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# Temperature differently affected methanogenic pathways and microbial communities in sub-Antarctic freshwater ecosystems

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ABSTRACT

Freshwater ecosystems are responsible for an important part of the methane ($CH_4$) emissions which are likely to change with global warming. This study aims to evaluate temperature-induced (from 5 to 20 °C) changes on microbial community structure and methanogenic pathways in five sub-Antarctic lake sediments from Magallanes strait to Cape Horn, Chile. We combined *in situ* $CH_4$ flux measurements, $CH_4$ production rates (MPRs), gene abundance quantification and microbial community structure analysis (metabarcoding of the 16S rRNA gene). Under unamended conditions, a temperature increase of 5 °C doubled MPR while microbial community structure was not affected. Stimulation of methanogenesis by methanogenic precursors as acetate and $H_2/CO_2$, resulted in an increase of MPRs up to 127-fold and 19-fold, respectively, as well as an enrichment of *mcrA*-carriers strikingly stronger under acetate amendment. At low temperatures, $H_2/CO_2$-derived MPRs were considerably lower (down to 160-fold lower) than the acetate-derived MPRs, but the contribution of hydrogenotrophic methanogenesis increased with temperature. Temperature dependence of MPRs was significantly higher in incubations spiked with $H_2/CO_2$ (c. 1.9 eV) compared to incubations spiked with acetate or unamended (c. 0.8 eV). Temperature was not found to shape the total microbial community structure, that rather exhibited a site-specific variability among the studied lakes. However, the methanogenic archaeal community structure was driven by amended methanogenic precursors with a dominance of *Methanobacterium* in $H_2/CO_2$-based incubations and *Methanosarcina* in acetate-based incubations. We also suggested the importance of acetogenic $H_2$-production outcompeting hydrogenotrohic methanogenesis especially at low temperatures, further supported by homoacetogen proportion in the microcosm communities. The combination of *in situ*-, and laboratory-based measurements and molecular approaches indicates that the hydrogenotrophic pathway may become more important with increasing temperatures than the acetoclastic pathway. In a continuously warming environment driven by climate change, such issues are crucial and may receive more attention.

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## 1. Introduction

Although freshwater ecosystems (i.e., lakes, reservoirs and ponds) represent 3.7% of the Earth's land surface (Verpoorter et al., 2014), they are a major natural source of biogenic methane ($CH_4$) (i.e., up to 43.1% of the natural $CH_4$ emissions), emitting up to 159 tons of $CH_4$ per year (Saunois et al., 2020).

Biogenic methane is produced through methanogenesis mainly by methanogenic archaea in freshwater sediments, and similar environments (Conrad, 2020), characterized by the accumulation of labile organic matter (OM) and anaerobic conditions. Methanogenesis is a process of OM degradation until mineralization (Schink and Stams, 2012). In freshwater ecosystems, the main reported methanogenic precursors are acetate and $H_2/CO_2$ for acetoclastic and hydrogenotrophic methanogenesis, respectively (Conrad, 1999; Kotsyurbenko et al., 2019). On the other hand, $CH_4$ can also originate to a lesser extent from methylated or methoxylated aromatic compounds (Conrad, 2020). For example, $CH_4$ production from trimethylamine has been identified in Arctic thermokarst lake sediments (de Jong et al., 2018).

For decades, methanogenic archaea were thought to be restricted to the phylum *Euryarchaeota* (Borrel et al., 2011). Recently, metagenomic studies evidenced new potential methanogens in other archaeal taxa, such as the phylum *Verstraetearchaeota* (Vanwonterghem et al., 2016) and the crenarchaeal *Bathyarchaeia* class (Evans et al., 2015). Methanogens, either acetoclasts, hydrogenotrophs or methylotrophs, share the methyl coenzyme M reductase (MCR), the final enzyme in the pathway of methanogenesis. The gene encoding for the α-subunit of this enzyme (*mcrA*) is considered as a molecular proxy for methanogens, and is widely used to study methanogenic community composition and dynamics (Luton et al., 2002; Steinberg and Regan, 2008).

Globally, together with the presence of electron acceptors and the OM content and quality, temperature is one of the main factors controlling $CH_4$ production in freshwater ecosystems (Conrad, 2020, 1996; Yvon-Durocher et al., 2014). In the context of global warming, *in situ* (Yvon-Durocher et al., 2017) and short-term incubations (Duc et al., 2010; Sepulveda-Jauregui et al., 2018) as well as long-term laboratory incubations (Knoblauch et al., 2018) have been commonly used to predict and understand the effects of temperature increase on $CH_4$ production rate (MPR). The temperature dependence value (energy of activation – $E_a$) of methanogenesis at the community level (evaluated through laboratory incubations) has been reported similar to the $E_a$ at the ecosystem level (evaluated through *in situ* $CH_4$ emissions) (Yvon-Durocher et al., 2014). Hence, laboratory incubations represent a valuable tool providing information about temperature dependence of methanogenesis than can be extrapolated to the ecosystem level (Yvon-Durocher et al., 2014). The effect of temperature on MPRs has been extensively reported in freshwater ecosystems from cold regions such as northern high-latitude zone (e.g., Blake et al., 2015; de Jong et al., 2018; Duc et al., 2010) and high-altitude zone (e.g., Deng et al., 2019). Despite the latter, only few studies have reported the total microbial diversity associated with MPR measurements (Deng et al., 2019; Tveit et al., 2015).

The sub-Antarctic Magellanic ecoregion (Cabrera and Willink, 1973) harbors the densest forest and freshwater ecosystems of the southern hemisphere (Bryant et al., 1997). As pointed out by Rozzi et al. (2012), there is no comparable forest biome at that latitude (40–60 °). In the sub-Antarctic zone, air and land surface temperatures are known to increase under global warming context, as well as temperature anomalies (IPCC, 2014; Olivares-Contreras et al., 2019). The outcome of temperature increase on methanogenic communities and processes as well as on the associated total microbial community is not well constrained and, to the best of our knowledge, has not been experimentally evaluated to date in southern high-latitude lakes. Hence, this work aims at determining the temperature-mediated changes in biogenic MPRs, in microbial community structure and in the relative importance of acetoclastic/hydrogenotrophic pathways in lake sediments from Magellanic sub-Antarctic ecoregion (Chile). The evaluation of MPRs using environmentally controlled incubations under current *in situ* temperatures (baseline level) and in response to 5 °C-increment of temperature (from 5 °C to 20 °C) was combined to insights from physicochemical and geochemical characterization, functional gene quantification and 16S rRNA gene metabarcoding.

## 2. Material and methods

### 2.1. Site description and sample collection

Five lakes were sampled during austral summer in January 2016. They represent different lake types that can be found in sub-Antarctic Magellanic ecoregion (Chile). The Lake G (Lake Hambre) and Lake R (Lake Lynch) are located in the Strait of Magellan region around the city of Punta Arenas (53°36.215′S; 70°57.145′W and 53°10.582′S; 71°0.486′W, respectively; Fig. 1) in a zone characterized by *Nothofagus* forest and grass steppe (Mancini et al., 2008; Markgraf and Huber, 2010).

The three *Sphagnum* peatland-associated lakes (Lake Castor: P1, Lake Fack: P2 and Lake Mejillones: P3) are situated on Navarino Island (Cape Horn), around the city of Puerto Williams (P1: 54°56.359′S; 67°38.348′W, P2: 54°55.885′S; 67°19.907′W and P3: 54°54.145′S; 68°0.686′W, respectively; Fig. 1). As a regional specificity, Navarino Island harbors a high diversity of non-vascular plants such as moss and liverwort species, composing the majority of the vegetation (Rozzi et al., 2008). The three studied peatland lakes are of unknown origin and are partially colonized by *Sphagnum* spp.. These ecosystems likely represent a transition between lake and *Sphagnum* peatland, progressively colonizing the water body through terrestrialization (Schumann and Joosten, 2014). Such *Sphagnum* peatlands represent 25% of the total Navarino and Dawson island's surface (Domínguez and Vega-Valdés, 2015).

Surficial sediments (approx. 0–10 cm below the sediment/water interface) were collected at the maximal depth zone near the center of the lake, using an Ekman dredge. Field duplicates (sampling points A and B) were collected at some meters of distance except for the peatland lake P3 were only point A was sampled. At each sampling point, sediment was collected in sterile 0.5 L-Nalgene bottles, fully filled, sealed and stored at + 4 °C until methanogenic incubation was set up. Subsamples were taken for physicochemical characterization and DNA extraction and stored according to the measured parameter (as detailed below).

### 2.2. Environmental physicochemical parameters

***Sediment.*** We determined dry weight from sediment stored at 4 °C (DW, overnight at 110 °C). Then, OM content was measured via loss on ignition (LOI) on dry samples (550 °C, until no change in mass). The pH of sediments was measured after resuspension in deionized water (1:5, (w/w)) and 1 h equilibration.

***Sediment porewater.*** Water was extracted from freshwater sediment through filtration to assess major anions and cations following the recommendations from Jones and Willett (2006). Briefly, back to the laboratory, the liquid phase was filtered through a microRhizon sampler (Rhizosphere, Netherlands) after an equilibration of 40 g of fresh sample with 200 mL deionized water agitated with a magnetic stirrer at room temperature for 1 h. Ammonium, nitrite, nitrate and sulfate concentrations were analyzed using a HPLC (Dionex, USA). Quantification limit was 0.050 mg $L^{-1}$ for nitrite and sulfate, 0.010 mg $L^{-1}$ for nitrate and 0.025 mg $L^{-1}$ for ammonium, and ion 915 (Natural water, National Laboratory for Environmental Testing, Canada) was used as reference.

***Water column.*** For each sampling point, temperature and dissolved oxygen (DO) were measured at the surface of the water column and near the sediments using a multi-parametric probe (HI 9828, Hanna Instrument, USA). Dissolved $CH_4$ concentration in the water column was

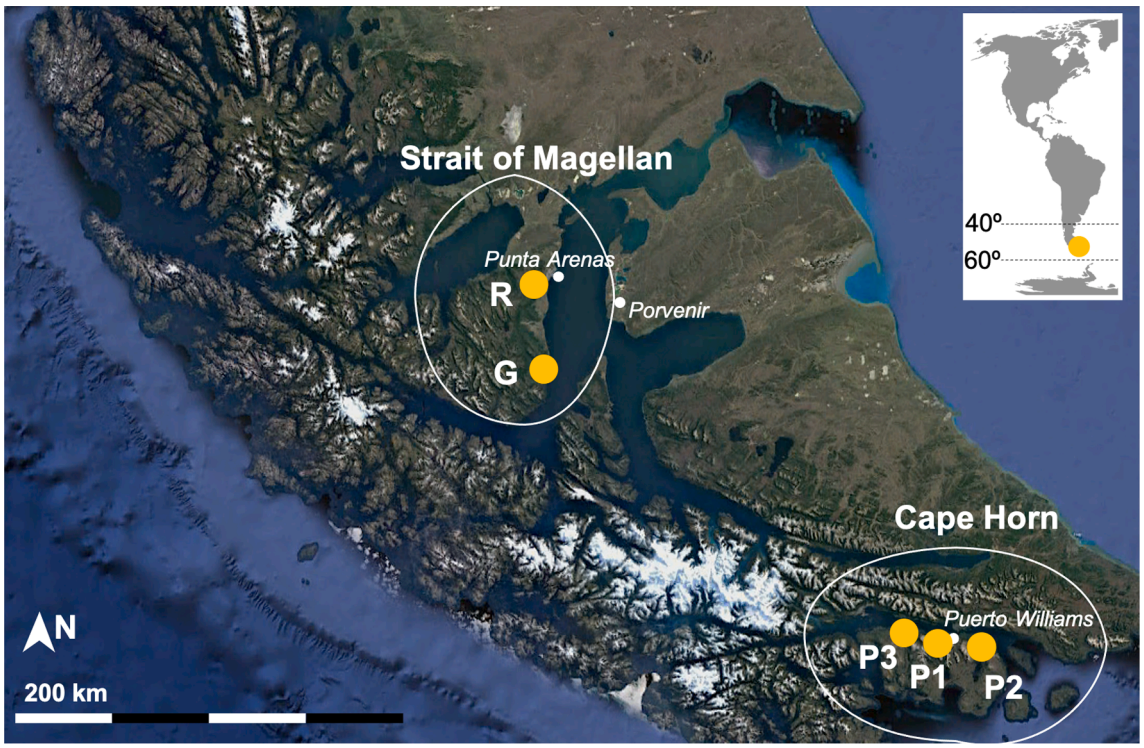


**Fig. 1.** Location of the five studied sub-Antarctic lakes from both Strait of Magellan and Cape Horn region. G, R, P1, P2 and P3 respectively refer to the post-glacial lake, reservoir and peatland lakes 1, 2 and 3 presented in Table 2. Map Source: Google Earth (2015).

determined at the surface and when oxygen stratification was observed-near the sediments, through membrane-integrated cavity output spectrometry method (M−ICOS) (Gonzalez-Valencia et al., 2014). Water was pumped with a peristaltic pump (12 V, Solinst, Mexico) through a gas–liquid membrane-based equilibrator and the $CH_4$ concentration in the gaseous phase was then continuously measured by an ultraportable greenhouse gas analyzer (UGGA, Los Gatos Research, USA). Net *in situ* $CH_4$ emission rates were determined in 6 to 24 locations using a floating static chamber connected to a UGGA, Los Gatos Research, USA) in closed circuit method as described in Gerardo-Nieto et al. (2017).

### 2.3. Set-up of methanogenic incubations

Sediment sample from each available field replicate (points A and B) of the five lakes was homogenized before incubation and introduced in sterile glass vials under anaerobic conditions ($N_2$ flush for 10 min) in an anaerobic chamber (Glove box, Captair, France). The vials were sealed with butyl rubber stopper and aluminum crimp caps. Triplicate vials were maintained in the dark at four different temperatures (i.e., 5, 10, 15 and 20 °C) in controlled temperature incubators (range ± 0.4–0.7 °C). These temperatures were chosen as representative of the *in situ* water temperature range observed over the year in the considered ecosystems (5 °C-15 °C) and of climate change scenario (+5 °C, highest predicted change under RCP8.5 scenario, IPCC (2014)). The MPR was measured without amendment (endogenic MPR), as well as after initial addition of acetate (acetate-based MPR) and $H_2/CO_2$ ($H_2/CO_2$-based MPR), as detailed in Table 1. The amendment concentration was fixed in order to set the incubations with similar feed to biomass ratio (i.e., 4.7 and 4.1 mmol C per g biomass, for acetate- and $H_2/CO_2$-based incubations, respectively; using OM as a proxy of biomass).

### 2.4. Analytical monitoring

The amount of accumulated $CH_4$ (mol) was monitored along time in the vial headspace by gas chromatography (Clarus 500, Perkin Elmer, USA) using a flame ionization detector (FID) as described in the Supplementary Material.

The produced $CH_4$ was the sum of gaseous $CH_4$ in the headspace and dissolved $CH_4$ in equilibrium in the liquid phase (calculated at each time using the Henry's law and considering the incubation temperature). Kinetics of $CH_4$ production were plotted against time and lag phase as well as substrate conversion yield were evaluated as described in the Supplementary Material. The MPR (μmol $CH_4$ $g^{-1}$ DW $d^{-1}$) was calculated as the maximal slope of accumulated $CH_4$ along time, by linear regression, normalized by the amount of sample in each incubation (expressed in dry weight). The calculation of MPR by regression was validated by fitting our data to the Gompertz model (**Supplementary**

**Table 1**
Summary of experimental protocols used for the determination of the $CH_4$ production rates in the studied lake sediments.

| | Acetate-derived MPR | $H_2/CO_2$-derived MPR | Endogenic MPR |
|---|---|---|---|
| Total vial volume | 12 mL | 55 mL | 55 mL |
| Ratio headspace/ total volume | 10% | 80% | 55% |
| Sediment inoculation | 8–10 g OM $L^{-1}$ | 12–47 g OM $L^{-1}$ | 20–110 g OM $L^{-1}$ |
| Mineral medium[a] | 4.5 to 7.5 mL | 5 mL | No[b] |
| Substrate | acetate (30 mM) | $H_2/CO_2$ (80/20; 1 bar)[c] | No |
| pH | Adjusted to *in situ* | Adjusted to *in situ* | *in situ* |
| Atmosphere | Anaerobic ($N_2$) | Anaerobic ($N_2$) | Anaerobic ($N_2$) |
| Technical replicates | n = 3 | n = 3 | n = 3 |
| Temperature | 5 °C – 10 °C – 15 °C – 20 °C | 5 °C – 10 °C – 15 °C – 20 °C | *in situ* and *in situ* + 5 °C |

[a] Slightly modified from Shelton and Tiedje (1984).
[b] For P3, 10 mL of sterile water was added to obtain a slurry, due to the high vegetal content.
[c] Spiked with 1 bar relative pressure of $H_2/CO_2$ (80:20 v/v) (Bialek, Cysneiros and O'Flaherty 2013), corresponding to similar feed-to-biomass ratios (mmol C $g^{-1}$ OM) compared to acetoclastic experiment.

**Figure S1**; Silverman and Silverman (2017)).

The temperature dependence of MPR was determined through the activation energy $E_a$ (kJ $mol^{-1}$), deriving from the Arrhenius equation according to Shelley et al. (2015) as follows: $k = Ae^{(\frac{-E_a}{RT})}$ were k is the MPR; A is the Arrhenius constant; R is the ideal gas constant (8.314 J $K^{-1}$ $mol^{-1}$), T is the experimental temperature (K). The $E_a$ was also indicated in electron volts (eV) for easier comparison with the values presented in Yvon-Durocher et al. (2014) by considering 96 kJ $mol^{-1} = 1$ eV. For acetate- and $H_2/CO_2$-based incubations, $E_a$ was calculated from MPRs measured at four temperatures (from 5 to 20 °C). For endogenic incubations, an approximation of $E_a$ was estimated using the MPRs recorded at the two studied temperatures (*in situ* and *in situ* + 5 °C).

At the end of each incubation (i.e., when kinetic curves reach a plateau), the samples were centrifuged at 6,000 rpm for 30 min. The pellet was stored at −20 °C for subsequent DNA extraction. In the supernatant, pH was recorded and the final remaining acetate concentration was measured using a HPLC (Series 200, Perkin Elmer, USA) equipped with HP 1100 RID detector (details are available in the Supplementary Material).

### 2.5. DNA extraction

Molecular analyses were performed on three ecosystems (i.e., lakes R, P2 and P3). From homogenized *in situ* sediment samples, DNA was extracted for each field duplicate as soon as possible (<4h) after sampling (i.e., field replicates A and B, note that no replicate B is available for lake P3). DNA was also obtained from post-incubation samples (i.e., from centrifuged pellets obtained at the end of the incubations) for each field duplicate sample from every amendment method (i.e., endogenic, acetate-based and $H_2/CO_2$-based) and at each incubation temperature.

In all cases, total genomic DNA was extracted from 0.5 g of sediment using the PowerSoil DNA isolation kit (Qiagen, USA) following manufacturer recommendations with slight modifications to maximize yields. The quantity and purity of DNA extracts was verified with a BioSpecnano micro-volume UV–Vis Spectrophotometer (Shimadzu, USA).

### 2.6. Quantification of methanogens, total archaea and bacteria

The quantifications of the *mcrA* gene, and the archaeal and bacterial 16S rRNA genes, were performed using the primer pairs mlas/mcra-rev (Steinberg and Regan, 2008), Arc-931F/M1100R (Einen et al., 2008; Jackson et al., 2001) and DGGE300F/Univ516 (Muyzer et al., 1993; Takai and Horikoshi, 2000), respectively. qPCR was done as described previously (Cabrol et al., 2020) and reaction mix preparation, thermal conditions, qPCR efficiencies as well as detection and quantification limits are presented in the Supplementary Material.

### 2.7. High throughput sequencing of 16S rRNA gene

Microbial diversity was characterized in three ecosystems (i.e., reservoir R, lake P2 and lake P3) by sequencing the V4-V5 region of the bacterial and archaeal 16S rRNA gene targeted by the primer pair 515F (GCGYCAGCMGCCGCGGTA) and 928R (CCCCGYCAATTCMTTTRAGT) (Wang and Qian, 2009). Amplicon libraries were prepared from 0.5 to 8 ng DNA template using MTP Taq DNA polymerase (Sigma Aldrich, France) following the procedure described in the Supplementary Material. The 16S rRNA amplicons were sequenced using Illumina MiSeq 2*250 bp technology (Genotoul platform, Genopole Toulouse, France).

Raw data were pre-processed using the FROGS pipeline (Escudié et al., 2017) implemented in Galaxy platform (Afgan et al., 2018). Operational taxonomic units (OTUs) were generated by a two-step SWARM clustering algorithm (first denoising with d = 1, and then d = 3 on the seeds of the first step, which helps removing false positives) (Mahé et al., 2015). Chimera were removed with Vsearch and sample cross-validation (Rognes et al., 2016). Singletons were removed before taxonomic affiliation against the SILVA 132 database (Quast et al., 2013). The generated '.biom' file was analyzed in R software version 4.0.2 (R Core Team, 2013) mostly using the 'phyloseq' package (McMurdie and Holmes, 2013). To avoid false positives, an additional filter was applied by removing OTUs representing<0.005% of the total sequence number (Bokulich et al., 2013), allowing to keep 84.8% of the total sequences. For multivariate analysis purpose, rarefaction was done with the *rarefy_even_depth* function of the 'phyloseq' package with a threshold of 11,945 sequences per samples (i.e., lowest sequence number retrieved in one sample). Raw sequence data are available in the European Nucleotide Archive (ENA) under the BioProject references PRJEB36733 (*in situ* samples) and PRJEB41082 (incubated samples).

### 2.8. Statistical and multivariate analysis

When more than three replicates are available, the results are presented as the mean ± standard error (SE) where the SE evaluates the mean estimation error. All statistical analyses were performed with R software version 4.0.4 (R Core Team, 2013) in R Studio environment Version 1.3.959 (R package versions available in the **Supplementary Table S2**). Plots were generated using the 'ggplot2' package (Wickham, 2016) and the 'ggordiplots' package (Quensen, 2018).

In order to link the contextual physicochemical data with the endogenic MPR, a multivariate principal component analysis (PCA) was done using the 'FactoMineR' package (Husson et al., 2013). The PCA was built with 14 variables, including incubation-related variables at *in situ* temperature (i.e., endogenic MPRs and the maximal $CH_4$ quantity accumulated in the incubations) and *in situ* measurements (i.e., the net $CH_4$ flux, the dissolved $CH_4$ and $O_2$ concentration in the water column, the sediment and water temperature, pH of the sediment, the organic matter content, the sulfate, ammonium and nitrate + nitrite concentrations, the total maximal depth of the water column and the area of the lake).

The significant difference between *in situ* and *in situ* + 5 °C endogenic MPR values was identified by the non-parametric Wilcoxon test because of the non-normal data distribution. The influence of the amendment type on $E_a$ was evaluated by a one-way ANOVA followed by a Tukey's HSD multiple comparison of means as this set of data passed for parametric test of variance (normal distribution and homoscedasticity of the residuals). The Welch's *t*-test for testing the mean equality of two samples with inequal variance was used to compare Bray Curtis distances for both microbial and methanogenic community structure. Significant correlation between endogenic MPR at *in situ* temperature (i.e., 5 °C for Lake G, 10 °C for reservoir R, lake P1 and P2, 15 °C for lake P3) and OM concentration was identified through the non-parametric Spearman test (ρ correlation coefficient) because of the non-normal data distribution (evaluated by Shapiro-Wilk's test).

To identify the best explanatory physicochemical variables for MPR under amended conditions at close-to-in-situ temperature, the physicochemical dataset was standardized (*decostand* function from 'vegan' package, Oksanen et al. (2013)) and forward selection analysis was performed (*forward.sel* function from the 'adespatial' package; Dray et al. (2013)). Briefly, the analysis begins with no variables selected in the forward selection model and significant explicative variables are added to the selection, based on stepwise regression, until no improvement is observed.

Multivariate analyses were also applied to identify the main driving factors of both the total microbial community structure and the methanogenic archaeal diversity. Methanogenic archaeal OTUs were selected from the unfiltered dataset on the basis of the known taxonomy of methanogenic archaea (Borrel et al., 2016; Evans et al., 2019). As *Verstraetearchaeia* and putative methanogenic *Bathyarchaeia* (i.e., *Ca.* Bathyarchaeota archaeon BA1 and BA2; Evans et al. (2015)) were absent from our dataset, the methanogenic archaeal diversity corresponded to 87 OTUs affiliated to orders *Methanobacteriales, Methanocellales, Methanofastidiosales, Methanomicrobiales* and *Methanosarcinales*. In order to

ensure that methanogenic archaeal communities were adequately represented, 5 samples with<100 methanogenic euryarchaeal sequences were removed from the analysis (they correspond to $H_2/CO_2$-based incubations at low temperature: R-A-5 °C, R-A-10 °C, R-B-5 °C, P2-B-5 °C and P3-A-5 °C; **Supplementary Figure S2**).

For both total and methanogenic community structure, non-metric multidimensional scaling (nMDS) analysis were performed based on Bray Curtis distance matrix computed at the OTU level. Permutational analysis of variance (PERMANOVA, *adonis* function, 'vegan' package; Oksanen et al., (2013)) tested the significance of different defined groups (i.e., temperature of incubation, ecosystems, amended conditions). A selection of discriminant OTUs was performed in two steps. First, we kept the OTUs (for the total community, only the 50% most abundant OTUs were considered; for the methanogens, all OTUs were considered) that best correlated with the nMDS ordination (i.e., with>50% axis fit score) using the *ordiselect* function ('goeveg' package; Goral and Schellenberg (2018)). The first step resulted in 45 and 60 pre-selected OTUs for the total dataset and the methanogenic archaeal dataset, respectively. Second, among the pre-selected OTUs, we only kept the OTUs that significantly correlated with the ordination (p-value < 0.01 and p-value < 0.05 for the total and methanogenic archaeal dataset, respectively; *envfit* function, 'vegan' package; Oksanen et al. (2013)). The selection finally resulted in 33 and 11 discriminant OTUs for the total microbial dataset and the methanogenic archaeal dataset, respectively.

Finally, a variation partitioning analysis was carried out to identify the proportion of variance explained by explanatory factors (pure and combined effects) for both total microbial and methanogenic archaeal community structure (Volis et al., 2011). Three explanatory matrices were considered in the variation partitioning analysis: "Ecosystem" containing the *in situ* OM concentration and the net *in situ* $CH_4$ fluxes; "Methanogenic conditions" containing the amendment type (i.e., none, acetate or $H_2/CO_2$) and the resulting MPRs at all temperatures; and "Temperature" containing the incubation temperature for each sample.

## 3. Results and discussion

### 3.1. Endogenic MPR levels and microbial communities in the studied freshwater ecosystems

#### 3.1.1. Endogenic MPR from unamended microcosms

Endogenic MPR measured in lake sediment samples at *in situ* temperature (5–15 °C) ranged from 0.03 to 1.22 μmol $CH_4$ $g^{-1}$ DW $d^{-1}$. The highest values were measured in the sediments from the peatland lake P1 (**Supplementary Table S3**). These values fall within the range of MPRs reported in the literature in slurry incubations from high-latitude and high-altitude freshwater ecosystems (Fig. 2). They are comparable to MPR measured from some Arctic Alaskan lake sediments (de Jong et al., 2018; Lofton et al., 2015) but are one to two orders of magnitude higher than values from lake sediments from high-altitude Yunnan plateau (Yang et al., 2017) and Japanese lake Biwa (Koizumi et al., 2004). Although $CH_4$ production was detectable after 8 days of incubation, the current unamended incubations have lasted for 100–300 days depending on the samples. Similar duration period was observed in subglacial sediments where $CH_4$ production was not measured until 374 days (Boyd et al., 2010). In a long term experiment using a similar protocol as in the current study, Knoblauch et al., (2018) were only able to detect $CH_4$ production after 2,500 days from thawed permafrost. This highlights the variability in methanogenic capacity according to sediment or soil origin and the presence of an active methanogenic community.

#### 3.1.2. Relationship between endogenic MPR and the physicochemical parameters of freshwater sediments

The principal component analysis (PCA, 72.7% of the total variation explained by the first two axis) built with physicochemical parameters, endogenic MPRs at *in situ* temperature and *in situ* $CH_4$ fluxes allowed to separate peatland lakes from glacial and reservoir lake along the first axis (Fig. 3b). Significant positive correlation was found between endogenic MPR and benthic OM ($\rho = 0.77$; p-value < 0.05). In eutrophic lake sediments, Berberich et al. (2019) emphasized the influence of both quality and quantity of OM on the endogenic MPR while in neotropical

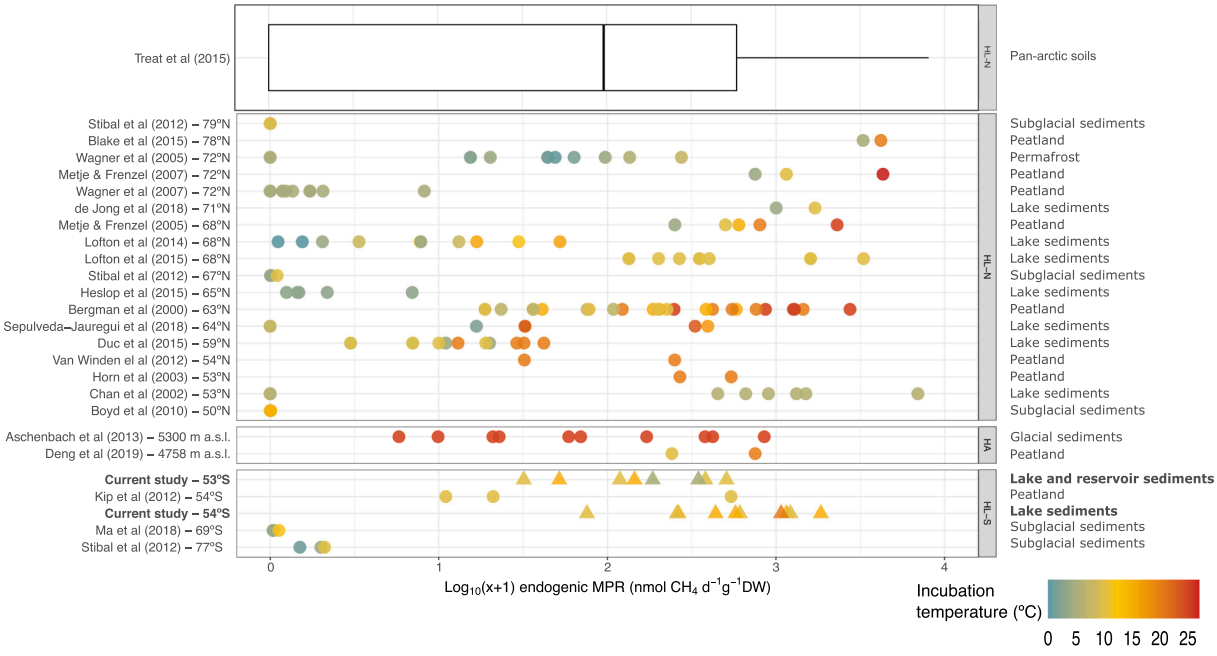


**Fig. 2.** Endogenic methane production rates (MPR) obtained in the current study (triangles and bold font) compared to literature values (circles) reported from cold high-latitude (north hemisphere: HL-N or south hemisphere: HL-S) or high-altitude (HA) ecosystems obtained from slurry incubations at different temperatures between 0 and 25 °C. Values are expressed in nmol $CH_4$ $d^{-1}$ $g^{-1}$DW and $\log_{10}(x+1)$-transformed. At the top of the plot, data from pan-Arctic soils incubated between 1 and 37 °C without addition of water or media (as reviewed in Treat et al. (2015), n = 96) are represented as a boxplot using the same x scale.

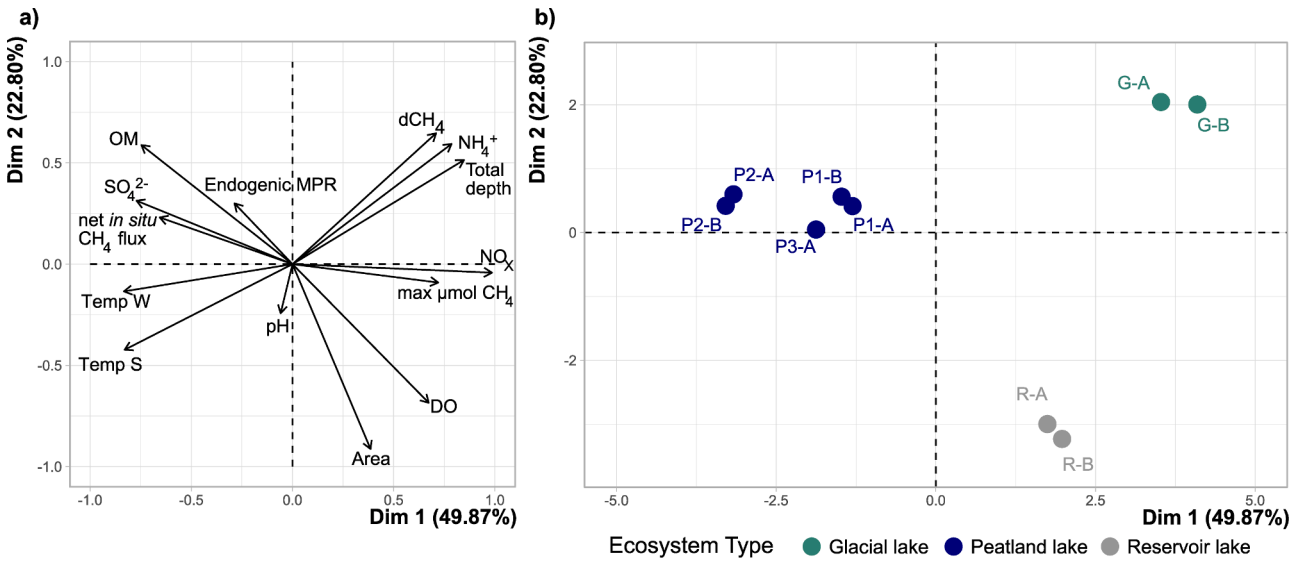


**Fig. 3.** Principal component analysis (PCA) of environmental data of the five lakes. a) The left panel represents the associated correlation circle allowing to visualize the contribution of each variable in the ordination. b) The right panel represents the map of individuals considered in the analysis corresponding to the 9 sediment samples from 5 lakes in duplicates (for P3 lake, only one sample was available). Samples are colored by ecosystem characteristic: in green post-glacial lake, in blue, the peatland lakes and in grey, the reservoir lake. The PCA was performed using the following variables: pH measured in the sediment (see Table 2), endogenic MPR measured in microcosm without amendment at the *in situ* temperature, Temp S = Temperature of the sediment, OM = organic matter content, $SO_4^{2-}$ = sulfates, net *in situ* $CH_4$ flux, $dCH_4$ = *in situ* dissolved $CH_4$ in water column, $NO_X$ = nitrite and nitrate concentration, $NH_4^+$ = ammonium, and max µmol$CH_4$ = maximum quantity of $CH_4$ accumulated at the end of the incubation, DO = dissolved oxygen, Temp W = temperature of water in surface, Total depth = Total water column depth, Area = Lake area in ha. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lakes, Hoyos-Santillan et al. (2016) showed that quality better controlled MPRs. In the current study, we did not assess the quality of the OM. Whatever the endogenic MPR value measured in the sediments, all five investigated lakes were net atmospheric sources of $CH_4$, with *in situ* $CH_4$ flux ranging from 2.5 to 64.5 mg $CH_4$ $m^{-2}$ $d^{-1}$ at the time of sampling (Table 2). These $CH_4$ flux values were neither correlated with the endogenic MPRs nor with the dissolved $CH_4$ concentration (Fig. 3a). This absence of correlation is not surprising, because (i) our atmospheric emission measurement was conducted upon a one-day basis, and (ii) $CH_4$ can be strongly oxidized in the water column, at a rate that mainly depends on $CH_4$ availability (Lofton et al., 2014; Martinez-Cruz et al., 2015). Interestingly, the dissolved $CH_4$ concentration ($dCH_4$) was positively linked to the maximum quantity of $CH_4$ that can be produced under unamended incubation at *in situ* temperature (max µmol $CH_4$) and negatively linked with the OM concentration (Fig. 3**a**). The data reported in the current study provide information about $CH_4$ production and fluxes from community to ecosystem level, respectively, and fit the *meta*-analysis about temperature dependence of methanogenesis by Yvon-Durocher et al. (2014).

The studied peatland lake sediments were characterized by higher OM concentration, greater endogenic MPR at *in situ* temperature and higher net $CH_4$ flux compared to lakes G and R. Beyond the highest OM concentration, the peatland lakes are strongly connected to the surrounding *Sphagnum* peatland constantly growing through the terrestrialization process (Domínguez and Vega-Valdés, 2015). This can result in an important input of OM with considerably different characteristics (such as *Sphagnum*-derived OM) compared to the OM that can enter lake G and R, that are only surrounded by broadleaf forest, and could sustain large part of the heterotrophic metabolism of the lake (Karlsson et al., 2012).

### 3.1.3. Microbial community diversity under unamended incubation conditions

The microbial diversity was evaluated for lakes R, P2 and P3, from *in situ* sediment samples and at the end of endogenic incubations at both *in situ* temperature (i.e., 10 °C for R and P2, 15 °C for P3) and *in situ* + 5 °C. Bray Curtis distances have been calculated between the community at the end of the incubation and the *in situ* community for each ecosystem (i.e., representing the incubation effect, **Supplementary Figure S6**). They were compared with Bray Curtis distances calculated for *in situ* communities between the different ecosystems (i.e., representing the ecosystem effect). The effect of ecosystems was significantly greater than the effect of incubation ($t = 2.01$, $df = 14.6$, p-value $< 0.05$). No strong changes of microbial community structure were observed between *in situ* and unamended conditions, with no emergence or disappearance of a specific group (Fig. 4). Noteworthy, some variations of proportions were observed over the course of the incubation, such as the 4-fold increase of class *Thermodesulfovibrionia* in peatland lake P2, the 4-fold increase of classes *Alphaproteobacteria* and *Woesearchaia* in peatland lake P3 and the 5-fold increase of *Aminicenantia* in the reservoir R (Fig. 4). Despite the long incubation times, these results confirmed that the "bottle effect" (as defined by Ionescu et al. (2015)) and the differential growth between taxa with time were low in these incubations compared to the effect of ecosystem origin.

The total microbial community in both endogenic incubations and *in situ* sediments from the three sequenced ecosystems was dominated by the bacterial classes *Bacteroidia* (15.3 ± 1.4% of total relative abundance), *Gammaproteobacteria* (10.9 ± 1.6%), *Deltaproteobacteria* (10.3 ± 0.7%) and *Chloroflexi* (10.3 ± 2.6%). On average, the most abundant OTUs (OTU 5, OTU 326, OTU 252) were affiliated to the fermentative *Prolixibacter* (*Bacteroidia*), the methylotrophic *Methylobacillus* (*Gammaproteobacteria*) and the anaerobic syntroph *Smithella* (*Deltaproteobacteria*), respectively. *Smithella* is known to be associated with acetoclastic or hydrogenotrophic methanogens retrieved from river, lake, peat or marine sediments (Holmes et al., 2007; Liu, 1999; Schmidt et al., 2016).

The relative abundance of methanogenic archaea did not exceed 0.062% of the total community in both *in situ* and endogenic incubation samples. The main methanogenic taxa retrieved in both *in situ* and endogenic incubation samples was the hydrogenotrophic *Methanoregula* (closest strain with 97.9% of sequence similarity: *Methanoregula formicica* SMSP; Yashiro et al. (2011)). In the lake R sediment,

**Table 2**
*In situ* physicochemical properties of lakes from Magallanes strait region (G and R) and Cape Horn (P1, P2 and P3). Note that P3 sample was not collected in duplicate. Sediment samples were collected at the center of the lakes in the zone of maximal depth. For contextualization, dissolved oxygen (DO) and dissolved methane ($dCH_4$) concentrations in the water column were also provided (measurement was done at the depth indicated in the column "Sampling depth"). OM: organic matter, DW: dry weight.

| | Sampling point | Ecosystem description | | | Net *in situ* $CH_4$ flux | | | Sediment Temperature (°C)[a] | pH | Dry weight (g $g^{-1}$ fresh sediment) | OM (g $g^{-1}$ DW) | $NH_4^+$ (mg $L^{-1}$) [b] | $NO_2$+$NO_3^-$ (mg $L^{-1}$) [b] | $SO_4^{2-}$ (mg $L^{-1}$) [b] | Water column | | DO (mg $L^{-1}$) | dCH4 (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Freshwater ecosystem type | Total area (ha) | Maximal depth (m) | Mean (mg $m^{-2}$ $d^{-1}$) | ±SE | *n* | | | | | | | | Oxic stratification | Sampling depth (m) | | |
| **G** | **A** | Post-glacial lake | 1.7 | 19 | 8.74 | 0.79 | 19 | 4.8 | 6.15 | 0.07 | 0.50 | 2.35 | 0.21 | 0.23 | Stratified | 14 | 8.10 | 163[c] |
| | **B** | | | | | | | | 6.30 | 0.05 | 0.36 | 2.14 | 0.24 | 0.30 | | | 7.30 | 213[c] |
| **R** | **A** | Reservoir lake | 45 | 6 | 2.45 | 0.13 | 24 | 9.9 | 6.34 | 0.29 | 0.09 | 0.49 | 0.18 | 0.15 | Unstratified | 1.5 | 11.78 | 3 |
| | **B** | | | | | | | | 6.65 | 0.27 | 0.09 | 0.45 | 0.18 | 0.17 | | | 11.72 | 3 |
| **P1** | **A** | Peatland lake | 2 | 4.5 | 6.56 | 0.97 | 8 | 11.0 | 6.65 | 0.06 | 0.85 | 0.87 | 0.07 | 0.33 | Unstratified | 3.75 | 4.43 | 1 |
| | **B** | | | | | | | | 6.66 | 0.06 | 0.83 | 0.51 | 0.06 | 0.34 | | | 4.26 | 2 |
| **P2** | **A** | Peatland lake | 0.7 | 3.1 | 64.49 | 11.47 | 9 | 11.0 | 6.34 | 0.04 | 0.75 | 0.31 | 0.05 | 0.89 | Unstratified | 1 | 4.71 | 16 |
| | **B** | | | | | | | | 6.49 | 0.04 | 0.77 | 0.02 | 0.04 | 0.83 | | | 4.74 | 23 |
| **P3** | **A** | Peatland lake | 0.2 | 4 | 9.06 | 0.56 | 6 | 13.69 | 5.70 | 0.05 | 0.85 | 0.49 | 0.06 | 0.41 | Unstratified | <1 | 6.71 | NA |

[a] temperature was measured at the interface water–sediment.
[b] measured by resuspension of sediment in distilled water and filtrated through Rhizon® filters.
[c] As Lake G was stratified, value provided came from the bottom water.

*Methanoregula* OTU reached 75% of the methanogenic archaeal community in unamended incubations (**Supplementary Figure S8**). Also, the acetate-using *Methanosaeta* (Smith and Ingram-Smith, 2007) was ubiquitous in the three studied lake sediments, and dominated in sediments from peatland lake P2 incubated under endogenic conditions (i.e., reached up to 48% of the methanogenic community). Interestingly, in the sediments of peatland lake P3, the hydrogenotrophic *Methanobacterium* represented up to 60% of the methanogenic archaeal community at the end of the endogenic incubations at both *in situ* and *in situ* + 5 °C temperature (relative abundance *in situ* = 5%; **Supplementary Figure S8**). As for total communities, Bray Curtis distances between methanogenic communities at the end of the incubation and *in situ* were significantly lower (range: 0.19–0.65) than the distances between methanogenic communities from different *in situ* ecosystems (range: 0.29–0.68) ($t = 1.84$, $df = 11$, p-value $< 0.05$). This reflected only slight changes in the methanogenic community structure between *in situ* and endogenic incubations particularly in lakes P2 and R (**Supplementary Figure S8**).

### 3.2. Effect of a 5 °C-increase on MPR and microbial community under unamended conditions

The endogenic MPR (i.e., unamended) increased significantly by almost 2-fold ($W = 2259$, p-value $< 0.001$) after a 5 °C increase of the incubation temperature (**Supplementary Table S3**). The mean estimated $E_a$ was $76 \pm 14$ kJ $mol^{-1}$ (i.e., $0.8 \pm 0.1$ eV) ranging from 27 to 171 kJ $mol^{-1}$. The few studies reporting temperature dependence of methanogenesis in southern high-latitude freshwater sediments have focused on subglacial sediments in Antarctica (Ma et al., 2018; Stibal et al., 2012) and have reported a positive effect of temperature (from 1 to 12 °C) on MPRs. Here, the temperature dependence of $CH_4$ production ($E_a$) measured at the community level can be extrapolated to $E_a$ at ecosystem-level, and thus provides relevant information in a global warming context (Yvon-Durocher et al., 2014).

Even though the endogenic MPR was affected by an increase of 5 °C, neither the associated total microbial nor the methanogenic community structure significantly changed with a 5 °C-increment in the three ecosystems studied (PERMANOVA, $df = 1$, $F = 0.34$ and 0.13, respectively, p-value $> 0.05$; Fig. 4). Hence, our results showed that under unamended conditions, a temperature increase of 5 °C doubled MPR ($E_a = 0.8$ eV) while microbial community structure was not affected. At temperatures below 25 °C, other studies have similarly reported the absence of temperature effect on microbial community structure (de Jong et al., 2018; Deng et al., 2019; Metje and Frenzel, 2005). However, at mesophilic to thermophilic temperatures (>45 °C), Deng et al. (2019) observed a sharp change of both active and present microbial community structure in high altitude wetlands. Also, short term (1.5 years) of winter warming (+1.5 °C) in northern tundra soils did not change the microbial composition (Xue et al., 2016), while long-term (5 years) winter warming modifies the microbial community structure and enhance both OM decomposition and methanogenesis gene abundance (Feng et al., 2020). In a climate change context, it is important to mention that other edaphic variables could be modified beyond the temperatures, like OM supply and quality, which are not assessed in the current study.

### 3.3. MPR and microbial community structure at close-to-in-situ temperatures under amended conditions

#### 3.3.1. Acetate- and $H_2/CO_2$-based MPR under close-to-in-situ temperatures

The acetate-amended MPR at close-to-*in-situ* temperatures were 7-fold to 127-fold higher than the endogenic MPR at the respective temperature (Fig. 5, **Supplementary Table S4**).

The differences of initial (i.e., *in situ*) physicochemical characteristics of the samples could partly explain the different MPR values. The acetate-amended MPRs at 5 °C were best explained by *in situ* pH

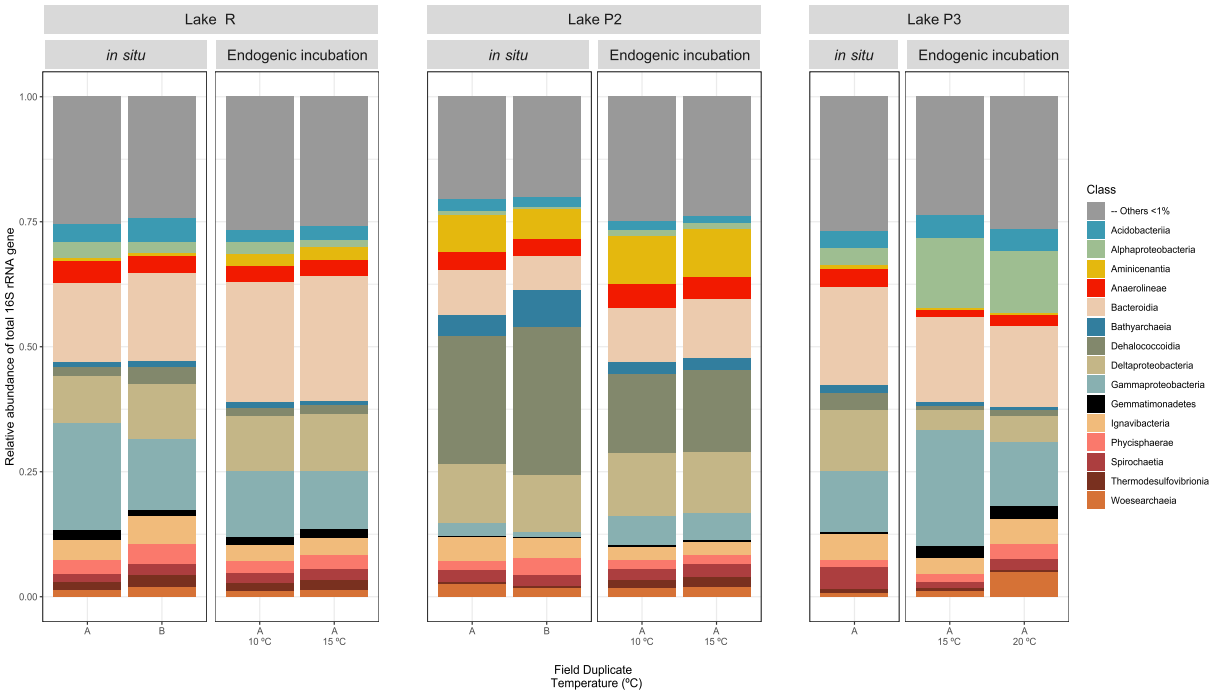


**Fig. 4.** Microbial community structure evaluated through 16S rRNA gene metabarcoding at the class level from three studied lakes (i.e., lake R and the peatland lakes P2, P3) both *in situ* and after endogenic incubation. Incubations were carried out at two different temperatures: *in situ* and *in situ* + 5 °C. On the x axis, the first line represents the field duplicate (A or B), the second line indicates the incubation temperature when applicable. The *in situ* samples were sequenced in field duplicates except for P3 for which no field replicate was available. Classes representing<1% of the total relative abundance were aggregated in a single group named "Others < 1%".

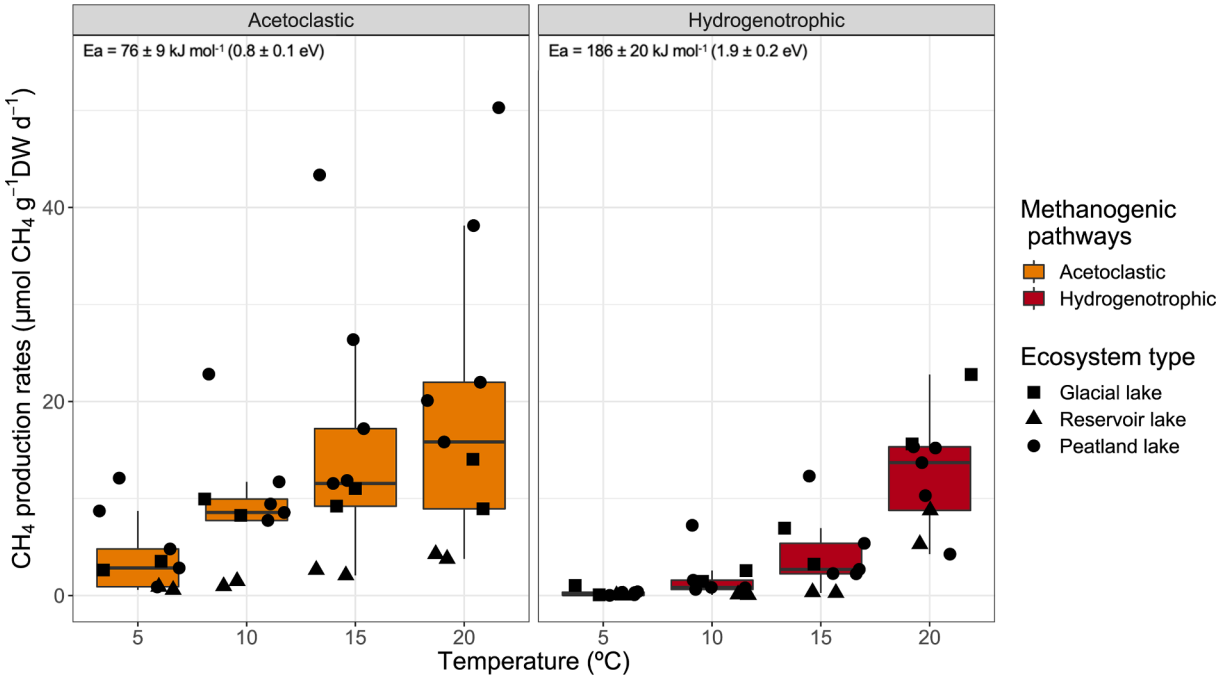


**Fig. 5.** Methane production rates in lake sediment microcosms incubated at different temperatures. Effect of temperature increase (from 5 to 20 °C) on acetate-derived (orange) and $H_2/CO_2$-derived (red) MPR, in sediments from lakes of the Strait of Magellan region (lakes G and R) and Cape Horn peatland lake sediments (P1, P2 and P3). The bold line of each boxplot shows the median, while upper and lower limits of the boxes represent the first and third quartiles respectively. The whiskers are maximum and minimum without outlier values, respectively. The mean of energy of activation ($E_a$) calculated by the Arrhenius equation for each sample is also annotated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(forward selection, $r^2 = 0.53$, F = 7.81, p-value < 0.05; **Supplementary Table S7**) and at 10 °C by OM concentration and pH (forward selection, $r^2 = 0.54$ and 0.37, respectively, F = 8.33 and 25.25, respectively, p-value < 0.05). Although incubated at similar feed-to-biomass ratios (4.4 mmol C $g^{-1}$ OM), the $H_2/CO_2$-amended MPR never exceeded > 19-fold the endogenic level (ranging from 0.2 to 19-fold), at the respective

temperatures. The $H_2/CO_2$-amended MPR at close-to-*in-situ* temperatures were not significantly explained by the physicochemical parameters suggesting that the measured *in situ* parameters were not the main drivers explaining the MPR through the use of $H_2/CO_2$ in microcosms.

At close-to-*in-situ* temperatures, MPRs using acetate as methanogenic precursor were 2.5 to 160-fold higher than the ones using $H_2/CO_2$ at the same temperature (median = 13-fold; Fig. 5, **Supplementary Table S4**). In the low temperature (5–10 °C) $H_2/CO_2$-based incubations, MPRs were always lower than 2.6 μmol $CH_4$ $g^{-1}$ DW $d^{-1}$ (except for the outlier P3 at 10 °C with 7.2 μmol $CH_4$ $g^{-1}$ DW $d^{-1}$), the lag phase was relatively long (ranging from 160 to 651 days) and the substrate conversion yields (i.e., mean: 11%) were on average 12-fold lower compared to acetate-based yields (**Supplementary Table S5 and S6**). This indicates that acetate was the major methanogenic precursor at close-to-*in-situ* temperatures in the studied ecosystems. Hence, it suggests that the native microbial communities were better adapted to the organic substrate acetate compared to the inorganic precursors $H_2/CO_2$. Our findings are supported by current knowledge. It has been established stoichiometrically (recently reviewed by Conrad, 2020) and experimentally (Kotsyurbenko et al., 2004) that when the degradation of OM is complete, the ratio of acetate and $H_2$ produced by fermentation eventually results in $CH_4$ derived at > 67% from acetoclastic pathway and at < 33% from hydrogenotrophic pathway.

Moreover, chemolithotrophic acetogenic bacteria consuming $H_2$ to produce acetate are favored at low temperatures (Conrad, 2020), which supports the important contribution of acetoclastic methanogens at low temperature. However, the uptake of one substrate or the other depends highly on temperature, redox conditions, pH and OM quality and can thus strongly vary according to the respective ecosystem. For example, below 1 °C, lower acetoclastic MPR was found in both Antarctic subglacial sediments (Ma et al., 2018) and Arctic permafrost (Wagner et al., 2007) compared to hydrogenotrophic MPR.

### 3.3.2. *Impact of substrate amendment on methanogenic community structure at close-to-in-situ temperatures*

When incubated at close-to-*in-situ* temperature with a methanogenic precursor (i.e., acetate or $H_2/CO_2$), both the microbial and the methanogenic community structure significantly changed compared to *in situ* sediments samples (comparison of all incubated samples with all *in situ* samples, PERMANOVA, df = 2, F = 1.66 and 3.72, for total and methanogenic communities, respectively, p-value < 0.05; **Supplementary Figure S8**). While almost absent *in situ* (i.e., 0.3 ± 0.3% of methanogenic community), the metabolically versatile methanogen *Methanosarcina* became dominant in the acetate-amended incubations (i.e., representing 62 ± 12% of methanogenic community). *Methanosarcina* outcompeted *Methanosaeta* (i.e., 13 ± 4% of methanogenic community) which was found ubiquitous in the different incubated samples. The dominance of *Methanosarcina* over *Methanosaeta* was also reported in peat soils (Schmidt et al., 2016).This is congruent with the metabolism of *Methanosarcina*, known to be more competitive at high acetate concentrations (i.e., > 4 mM) compared to *Methanosaeta* (De Vrieze et al., 2012; Jetten et al., 1992).

In the $H_2/CO_2$-amended incubations at close-to-in-situ temperature, the discriminant OTUs driving the community clustering (OTU 19, OTU 103, OTU 17,797 and OTU 27491; Fig. 6**a**) belonged to the hydrogenotrophic genus *Methanobacterium*. For example, OTU 19 and OTU 27,491 were closely related to *Methanobacterium paludis* SWAN1 (99.5% of sequence identity; NR_133895, (Cadillo-Quiroz et al., 2014)) isolated from northern peatlands in Alaska.

## 3.4. *Contrasting response of methanogenic pathways and microbial community structure to temperature increase*

### 3.4.1. *Stronger temperature effect on $H_2/CO_2$-amended MPRs*

In all cases, the MPRs (ranging from 0.59 to 50.28 μmol $CH_4$ $g^{-1}$ DW $d^{-1}$ in acetate-amendments and from 0.01 to 22.78 μmol $CH_4$ $g^{-1}$ DW $d^{-1}$ in $H_2/CO_2$-amendments) increased with increasing temperature, which is a commonly reported pattern in lake sediments under global warming (Glissmann et al., 2004). However, the effect of increasing temperatures was different for the two methanogenic pathways. The $E_a$ values ranged from 40 to 132 kJ $mol^{-1}$ (average: 76 ± 9 kJ $mol^{-1}$ corresponding to 0.8 ± 0.1 eV) in acetate-amended incubations and from 130 to

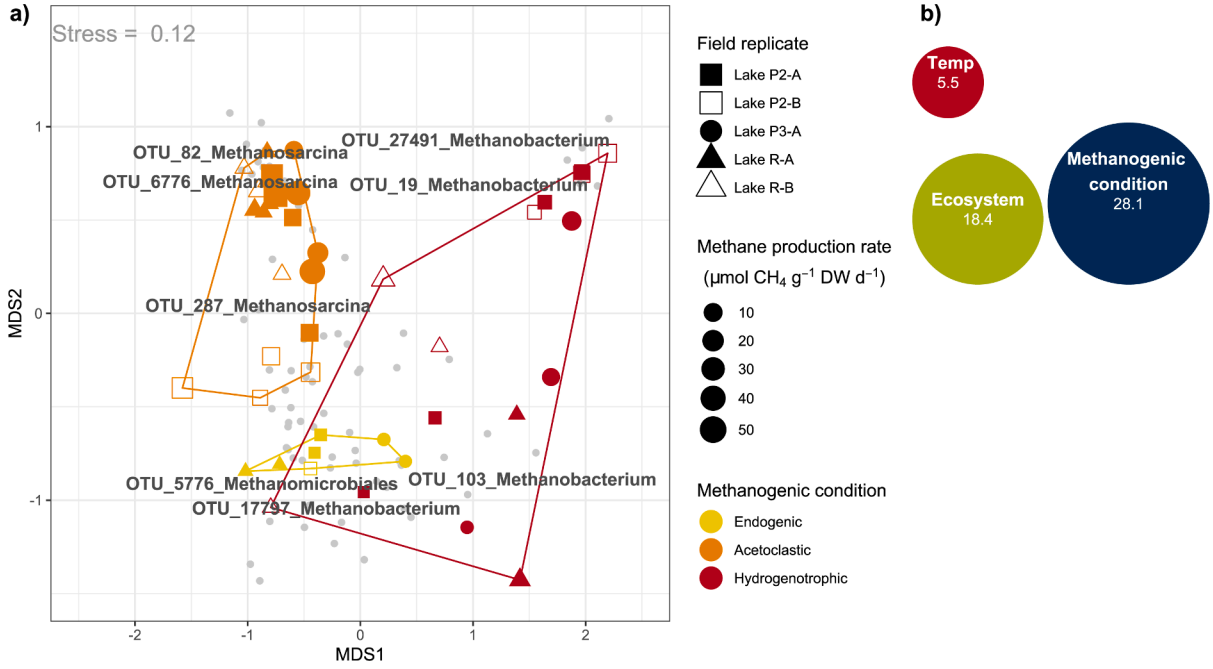


**Fig. 6.** Structure of methanogenic archaeal community. a) non-metric multidimensional scaling (nMDS) based on Bray Curtis distance of the 87 methanogenic archaeal OTUs abundances. The polygons visualize the dispersion of the samples from the different incubation types. A selection of the most discriminant OTUs was plotted (n = 14). b) A variation partitioning allowed to determine the percentage of methanogenic archaeal community structure variation explained by "Ecosystem" (i.e., the three studied lakes), Methanogenic condition (Endogenic, Acetoclastic and Hydrogenotrophic) and Temp (Temperature).

266 kJ mol$^{-1}$ (average: 186 ± 20 kJ mol$^{-1}$ corresponding to 1.9 ± 0.2 eV) in $H_2/CO_2$-amended incubations (Fig. 5). The temperature dependence of MPR was significantly higher in experiments spiked with $H_2/CO_2$ compared to acetate and unamended incubations (Tukey's HSD, p-value < 0.05, Fig. 5). Yvon-Durocher et al. (2014) previously combined results of $CH_4$ production from culture-, community- and ecosystem-based experiments to study the temperature dependence of amended and unamended methanogenesis ($E_a$). Independently of the methanogenic pathway, the authors found a universal $E_a$ for methanogenesis in a large temperature range (from 1 to 60 °C), between 0.82 and 1.27 eV. The temperature dependence of methanogenesis from stimulated natural communities were not considered in the *meta*-analysis of Yvon-Durocher et al. (2014). Here we reported that MPRs from acetate-stimulated natural communities exhibit $E_a$ within the universal range proposed by Yvon-Durocher et al. (2014). However, our results point to higher temperature dependence value for $H_2/CO_2$-stimulated sub-Antarctic lake sediments, thus nuancing the general findings of Yvon-Durocher et al. (2014).

The higher temperature dependence of the $H_2/CO_2$-stimulated methanogenesis is not likely supported by a differential substrate availability because amended incubations were set up at similar feed-to-biomass ratio. It might not either be controlled by methanotrophy at low temperature in the $H_2/CO_2$-stimulated incubation since methanotrophs never exceeded 0.6% of the total community. Also, thermodynamically, hydrogenotrophic methanogenesis is more exergonic than acetoclastic one (Hedderich and Whitman, 2006) and higher temperatures tend to reduce the solubility of gaseous substrates. Nonetheless, the $E_a$ is an integrative value of temperature dependence that might rather reflect very low MPRs at 5 and 10 °C. As discussed above, the low MPRs in $H_2/CO_2$-stimulated incubations may strongly rely on OM sources and microbial community composition. Especially, the *in situ* conditions in the studied lakes may have favored both acetoclastic methanogens (because of the acetate-producing OM-degrading routes), and $H_2$-consuming acetogens outcompeting hydrogenotrophic methanogens, thus leading to the limitation and/or inhibition of the hydrogenotrophic pathway at low temperatures, as previously reported (Conrad, 1999; Kotsyurbenko et al., 2001; Liu and Whitman, 2008; Nozhevnikova et al., 2007).

Hence, our data suggest that under a climate change scenario (+5 °C, highest predicted change under RCP8.5 scenario, IPCC (2014)), microbial community composition and substrate availability may be modified and the hydrogenotrophic pathway may dominate in freshwater sediments.

#### 3.4.2. Response of the methanogens to temperature increase and methanogenic substrate type

First, the abundance of the methyl coenzyme M reductase (*mcrA*) was assessed in every sample *in situ* and at the end of the incubations. The *in situ* abundances of the *mcrA* gene ranged from 1.32 x10$^6$ gene copies g$^{-1}$ DW in sediments from Lake R to 2.68 x10$^9$ gene copies g$^{-1}$ DW in sediments from Lake G (**Supplementary Table S9**). Compared to this baseline, *mcrA* gene abundance was further enriched after incubation by a factor of 31 ± 13, 197 ± 42 and 5 ± 1, in endogenic, acetate-based and $H_2/CO_2$-based incubations, respectively (**Supplementary Table S9-11**). Even if the active methanogenic fraction was not targeted, present methanogens were probably effectively active in our incubations. First, because of the significant correlation between *mcrA* gene abundances and amended MPRs (either in acetate-based and $H_2/CO_2$-based incubations; Supplementary Figure S7). Second, because methanogenesis is an obligate process as both energy and carbon source for methanogen growth (Deppenmeier, 2002; Lyu et al., 2018). Hence, the abundance of *mcrA* gene could be considered as a good molecular proxy of MPRs and represents a satisfactory way to better understand the potential of environmental microbial process rates at the molecular level, as previously suggested by Smith and Osborn (2009). Even though *mcrA* transcript/gene ratio could be a preferable indicator of process rate, the functional marker alone is not the only factor explaining $CH_4$ production (Freitag and Prosser, 2009). Interestingly, our forward selection analysis highlighted that 61% of variation of $H_2/CO_2$-amended MPRs can be explained by incubation temperature (**Supplementary Figure S7c**). This result reinforced the strong temperature dependence of $H_2/CO_2$-based MPRs previously observed with high $E_a$ values.

Second, when considering only methanogen-affiliated sequences in the 16S rRNA gene dataset, we showed that temperature only explained 5.5% of the variation of the methanogenic community structure (variation partitioning; "Temperature", Fig. 6**b**). Temperature was expected to have an effect on the methanogenic community structure (Blake et al., 2015; Fey and Conrad, 2000). We however found that the amended methanogenic precursor and the ecosystem of origin were stronger drivers of the methanogenic community structure than temperature (28.1% and 18.4% of variation explained, respectively, Fig. 6b). Hence, as mentioned above, it results in a global dominance of *Methanosarcina* (OTUs 82, 6776 and 287) in the acetate-stimulated incubations and of *Methanobacterium* (OTUs 19, 103, 17,797 and 27491) in the $H_2/CO_2$-stimulated incubations with almost no change with the increase of temperature (Fig. 6**a**).

#### 3.4.3. Response of the microbial community structure to temperature increase and methanogenic substrate conditions

Temperature explained only 1.8% of the variation of the total microbial community structure, when considering all samples together (Fig. 7b). However, temperature had a significant effect on microbial composition when considering each ecosystem separately, as a *strata* (PERMANOVA; df = 1; F = 0.887; p-value < 0.05). Similarly, Samad et al. (2020) identified that temperature significantly shaped microbial community structure from lakes and affected differently bacterioplankton or particle-associated microbial communities. Hence, distinct microbial assemblages from different ecosystems reacted in a different way to temperature.

As indicated by the significant clustering of samples according to their ecosystem of origin (PERMANOVA; df = 2; F = 11.60; p-value < 0.05; Fig. 7a) and by the variation partitioning analysis (35.5% of variation explained by the "Ecosystem" matrix, Fig. 7b), each studied sub-Antarctic lake sediment exhibited a site-specific microbial community structure. The most discriminant OTUs driving this ecosystem-based segregation (**Supplementary Figure S10**) were mainly close relatives (as indicated by blastn search) with environmental sequences retrieved in lake sediments (Watanabe et al., 2016), permafrost (Hansen et al., 2007), periglacial environment (Vimercati et al., 2019) and northern peatlands (Cadillo-Quiroz et al., 2014; Schmidt et al., 2016).

The methanogenic conditions (i.e., represented by the type of amended substrate and resulting MPR) significantly explained 14.2% of the total microbial community structure (variation partitioning; "Substrate" matrix, Fig. 7**b**). As expected, different substrate amendment resulted in different microbial assemblages within each ecosystem. Bray Curtis distances were calculated between the microbial community structures at the end of the incubations, comparing 1) acetate-amended versus endogenic conditions and 2) $H_2/CO_2$-amended versus endogenic conditions (for the same incubation temperature) (**Supplementary Figure S6**). Stimulation of methanogenesis by $H_2/CO_2$-amendment resulted in higher dissimilarity from endogenic conditions than the acetate-amendment did (t = -2.58, df = 15.42, p-value < 0.05). The $H_2/CO_2$-amended communities appeared to be the most dispersed and most different from the endogenic communities, while the acetoclastic communities were more similar to endogenic communities, suggesting a distinct selective pressure of $H_2/CO_2$ on microbial community structure.

### 3.5. Production of acetate in $H_2/CO_2$-amended incubations

At 20 °C, in sediments from lakes G and R, $H_2/CO_2$-based MPR exceeded the acetate-based MPR values (**Supplementary Table S4**). At the beginning of the $H_2/CO_2$-amended incubations, we observed a decrease of the gaseous substrate indicated by the decrease of the

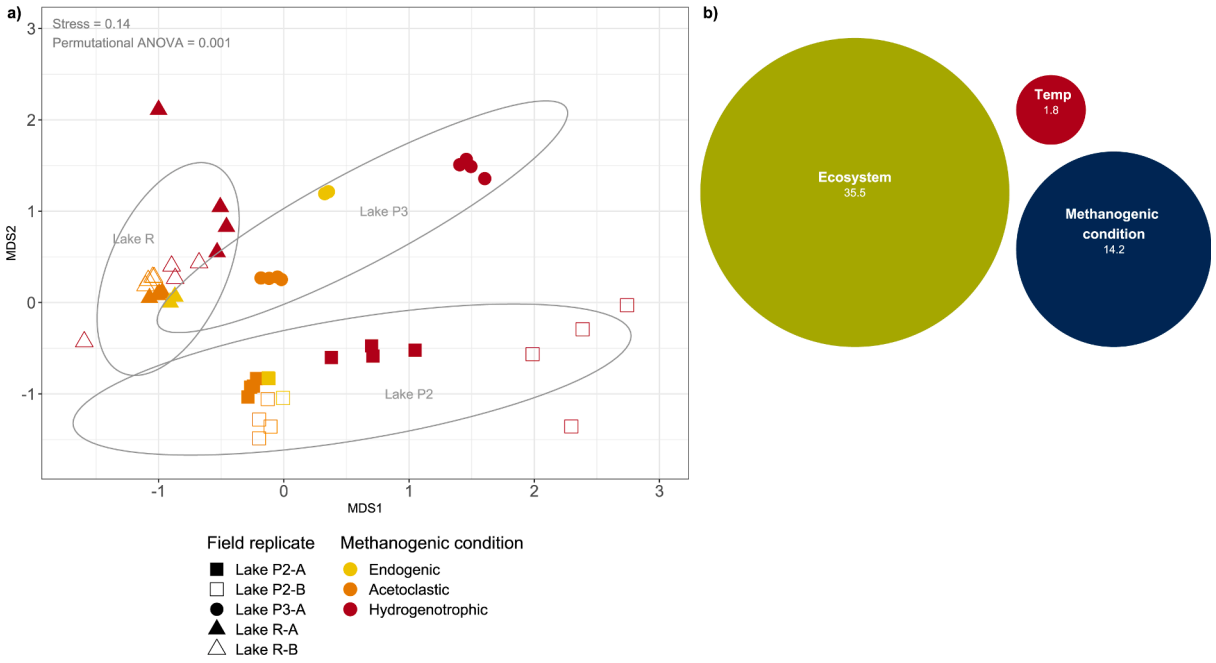


**Fig. 7.** Variation of the microbial community structure in lake sediments from methanogenic incubations. For the non-metric multidimensional scaling (nMDS) based on non-transformed Bray Curtis distance matrix (calculated from the relative abundance matrix), samples are represented with different shapes and colors for field replicate and type of methanogenic incubations, respectively (a). The 90% confidence ellipses were plotted using the *gg_ordiplot* function ("ggordiplots" package; Quensen (2018)) and significance was tested using permutational ANOVA (PERMANOVA; df = 2; F = 11.599; p-value < 0.005). Variation partitioning (b) identified the percentage of pure variation (of non-transformed Bray Curtis distance matrix) explained by each factor: Ecosystem (i.e., the three studied lakes), Methanogenic condition (Endogenic, Acetoclastic and Hydrogenotrophic) and Temp (Temperature).

headspace pressure over time, together with the accumulation of acetate ranging from 12.77 to 47.9 mM with a tendency to increase with temperature, and no $CH_4$ production (**Supplementary Table S6**). Moreover, we detected fermentative order *Clostridiales* (e.g., OTU 18, OTU 183, OTU 394) known to generate acetate within the main end products of fermentation (Lanjekar et al., 2015). Also, the homoacetogen *Acetobacterium* spp. was retrieved (i.e., OTU 233 and OTU 668) and produce only acetate as a fermentative product (Drake et al., 2013). OTU 233 represented up to 6.5% of the total community in lake R sediment samples incubated at 20 °C with $H_2/CO_2$ (**Supplementary Figure S11**). As also recorded by Nozhevnikova et al. (2007), acetogenesis and homoacetogenesis seem to occur in the $H_2/CO_2$-amended incubations at higher rates than hydrogenotrophic methanogenesis, explaining the low calculated substrate conversion yields of 29 ± 4% (**Supplementary Table S6**). While Fey and Conrad (2000) reported that homoacetogenesis played only a minor role in rice field soils, our results suggest that acetogenesis and homoacetogenesis may be important processes in the studied sediments.

## 4. Conclusion

Our results showed that under unamended conditions, a temperature increase of 5 °C doubled methane production rates, while microbial community structure was not affected. The acetoclastic pathway seemed to be more efficient to produce $CH_4$ in the studied freshwater sediments. The methanogenic archaeal community structure changed when different methanogenic precursors were added, favoring *Methanosarcina* and *Methanobacterium* with acetate and $H_2/CO_2$ amendments, respectively. The total microbial community structure revealed a strong variability due to the ecosystem, overpassing the amendment and the temperature effects. Temperature dependence of acetate-amended and unamended methane production was equivalent. However, $H_2/CO_2$-stimulation of methanogenesis resulted in greater temperature dependence of both the abundance of methanogens and methane production rate. The combination of *in situ* physicochemical and geochemical characterization, microcosm incubations, gene abundance quantification and microbial community structure analyses allows a better understanding of temperature influence on methanogenic pathways and microbial communities, opening a new frontier in methane cycle-research in austral freshwater ecosystems.

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## CRediT authorship contribution statement

**Céline Lavergne:** Conceptualization, Investigation, Methodology, Supervision, Writing - original draft. **Polette Aguilar-Muñoz:** Investigation. **Natalia Calle:** Investigation. **Frédéric Thalasso:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Maria Soledad Astorga-España:** Conceptualization, Investigation, Resources. **Armando Sepulveda-Jauregui:** Methodology, Investigation, Writing - review & editing. **Karla Martinez-Cruz:** Methodology, Investigation, Writing - review & editing. **Laure Gandois:** Methodology, Investigation, Writing - review & editing. **Andrés Mansilla:** Resources. **Rolando Chamy:** Resources. **Maialen Barret:** Conceptualization, Investigation, Methodology, Supervision, Writing - original draft. **Léa Cabrol:** Conceptualization, Investigation, Methodology, Supervision, Writing - original draft.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106575.

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