteria were a dominant fraction of prokaryotic microorganisms in the samples, and archaea rarely exceeded 1% of the total prokaryotic community. Exceptions were lakes ESM and LM1 with values to be 8.8 % and 1.2%, respectively.

Based on the sequencing of the *mcrA* gene, a high proportion of methanogens was detected in lakes BLA and SOL42. These samples, together with samples from LM1 and WHI (J) and WHI (S), also showed the highest *mcrA* gene copy numbers (*see* Fig. 2). However, final sequencing of the *mcrA* gene was not performed at the latter sites due to the lower quality of the DNA. Similarly to qPCR, gene sequencing for 16S rRNA also generally detected a higher proportion of potential methanogens in lakes from the Lagoons mesa and Solorina Valley regions. This is probably due to their common characteristic - they are stable shallow lakes near the sea with the presence of algae growths, where methanogens find optimal conditions. At the time of sampling, these lakes also had the highest surface water temperature, which may have had an impact on the resulting activity of methanogenic archaea in the sediments and their subsequent detection.

The only sample that did not correlate with the qPCR results was ESM, where few copies of the *mcrA* gene were detected compared to the sequencing with the highest proportion of methanogens. The higher proportion of archaeal, namely methanogenic sequences in the lake Esmeralda (ESM) has been caused probably due to the lower proportion of Deltaproteobacteria (data not shown), which usually compete with methanogens for H_2 (Karr et al. 2006). Generally, the low contribution of archaeal sequences has also been observed in many other studies of Antarctic freshwater lakes (Bowman et al. 2000, Mulyukin et al. 2014, Gugliandolo et al. 2016, Chaya et al. 2019).

On the other hand, due to the development of next-generation sequencing, along with using more effective primers and a growing dataset of newly described sequences we were able to detect the prevalence of two relatively newly proposed archaeal classes Woesearchaeia (Woesearchaeota) and Nitrososphaeria (Thaumarchaeota) representatives in some lakes. Currently, Woesearchaeota is widely found in diverse environments (Liu et al. 2018) and belong usually among the most frequent and abundant archaea found in lakes (Ortiz-Alvarez and Casamayor 2016, Juottonen et al. 2020, Tóth et al. 2020). Woesearchaeota can enable carbon and hydrogen metabolism under anoxic conditions, which might associate with symbiotic and/or fermentation-based lifestyles (Castelle et al. 2015, Gründger et al. 2019).

Most members of the Thaumarchaeota are chemolithoautotrophic ammonia-oxidizers and may play important roles in nitrogen and carbon biogeochemical cycles (Pester et al. 2011, Stieglmeier et al. 2014). Nitrification by ammonia-oxidizing archaea (AOA) contributes to N_2O production which might have implications for climate change (Santoro et al. 2011). Many Thaumarchaeota are marine and live in the open ocean (Schleper and Nicol 2010), however, AOA representatives were also found in freshwater aquaculture ponds (Lu et al. 2021), rice paddy soils, reservoir sediments (Wang et al. 2014), rivers (Li et al. 2018) and lakes (Ortiz-Alvarez and Casamayor 2016, Juottonen et al. 2020).

Although the qPCR approach was not used in Antarctic lake sediment analyses until now, our results are comparable with those from shallow freshwater arctic lakes in Alaska where the *mcrA* gene occurred in orders of 10^2-10^4 the gene copies per one gram of the sediment (Matheus Carnevali et al. 2015). Slightly higher densities of the gene copies (10^5-10^6) were found in freshwater lakes in plateau Yunnan in China (Yang et al. 2020).

Results of 16S rRNA sequencing also showed that methanogenic archaea form not only a small part of the total prokaryotic diversity but are also within the archaeal community in the most of studied lakes. This trend has been reported already before in some papers on prokaryotic communities in the sediments of the Antarctic lakes (Sjöling and Cowan 2003, Tang et al. 2013, Gugliandolo et al. 2016). The

smaller proportion of methanogens within the prokaryote/archaeal community may probably indicate the prevalence of terminal acceptors used by other microorganisms (Purdy et al. 2003).

Contrary to the sequences retrieved by 16S rRNA analysis, many more reads belonging to methanogens were found with *mcrA* gene sequencing. This finding is, however, not surprising because it targets the specific function gene which is common for methanogenic and methane-oxidizing archaea (Steinberg and Regan 2009). Moreover, the identification of uncultivated methanogens based on 16S rRNA (or its gene) as a marker is generally limited by the fact that methanogenic Archaea are not monophyletic. Methanogens rather form several different major lines of descent within the kingdom Euryarchaeota, some of which are interspersed by lines of descent harboring nonmethanogenic *Archaea* only (Friedrich 2005). Nevertheless, the presence of taxa was almost the same in both of the used approaches except for the detection of *Methanothermobacter* sp. and *Methanoculleus* sp. sequences, and the absence of the *Methanolobus* sp. in *mcrA* gene sequencing.

On the other side, the *mcrA* gene sequencing confirmed a high proportion of sequences belonging to Methanoperedens-like archaea ANME-2d the anaerobic methanotroph mostly in BLA and SOL42 lake sediments. "*Candidatus* Methanoperedens nitroreducens" is a candidate species of methanotrophic archaea that couples anaerobic methane oxidation to denitrification (DAMO archaea) (Raghoebarsing et al. 2006, Haroon et al. 2013). This high detection sensitivity is a somewhat surprising finding since the available general *mcrA* primers are not well suited to capturing *mcrA* sequences of '*Candidatus* M. nitroreducens' in the environment, potentially resulting in underrepresentation in molecular surveys (Vaksmaa et al. 2017). *Ca*. Methanoperedens nitroreducens" carries out reverse methanogenesis and harbors the key enzyme methyl-coenzyme M reductase (MCR), which catalyzes either the last step of methane production in methanogens or the first step of methane oxidation in anaerobic methanotrophic archaea (Guerrero-Cruz et al. 2018). This microorganism is present at oxic-anoxic interfaces in a wide range of aquatic environments and man-made ecosystems, such as lakes, rivers, paddy fields, and wastewater treatment systems (Ding et al. 2015, Guerrero-Cruz et al. 2018, Ding and Zeng 2021) but no presence of this archaeal organism has been reported for Antarctic lakes until now. To our current knowledge, the only ANME-3 sequences typical for sulfate-dependent anaerobic alkane-oxidizing archaea (Wang et al. 2021) were detected in cold bottom seepages in the Weddel sea surrounding James Ross Island (Niemann et al. 2009). Both the ANME-2d and ANME-3 clades harbor *mcrA* gene (Cui et al. 2015) and belong to the same phylum Euryarchaeota and order Methanosarcinale, however, most environmental sequences of the ANME-2d group are derived mostly from freshwater sediments while members of the ANME-3 clade dominate methane-rich arctic mud volcanoes (Wang et al. 2021). The occurrence of this genus might indicate higher concentrations of nitrates in the water of the particular lakes, however, our analyses have shown almost zero concentrations of nitrates in all studied lakes (*see* Table 2).

Methanothrix and *Methanosarcina*, as the most dominating methanogenic genera in our samples, have also been found to be dominant in other antarctic lakes (Purdy et al. 2003, Karr et al. 2006, Chaya et al. 2019). Genus *Methanobacterium* occurs mostly in an active layer of permafrost (Rivkina et al. 2007, Barbier et al. 2012, Wang et al. 2020), however, in Antarctica, this taxon has been captured for the first time to such a large extent.

Similarly, sequences of potentially thermophilic representatives belonging to *Methanothermobacter* and Methanomas-

siliicoccales (Thermoplasmata) were recorded for the first time in Antarctic lakes. The species within the genus *Methanothermobacter* (Euryarchaeota, Methanobacteriales) are thermophilic, hydrogenotrophic methanogens and grow best at temperatures between 55°C and 65°C. However, Mickol et al. (2018) have suggested that thermophilic representatives of the *Methanothermobacter* genus can survive long-term extreme conditions in permafrost, reducing their metabolism while enduring the more convenient conditions. Members of both orders Methanobacteriales and Methanomassiliicoccales were already found in peat bogs in the Siberian tundra (Grodnitskaya et al. 2018).

It is too difficult to say which kind of methanogenesis could be prevalent in the studied lakes because many sequences were not aligned with any genus level. Generally, sequences of the methanogens which might be involved in all three types of methane production were found in our samples. Genera *Methanobacterium*, *Methanothermobacter*,*Methanoculleus* and partially *Methanosarcina* are involved in hydrogenotrophic methanogenesis, while acetoclastic methanogenesis is mainly dominated by species of *Methanosarcina* and *Methanothrix* genera, respectively (Garcia et al. 2000). Based on the detected sequences we can assume the prevalence of hydrogenotrophic methanogenesis in lakes LA1, KAT1, and ESM. On the other hand, the acetoclastic pathway should dominate in the lakes LM2 and LM3 where the dominance of the genera *Methanohrix* a *Methanosarcina* was found.

Methylotrophic methanogens all belong to the family Methanosarcinaceae, except the genus *Methanosphaera*, which belongs to the order Methanobacteriales. The methylotrophic growth of *Methanosphaera* species is H_2-dependent - they are obligate methylotrophic and hydrogenotrophic methanogens that are specialized to reduce methyl groups with H_2. The metabolism of *Methanosphaera* sp. is restricted to methanol (Liu and Whitman 2008). More recently, methanogens that also reduce methanol with H_2, belonging to the order Methanomassiliicoccales have been described (Dridi et al. 2012, Paul et al. 2012). These methanogens occur in animal gut systems but also have frequently been detected in other anoxic environments such as fens (Söllinger et al. 2016), mangrove sediments (Li et al. 2016), digesters (Wilkins et al. 2015), and anoxic paddy soils (Ji et al. 2018). Methanogens of the order Methanomassiliicoccales are probably even more widespread since they have only recently been added to sequence databases and probably were previously recorded as Thermoplasmatales-related Euryarchaeota. However, their role in carbon flux is presently unknown (Conrad 2020). Due to the higher abundance of sequences belonging to Methanomassiliicoccales and *Methanosarcina* sp., methylotrophic methanogenesis is suggested to be prevalent in Lake SOL42. Nevertheless, as methanol is a product of pectin degradation, and pectin turnover in lake sediments is slow, methanol is a marginal methanogenic precursor in these sediments (Lovley and Klug 1983) and also in anoxic rice field soils (Conrad and Claus 2005). Moreover, *Methanosarcina* species can produce methane by using all three above-described pathways (Whitman et al. 2014).

Several factors like temperature, incomplete organic matter degradation, or the presence of syntrophic acetate oxidation may cause a higher proportion of hydrogenotrophic methanogens. In Yamal tundra lakes (Subarctic Eurasia), the rates of hydrogenotrophic methanogenesis appeared to be higher in the sediments of deep lakes than in those of the shallow ones (Savvichev et al. 2021). On the contrary, acetoclastic methanogenesis flourishes at a lower temperature, complete degradation of the organic matter, and the presence of acetogenic bacteria (Conrad 2020). Prevalence of the hydrogenotrophic methanogenesis in freshwater lakes of mar-

itime Antarctica has already been suggested in a study by Ellis-Evans (1984). Recent studies from subglacial Antarctic sediments and sub-Antarctic lake sediments confirm this prevalence (Ma et al. 2018) and suggest that the hydrogenotrophic pathway may become more important with increasing temperatures than the acetoclastic pathway due to global warming (Lavergne et al. 2021). However, it would require culturing the microbes as suggested by Laskar et al. (2018) to better understand the physiological pathways, ecological interactions, and ecological roles of the present methanogens. Diversity analysis reveals a similarity between lakes located in two areas: Lagoons Mesa and Solorina Valley. Individual lakes from one area, however, shared much higher similarity with the lakes from another area rather than with the lakes within the same area. This finding is supported further by a comparison of samples from Esmeralda and Katia 1 lakes which are several kilometers apart on different islands, suggesting that a lake location will be not the main factor influencing the diversity of the methanogens.

Species diversity can differ even in samples taken from one lake as evident from prokaryote composition and *mcrA* gene copies number in samples taken from two sites in the White lake - WHI (J) and WHI (S). To get more complex information about the microbial diversity would require taking much more samples during various periods of the year from one lake. In Antarctica, however, this approach is hardly possible, at least due to financial reasons and impossible sampling during the rest of the year.

Nevertheless, for further investigation of the methanogenic diversity, we recommend sampling lakes from Lagoons Mesa and Solorina Valley that showed both the highest diversity and number of *mcrA* gene copies. Many lakes located within these two areas allow for collecting numerous samples. In addition, Esmeralda lake at Vega Island could be a further subject of the microbiological research due to distinct prokaryotic diversity and potentially high diversity of methanogens.

Concluding remarks

This study represents only an initial phase of the methanogens investigation in the antarctic lakes of the James Ross Island. Therefore, there is a need of follow-up studies focused on further explorations of the lakes involved into this study which should clarify the role of various environmental factors in the composition of methanogenic assemblages. Another task will be to gain more information on how the factors influence real (*in situ*) rates of methane production in the lake sediments and to evaluate the amounts of methane emissions released to the atmosphere. Recent view is that methanogenic potential of Antarctic lake sediments is still understudied in comparison with those from Arctic and sub-Arctic regions. Therefore, the need for further study of methanogens from Antarctic lakes is obvious. Our research reported in this study unambiguously provides evidence that the lakes of the JRI and surrounding islands are a source of new archaeal species and potential metabolic pathways for future biotechnologies based on anaerobic digestion.

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