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The biotechnological potential of microbial communities from Antarctic soils and sediments: application to low temperature biogenic methane production

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Abstract

Anaerobic digestion (AD) is an attractive bioprocess for waste treatment and energy recovery through methane-rich biogas production. Under temperate to cold climate, the implementation of AD for low-organic load wastewater treatment has been limited to date, due to the energetic and economic cost of maintaining optimal mesophilic temperature. Hence, we aim at (i) exploring the biotechnological potential of a microbial inoculum from Antarctic soils and sediments to run AD at low temperatures; and (ii) evaluating the effect of temperature over a psychrophilic-mesophilic range on both methane production rates and microbial community composition. Methane production stimulated by acetate amendment was detected from 5 to 37 °C, with a maximum at 25 °C, corresponding to the highest relative abundance of methanogenic archaea (c. 21.4% of the total community). From 5 to 25 °C, the predominant methanogen was *Methanosaeta*, while it

shifted to *Methanocorpusculum* at 30 °C. Compared with an industrial mesophilic sludge, the relative methane production rate at 5 °C (compared to the maximum) was 40% greater in the Antarctic inoculum. Microbial communities from permanently cold Antarctic sediments efficiently produce methane at low temperatures revealing a biotechnological potential for the treatment of low-organic load residues in cold regions.

Keywords

Polar region, bioprocesses, archaea, bacteria, high throughput sequencing, qPCR, microcosms

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1. Introduction

Anaerobic digestion (AD) is a widespread bioprocess used for organic waste treatment and energetic valorization, leading to biogas (methane - CH_4 and carbon dioxide – CO_2) formation (Kelleher et al., 2002). Temperature is a critical parameter for this process. As a mesophilic process, AD provides optimal performance around 37 °C. At lower temperatures, the biogas production is drastically affected (McKeown et al., 2012). For example, below 15 °C, anaerobic reactors inoculated with mesophilic biomass lose more than 90% of their methanogenic activity (Lettinga et al., 2001). For that reason, almost all large-scale AD applications for wastewater are operated in a mesophilic range (Van et al., 2020). In temperate to cold climate regions, the energetic requirement to heat and maintain anaerobic digesters for wastewater at mesophilic temperature represents up to 30% of the biogas produced if the chemical oxygen demand (COD) is low, which makes the overall economic yield marginal or negative (Lettinga et al., 2001; McKeown et al., 2012). The low-temperature limitation is even more pronounced for residues with low organic matter content, such as domestic and some industrial wastewater (Lettinga et al., 2001). The AD operation at ambient to cold temperature can alleviate this requirement, through technological improvement of reactor configuration (e.g. using expanded granular sludge bed, EGSB), or by using a psychrophilic or cold-adapted microbial consortium ensuring stable biogas production (McKeown et al., 2009a). The choice of inoculum has been recognized as a key factor enabling the feasibility of low-temperature AD (McKeown et al., 2009b).

AD involves a complex microbial consortium performing a set of reactions, from hydrolysis to methanogenesis. While hydrolysis is limiting for complex and/or solid substrates (Donoso-Bravo et al., 2009), the methanogenesis is commonly recognized as the limiting step in the treatment of low organic load wastewater at low temperature. Two main metabolic pathways exist for the generation of CH_4 and CO_2 in AD, using either acetate or H_2/CO_2, however, methanogenesis from

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methanol, methylamines, and formate has also been observed especially in marine environmental (Conrad, 2020). The acetoclastic methanogens generate CH_4 and CO_2 from acetate in such a way that carboxyl group of acetate is oxidized to CO_2 so the methyl group is reduced to CH_4 (Náthia-Neves et al., 2018; Whitman et al., 2006). The only archaea capable of performing this conversion are those belonging to the order Methanosarcinales, and more specifically to the genus *Methanosarcina* and *Methanosaeta* (Evans et al., 2019; Zeikus, 1977). In contrast, hydrogenotrophic methanogens generate CH_4 by reducing CO_2 using H_2 as an electron donor (Náthia-Neves et al., 2018; Whitman et al., 2006) and belong to the order *Methanomicrobiales*, *Methanobacteriales*, *Methanocellales*, and *Methanosarcinales* (Conrad, 2020; Evans et al., 2019; Zeikus, 1977).

While hydrogenotrophic methanogenesis is thermodynamically more favorable compared to acetoclastic methanogenesis (Conrad, 2020), in mesophilic AD, approximately 60 to 70 % of CH_4 is produced from acetate, because the stoichiometry of the fermentation reactions result in the production of acetate in a greater proportion than H_2 (Conrad, 2020; Nozhevnikova et al., 2020; Venkiteshwaran et al., 2015). The effect of low temperature on metabolic pathways for methane production is not completely resolved (Horn et al., 2003). However, generally, it is considered that at low temperature, about 70% of the produced CH_4 originates from acetate as the main precursor, the remaining originating from H_2 and CO_2 (Conrad, 2020).

Among natural ecosystems, wetlands are the major source of CH_4 emissions (Reay et al., 2010; Saunois et al., 2020). In permanently cold natural environments, such as high Arctic peat, methanogenic archaea putatively adapted to low temperature (1-5 °C) have been detected using molecular techniques (Høj et al., 2008). The different functional groups involved in AD reactions have psychrophilic or psychrotrophic representatives (Dhaked et al., 2010). A study of microbial communities and methanogenic potential in wetland sediments from high Arctic regions

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concluded that temperature was the dominant factor determining the rates of methanogenesis and structuring the methanogenic community (Blake et al., 2015). Although CH_4 production has been demonstrated in cold ecosystems, the inhabiting methanogenic archaea are generally considered as psychrotolerant. Indeed, the CH_4 production rate (MPR) usually increases when the temperature increases until reaching at least a temperature of 20 °C (de Jong et al., 2018; Lavergne et al., 2021). However, in constantly low-temperature anoxic sediments from profundal lakes in Switzerland, maximal methanogenic activity was observed at low temperature (6 °C) within a range from 2 °C to 70 °C, demonstrating the psychrophilic activity of this methanogenic community (Nozhevnikova et al., 2003). Psychrophilic methanogenic archaea species have been reported, such as *Methanogenium frigidum* (optimal 15 °C) isolated from Ace Lake, Antarctica (Franzmann et al., 1997) and *Methanolobus psychrophilus* (optimal 18 °C) isolated from a Tibetan Plateau wetland (Zhang et al., 2008).

The Antarctic region harbors one of the least human-impacted environments and provides a model for low-temperature systems (Purdy et al., 2003). Due to their unique extreme conditions, Antarctic ecosystems can be considered as a reservoir of psychrophilic biodiversity for potential biotechnological applications (Bowman et al., 2005; Yarzábal, 2016). During the short austral summer, ice-free areas appear in the coasts and islands of Antarctic Peninsula. Due to ice melting and glacier runoff, these ice-free areas are usually saturated in water, impacted by marine animal colonies, and sparse biological cover develops including microbial mats, lichens, mosses, and rare vascular plants (Casanova-Katny et al., 2016; Putzke and Pereira, 2020). Until now, the acetoclastic methanogenic potential of Antarctic soils and lake sediments has been understudied in comparison with those obtained in Arctic and sub-Arctic regions.

Few reports using sediments/soils from permanently cold regions for waste treatment at low temperatures show high rates of CH_4 production (Petropoulos et al., 2021, 2019; Xing et al., 2010)

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suggesting their potential biotechnological application. Hence, the objectives of this work are (i) to determine the feasibility of low-temperature AD from cold-adapted microbial communities from Antarctic soils and freshwater sediments, without the need for sophisticated reactor configuration change, and (ii) to evaluate the effect of temperature (i.e., within a psychrophilic-mesophilic range) on CH_4 production rate (MPR) and microbial community composition. This work will lead to the generation of a consortium with a biotechnological potential for anaerobic wastewater treatment at low temperatures.

2. Materials and Methods

2.1. Inoculum origin. The studied inoculum is a composite of nine sediment and soil samples (physicochemical characteristics available in Supplementary Table S1: temperature; pH; nitrate, nitrite, ammonia, sulfate, and phosphate concentrations; volatile solid content) mixed and homogenized, so that each individual sample contributed the same proportion of volatile solid content (i.e. organic matter proxy) to the composite sample. The samples were collected in February 2013 (Table S1) from the Antarctic peninsula and South Shetland islands, in water-saturated and organic matter rich areas such as moss wetlands or animal colony ponds (Table S1). All samples were strongly impacted by penguin and/or sea lion excrements, resulting in high organic and nutrient contents (Table S1). The nine samples were selected among a preliminary screening of 43 Antarctic samples, as the ones presenting the highest methanogenic activity at both 5 and 37 °C and the shortest latency time before methane production. The dry weight (DW) of all samples was determined by drying overnight at 110 °C. Then, volatile solid (VS) content was measured via loss on ignition (LOI) on dry samples (550 °C, at least 6h, until no change in mass).

2.2. Experimental setup for MPR measurement. The determination of MPR was carried out in triplicate 12 mL glass vials with 2 mL of headspace, inoculated with a similar volatile solid concentration (*i.e.,* 5.9 g VS L^{-1}). Each vial contained 8 mL of mineral nutrient solution specifically

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developed for anaerobic biodegradation (Shelton and Tiedje, 1984), 2 g of fresh soil/sediment inoculum and acetate substrate at 30 mM final concentration, as previously described (McHugh et al., 2004). The vials were sealed with butyl septa (Plasticoid, USA) and the headspace was initially flushed with N_2 for 15 minutes to ensure anaerobic conditions. Microcosms were incubated in parallel at seven controlled temperatures: 5, 10, 15, 20, 25, 30 and 37 °C (± 1 °C precision), setting up triplicate microcosms at each temperature.

2.3. Analytical monitoring for MPR determination. The biogas production was quantified periodically by measuring the headspace pressure with a manometer (PCE-P05, PCE Instrument, Germany). The biogas composition was determined by gas chromatography (Clarus 500, Perkin Elmer, USA), equipped with a VICI Hayesep Q (4m x 1/8" OD) column (80 °C) and thermal conductivity detector (TCD, 120 °C), on a gas sample collected from the headspace with gas-tight syringe (SGE Analytical Science, Australia). The cumulated amount of moles of CH_4 in the gas phase was calculated using the Ideal gases law from headspace pressure and CH_4 proportion. The concentration of CH_4 in the liquid phase was determined by Henry's law considering the incubation temperature. The amount of total cumulated CH_4 (in the gas phase and the liquid phase) was plotted against time (Figure S2). The conversion from moles to volume of CH_4 was performed using normal pressure and temperature conditions (P= 1 atm, T= 273 K). The MPRs were computed as the maximum slope of the linear phase (at least four points) of CH_4 accumulation kinetics over time, normalized by dry weight (DW) or volatile solid (VS). The conversion yield of the substrate was calculated to the end of the exponential phase of production with respect to the fed substrate.

The temperature constant k (K^{-1}) was defined by the equation: $\frac{r_{X,T1}}{r_{X,T2}} = e^{k(T1-T2)}$ were T1 and T2 are temperatures (K); $r_{X,T1}$ and $r_{X,T2}$ are MPR at temperature T1 and T2 respectively (Henze and Harremoës, 1983). The activation energy Ea (kJ mol^{-1}) was derived from the Arrhenius

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equation: $k = Ae^{\left(\frac{-E_a}{RT}\right)}$ 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) (Shelley et al., 2015).

To determine the final acetate concentration, at the end of the incubation (i.e., when kinetic curves reached a plateau), the content of two vials was centrifuged (10 000 rpm for 15 min) and supernatant was filtered through a 0.22 µm membrane. Acetate concentration was measured in the filtered supernatant by HPLC (Series 200, Perkin Elmer, USA) equipped with HP 1100 RID detector, through an AMINEX column (HPX-87H, 300 * 7.8mm * 9 µm, BioRad, USA) heated at 50 °C, setting the mobile phase flow rate at 0.5 mL min^{-1}; allowing a detection limit of 2.85 mM of acetate.

2.4. DNA Extraction. At the end of the exponential production phase of CH_4, duplicated incubation samples were centrifuged (10 000 rpm for 15 min) and the pellet was stored at -20 °C. Total genomic DNA was extracted from approximately one gram of the pellet with the PowerSoil DNA isolation kit (Qiagen, USA), following the recommendations of the supplier. DNA quantity and quality were verified by UV spectrometry (BioSpec-nano, Shimadzu Scientific, Japan).

2.5. Quantitative PCR (qPCR). The copy number of 16S rRNA genes from total bacteria and total archaea was determined through quantitative PCR (qPCR). The amplification was carried out in duplicate using the SYBR Select Master Mix kit (Applied Biosystem) with the AriaMx thermocycler (Agilent Technologies, USA). The thermal program consisted of one cycle at 95 °C for 20 sec and 44 cycles at 95 °C for 3 sec for denaturation and at 60 °C for 30 sec for hybridization and extension. Each reaction consisted in 2 µL of DNA sample (DNA amount ranging 2.4 – 0.7 ng) in a total reaction volume of 20 µl containing 750 mM of each primer (Supplementary Table S2). For each group, standard curves were made with concentrations between 10^1 and 10^9 gene copy number μL^{-1} using pure strains *Bacillus subtilis*, DSM ref 10, for bacteria and *Methanosarcina mazei*, DSM ref 3647, for archaea. The average amplification efficiency was higher 80% in the

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linear range of standard curves (R^2 > 0.99). The results obtained by qPCR were expressed in number of gene copies per dry weight of sediment/soil.

2.6. High-throughput sequencing of bacterial and archaeal 16S rRNA genes. The diversity of the bacterial and archaeal community was determined by high-throughput sequencing (Illumina MiSeq, MrDNA Lab, Shallowater, USA) of the hypervariable region V4-V5 of the 16S rRNA gene, using the universal primers 515F (GCGYCAGCMGCCGCGGTA) and 928R (CCCCGYCAATTCMTTTRAGT) (Wang and Qian, 2009). The sequences were cleaned with the pipeline of MrDNA (elimination of barcodes, sequences <150bp, chimeras removal, sequences with ambiguous bases). The OTUs were defined at 97% similarity using MOTHUR (Schloss et al., 2009) and taxonomically classified by comparison (BLASTn) with the cured databases derived from GreenGenes, RDPII and NCBI. Raw sequence data are available in the European Nucleotide Archive (ENA) under the BioProject reference PRJEB52010.

2.7. Data processing. A total of 418 035 sequences were obtained corresponding to 15 045 OTUs. After removal of singletons, the dataset resulted in 416 338 sequences corresponding to 99.6% of the whole dataset (Supplementary Table S3). The final dataset contained 5 386 OTUs (35.8% of the pre-processed OTUs number). Number of sequences in each sample ranged from 41 658 (at 5 °C) sequences to 84 278 sequences (at 25 °C). The clean and trimmed sequences were analyzed using R version 3.6.2 software (R Core Team, 2013). The alpha diversity indices were calculated before rarefaction. For subsequent multivariate analyzes (using the 'vegan' package; Oksanen et al. (2018)), a rarefaction was performed (function *rarefy_even_depth* of the 'phyloseq' package; McMurdie and Holmes, (2013)) resulting in 41 658 sequences per sample.

2.8. Statistical analyses. The influence of temperature on MPRs was evaluated by one-way analysis of variance (ANOVA) followed by a Post-hoc test (Tukey HSD), making sure that ANOVA

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assumptions were met (normality through Shapiro test and homoscedasticity through Levene test). Spearman correlation was used to correlate methanogenic activity and latency time.

The differences of microbial community structure between samples were compared through hierarchical clustering (UPGMA method) and principal component analysis (*PCA_ordinate* function, 'phyloseq' package) based on Bray Curtis dissimilarity. Discriminating OTUs for temperature were defined by a contribution on the first two PCA dimensions greater than 2% ('FactoMineR' package).

3. Results

3.1. CH_4 production rates (MPRs). MPRs from the mixed Antarctic consortium were significantly affected by a temperature increase from 5 to 37 °C (ANOVA, df= 6, F = 195.6, p-value < 0.001), with the maximum MPR reached at 25 °C (Table 1, Figure 1A). The MPR value at 25 °C was higher and significantly different from those obtained at other temperatures (Tukey Post-Hoc test, p-value < 0.05). When the temperature decreased from 25 °C to 5 °C, the MPRs decreased significantly and gradually (Tukey Post-Hoc test, p-value < 0.05), being reduced by up to 52-55% at 5 °C. Similarly, when the temperature increased from 25 °C to 37 °C, a decrease in the MPR was observed compared to 25 °C (Tukey Post-Hoc test, p-value < 0.05) with 27-31% of activity loss. Interestingly, the MPRs at 20 °C were not significantly different compared to the values at 37 °C (Tukey Post-Hoc, p-value > 0.05). The reproducibility of the MPR measurements between duplicates was robust as revealed by a relative coefficient of variation always lower than 2.3%. The maximum substrate conversion yield was also obtained at 25 °C, with a value of 11.19 mmol CH_4 g^{-1} COD (250.7 mL CH_4 g^{-1} COD), which is significantly higher than the yield obtained at lower temperatures (Tukey Post-Hoc test, p-value < 0.05). Between 5 and 20 °C, substrate conversion yield remained stable with no significant differences (Tukey Post-Hoc test, p-value > 0.05). At higher temperatures, the yield was not significantly different from that of 25 °C (Tukey Post-Hoc

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test, p-value > 0.05). At the end of the incubation (as defined in the Material and Method section), the residual acetate was always undetectable (below the detection limit).

The shortest latency time (i.e., 10 days) was found between 20 °C and 30 °C, while 37 days were needed to detect first CH4 production at 5 °C. Interestingly the latency time at 10-15 °C were comparable to the latency time at the highest tested temperature (37 °C, usually considered as optimal for AD), and was twice as high as the optimal one (Table 1). MPR values were negatively correlated with latency time (Spearman correlation; R^2=-0.89; p-value < 0.05).

Table 1

The values of MPRs were expressed as a percentage of the maximum MPR (measured at 25 °C) and compared with an empiric model from a compilation of experimental data given in Henze and Harremoës (1983) for 32 reactors inoculated with mesophilic sludge (Figure 2). The optimal temperature was 25 °C for Antarctic inoculum and 35 °C for mesophilic sludge. At the lowest temperature (i.e., 5 °C), the MPR of the conventional reactors inoculated with mesophilic sludge drastically dropped, down to 5% of their maximum MPR. By contrast, the studied Antarctic inoculum enabled to maintain at 5 °C 50% of its maximum MPR. Between 10 and 20 °C, the MPR of the mesophilic reactors was as low as 8 to 22% of the maximum MPR, while the Antarctic inoculum maintained from 46% to 70% of its maximum MPR. At 25 °C, which is the optimal temperature of the Antarctic inoculum, the mesophilic reactors present only 37% of their maximum MPR. Using the Arrhenius model, the MPR temperature dependency in the Antarctic inoculum was characterized by a calculated temperature constant of 0.047 $°C^{-1}$ and an activation energy E_a = 26.23 kJ mol^{-1}.

3.2. Proportion of archaea and bacteria. The abundance of bacteria was stable between 5 and 25 °C with an average of 6.44 ± 1.47 $x10^8$ 16S rRNA gene copies g^{-1} DW (Turkey Post-Hoc test, p-value > 0.05). As the temperature increased (30-37 °C), the abundance of bacteria decreased significantly compared to the range 5-25 °C (Turkey Post-Hoc test, p-value < 0.05) with an average

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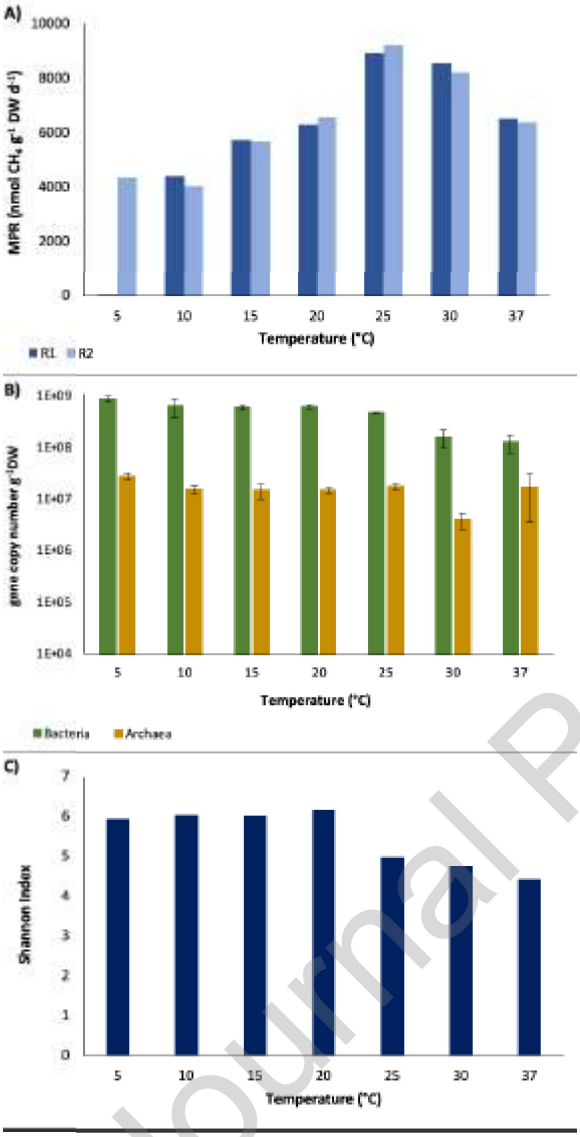
value of 1.44 ± 2.21 x10^8 16S rRNA gene copies g^{-1} DW (



3.3. Figure *1*B).

Between 5 and 30 °C, archaea represented 2.8 ± 0.6% of the total archaeal and bacterial 16S rRNA gene copies. The lowest archaeal abundance was recorded at 30 °C with on average 4.13 x106 16S

Figure 1

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rRNA gene copies g-1 DW (2.6% of the total archaea and bacterial 16S rRNA gene copies) (

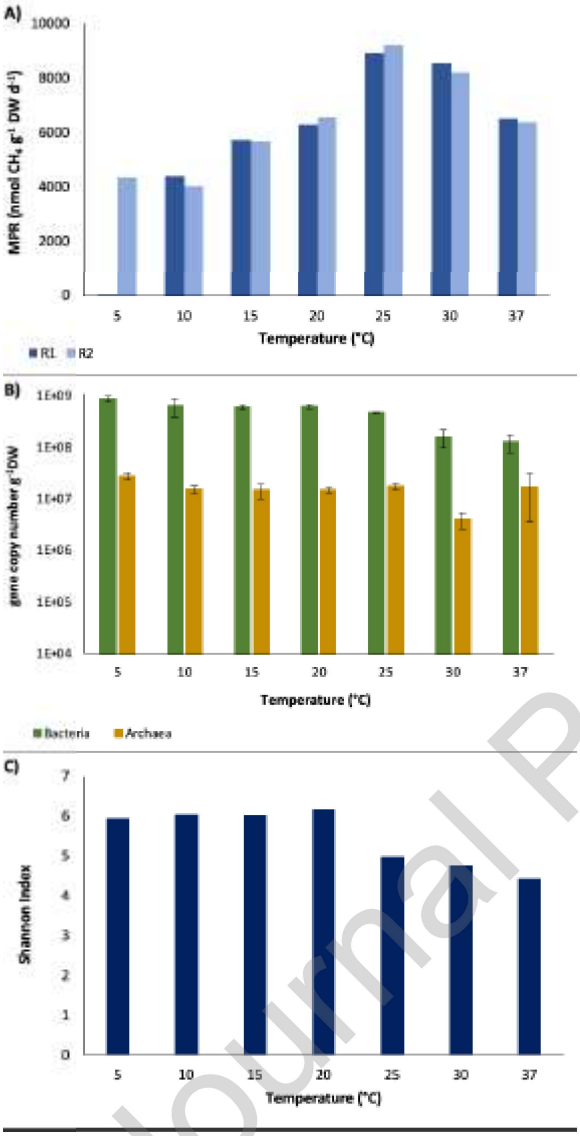

Figure 1B). At 37 °C, the relative abundance of archaea reached 13.6% of the total prokaryotic abundance (mainly because of the lower bacterial absolute abundance).

3.4. Microbial community composition. The OTU number remained relatively constant in all the samples from 5 to 20 °C (in average 2590 ± 109 OTUs), and then decreased by 25%, 30% and 37%, at 25, 30 and 37 °C, respectively. The alpha diversity indices decreased with increasing temperature (Table S4). More specifically, the Shannon index was stable within the range 5-20 °C

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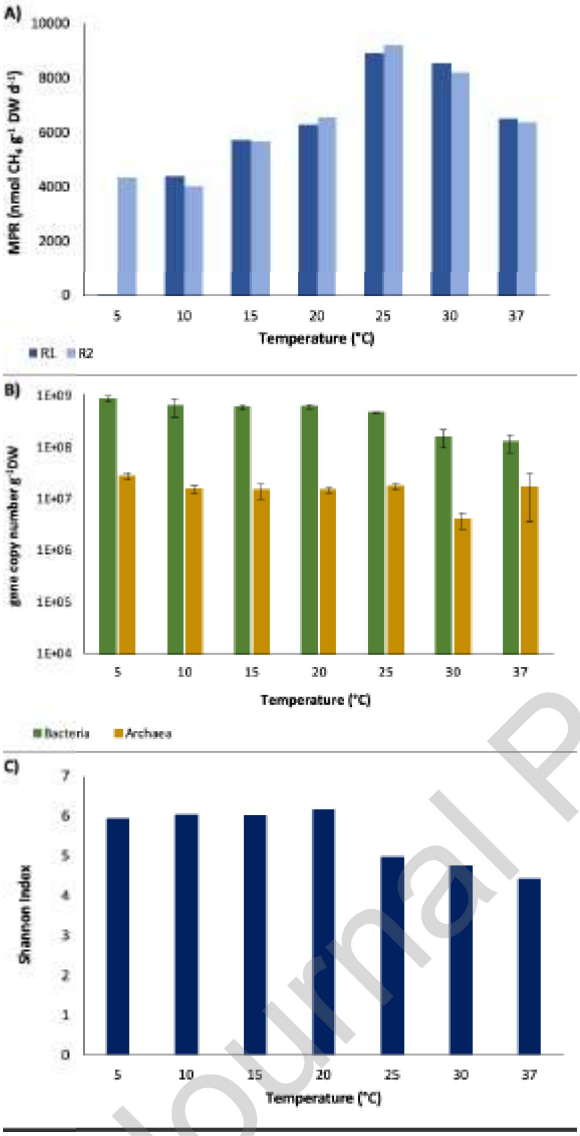
(6.0 ± 0.1) and showed a slight and progressive decrease above 25 °C until a value of 4.4 at 37 °C (



3.5. Figure *1*C). Interestingly, at higher temperature, the diversity was low suggesting that higher temperatures applied a strong selective pressure on cold-adapted Antarctic microbial communities.

Analysis of microbial structure showed that the most abundant phyla were *Bacteroidetes* (28.5% to 61.0% of total sequences), *Proteobacteria* (20.9% to 34.9% of total sequences) and *Firmicutes* (2.9% to 31.1% of total sequences) (Figure 3A). At low temperature (5-20 °C), the community was strongly dominated by two phyla, while the community was more uniformly distributed when the

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temperature increased above 25 °C. The samples clearly clustered according to incubation temperature, revealing the strong influence of temperature on microbial community structure.

The first two dimensions of the PCA (Figure 3B) allowed to visualize 71.6 % of variation in the total microbial community structure. The horseshoe distribution of the samples traduced the progressive changes of community structure with the gradual increase of temperature. The conformations of microbial community were structured in two groups driven by the temperature of incubation, distinguishing (i) low and medium temperature (5-20 °C) and (ii) high temperature (30-37 °C). Interestingly, at 25 °C (corresponding to the optimal MPR), the community composition was intermediate between the two temperature ranges.

Interestingly, 15 microbial OTUs were found discriminant for the construction of the PCA (Figure 3B). Between 5-25 °C, the discriminating OTU_53 was affiliated to methanogenic archaea *Methanosaeta*, representing 1.0 ± 0.4% of the total community between 5-20 °C and 18.1% of the community at 25 °C, and the discriminating OTU_10 was affiliated to the bacterial genus *Parabacteroides*, representing 7.5% to 19.9% of the total community. At 30 to 37 °C, the methanogenic community clearly changed with the highest relative abundance of the genera *Methanocorpusculum* (0.3% of total community) and *Methanosarcina* (4.0% of total community) respectively. In the bacterial community, most of the discriminant genera were fermentative bacteria (e.g., *Clostridiisalibacter*, *Bacteroides*, *Candidatus Cloacimonas*).

Methanosaeta dominated the archaeal community from 5 to 25 °C. It showed a constant abundance of 58.7 ± 5.1% of the archaeal community from 5 to 20 °C, and increased at 25 °C reaching 84.2% of archaeal community. At higher temperatures, the archaeal community composition shifted from *Methanosaeta*-dominated to a co-dominance of genera *Methanocorpusculum* and *Methanosarcina* representing 44.5% and 68.8% of archaeal community at 30 °C and 35 °C, respectively (Figure 2).

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4. Discussion

In cold and temperate regions, the development of AD processes at ambient (low) temperature for organic waste treatment is of major economic and ecological interest (Lettinga et al., 2001). In this study, a new microbial consortium from Antarctic soils and sediments, able to produce CH_4 at low temperature, was enriched and characterized for both methane production efficiency and microbial diversity, over a temperature gradient from 5 to 37 °C.

4.1. High CH_4 production rates in the newly generated Antarctic enrichment. Comparing data of CH_4 production with literature is challenging since MPRs are reported either in natural environments or in bioreactors, each field using different conventional units and protocols. The main variations can be 1) the liquid and/or headspace volume, 2) the inoculum history and concentration, 3) the addition of substrate and its concentration and 4) the incubation temperature, among others. Considering these discrepancies, we set up our incubations and expressed our results in such a way to be broadly comparable with both ecological studies (MPRs expressed in nmol CH_4 d^{-1} g^{-1} DW, Table 2) and bioengineering studies (MPRs expressed in mL CH_4 g^{-1} VS d^{-1}, Table 3).

Table 2

The acetate-derived MPRs of the newly generated consortium at low temperatures (5-15 °C) ranged from 4 200 to 5 600 nmol CH_4 d^{-1} g^{-1} DW. These values are 5 to 10-fold higher than published data from cold natural environments with acetate amendment at comparable incubation temperatures, mainly recorded from the Arctic region (Table 2 and Table S5). Different works conducted in Antarctica demonstrated the presence of methanogenic activity in soils and lake sediments. In Wilson Piedmont glacier samples, Stibal et al. (2012) reported unamended MPR of 0.5, 1, and 1.1 nmol CH_4 d^{-1} g^{-1} FW (fresh weight) at 1 °C, 4 °C and 10 °C, respectively. Our consortium presented an optimal temperature of 25 °C, with acetate-derived MPR reaching 9 055 nmol CH_4 d^{-1} g^{-1} DW. The optimal temperature for methanogenesis can vary in different soils from

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cold environments. In general, in low temperature ecosystem, the optimal MPR is reported at mesophilic temperatures (25 °C - 30 °C). Metje and Frenzel (2005) incubated northern peatland soils (68 °N) between 4 °C and 40 °C without amendment and concordantly found an optimum at 25 °C (i.e., 2 300 nmol CH_4 d^{-1} g^{-1} DW). However, Nozhevnikova et al. (2003) reported true psychrophilic methanogenic communities exhibiting an MPR optimum at 6 °C (i.e., 73.4 nmol CH4 d-1 g-1 DW) when comparing unamended incubations between 4 and 60 °C of anoxic sediments from permanently cold deep lakes (4 to 5 °C). Interestingly, the maximal MPRs were reported from lakes and reservoir sediments of Chilean Patagonia (Lavergne et al., 2021), exhibiting higher content of organic matter, which can potentiate CH_4 production and influence the composition of the methanogenic community.

4.2. High performance at low temperature compared with mesophilic sludge. By fitting the model proposed by Henze and Harremoës (1983), we determined that for the Antarctic inoculum, the constant traducing the temperature effect on MPR was 0.047 °C^{-1}, i.e. two times lower than the constant reported for mesophilic sludge exposed to low temperatures (0.1 °C^{-1}). This means that the detrimental temperature effect is much stronger on conventional AD, and our Antarctic inoculum was more efficient for producing CH_4 at low temperature compared to mesophilic ones, certainly thanks to active psychrophilic microorganisms able to produce CH_4 at low temperature (Figure 2).

4.3. A biotechnological potential for AD at low temperatures. The MPRs of continuous low temperature anaerobic digesters (LTAD) operated with adapted and non-adapted mesophilic inocula have been extensively studied, mostly in state-of-the-art technological configurations such as UASB or EGSB (as reviewed in Table 3 and Table S6). They are measured in batch assays outside the reactor, after long adaptation times in the reactor (app 100-1000 days). By contrast, in the present study, the Antarctic inoculum was active after 10 to 37 days between 5-25 °C, in a simple

Table 3

Table 3

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batch configuration. The acetoclastic MPRs from our natural consortium (i.e., 2.65 and 5.71 mL CH_4 g^{-1} VS d^{-1}) are comparable with non-adapted seed inocula and approximately 10 times lower than those obtained in long-term LTAD reactors. This difference can be explained by the fact that the biomass coming from anaerobic digesters is considerably more concentrated and enriched in active methanogens than the biomass from natural soil environments, especially in Antarctic soils and sediments poor in organic content. Notably, the reactor configurations used for LTAD and reported in Table 3, where biomass is granulated or attached to a growth-support through a biofilm, lead to considerably enhanced biomass retention in the reactor, thus facilitating the treatment of higher flow rates and lower substrate concentrations and enabling the operation at low temperature. Furthermore, organic matter from mosses can overestimate our estimation of biomass by volatile solids in Antarctic sediments and soils.

Hence, when available, the normalization of MPRs by the abundance of archaea (quantified by qPCR) allows more robust comparisons between different types of inocula. Here, unit conversions were performed assuming that there is on average 2.4 copies of 16S RNA per cell for methanogenic archaea (Sun et al., 2013). The specific MPRs of the Antarctic inoculum at temperatures below 25 °C ranged between 8 and 28 x10^{-8} mL CH_4 d^{-1} $cell^{-1}$, which is significantly higher than previously reported rates. In reactor treating synthetic sewage, the specific MPR at 12 °C ranged from 0 to 6 x10^{-8} mL CH_4 d^{-1} $cell^{-1}$ depending on the adaptation time (from 105 to 889 days) (Keating et al., 2018). From synthetic wastewater, McAteer *et al.* (2020) found specific MPRs between 3 and 29 x10^{-8} mL CH_4 d^{-1} gene $copy^{-1}$, with the greatest rates measured after long acclimatization times of the microbial community (from 110 to 443 days). Petropoulos et al. (2017b) used a mixture of lake sediment (temperature between -11 to 17 °C) and high Arctic soil (temperature between -16 to 6 °C) to treat sterile complex wastewater from a treatment plant and reported specific methanogenic activity of 4, 8 and 15 °C of 14, 17 and 23 x10^{-11} mL CH_4 $cell^{-}$

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$^{1}day^{-1}$ respectively. When the mixture was previously acclimatized to 4 °C, the specific methanogenic activity increased to 88 x10^{-11} mL CH_4 $cell^{-1}day^{-1}$ at 15 °C (Petropoulos et al., 2017a). The difference between MPRs may be due to the substrate used.

All together, these results show that the Antarctic inoculum obtained in this study (i) is highly active at low temperature, with specific methanogenic rates similar or higher than those reported in LTAD reactors; (ii) enables to reduce considerably the biomass adaptation time to reach high activity at low temperature; (iii) is active in a simple batch configuration mode which can be easily scaled to a ASBR reactor; (iv) could be further enriched in anaerobic granules and/or biofilm grown on a fixed support. In the present work, acetate was used as a proxy to target the methanogenic step activity which is usually limiting for low strength wastewater. Further work should evaluate the efficiency of this inoculum to treat more complex substrates, since lipolysis has been identified as the rate limiting step on the treatment of real waste by AD at low temperature (Petropoulos et al., 2018).

4.4. Effect of increasing temperature on methanogenic community composition. As expected, the addition of acetate as a substrate, led to a dominance of acetoclastic methanogens (Figure 2). While a dominance of *Methanosaeta* between 5-25 °C was associated with gradual increment of MPR until a maximal value at 25 °C, we observed a shift in the methanogenic composition above 30 °C with a dominance of *Methanocorpusculum* and *Methanosarcina.*

Figure 2

It suggests that *Methanosaeta* would be more tolerant/adapted to low temperatures below 25 °C with a slow growth rate, enabling to maintain significant MPR up to the highest measured CH_4 production efficiency. On the other hand, *Methanosarcina* should be more competitive at higher temperature (37 °C) with faster growth rate but exhibiting lower MPR than *Methanosaeta* at 25 °C; moreover, *Methanosarcina* was overcompeted at low temperatures. The presence of the genus *Methanosaeta* is consistent with the addition of acetate in the experiment. However, its

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dominance over *Methanosarcina* at low temperatures (below 25 °C) in the Antarctic inoculum incubated with 30 mM acetate can be more surprising since at high (> 4 mM) acetate concentrations the r-strategist *Methanosarcin*a is usually considered as more competitive than the K-strategist *Methanosaeta* (Jetten et al., 1992). This could be explained by the capacity of *Methanosarcina* to use methanol and/or H_2/CO_2 with no acetate dependence for growth under low temperature conditions (Gunnigle et al., 2013).

A large diversity of *Methanosarcin*a species has been reported in cold regions. For example *Methanosarcina lacustris* has been described in anoxic sediments from Switzerland growing between 1 and 35 °C with optimum at 25 °C (Simankova et al., 2001). Also, *Methanosarcina baltica* was isolated from sediment in the Baltic Sea and in anoxic marine sediments in Alaska as capable to grow from -2 to 28 °C, with an optimum at 21 °C (Von Klein et al., 2002). *Methanosarcina* sp. was also found in the frozen Antarctic lake Fryxell (Karr et al., 2006). Finally, *Methanosarcina subterranea* was the predominant methanogenic archaea in sub-Antarctic freshwater sediments incubated with acetate at low temperature (5-20 °C) (Lavergne et al., 2021). On the other hand, in organic-rich Antarctic marine sediments, *Methanosaeta* was reported as the predominant and active methanogenic archaea, highlighting their role in CH_4 production and overall carbon cycling within sedimentary environments (Carr et al., 2018).

Figure 3

In LTAD, *Methanosaetaceae* are usually predominant and positively correlated to process efficiency, whereas low levels of *Methanosarcinaceae* have been reported in well-functioning LTAD systems (Gunnigle et al., 2013). Ribera-Pi et al. (2020) operated three configurations of reactors and concluded that *Methanosaeta* was by far the most abundant genus (> 50%) and suggested the importance of the acetoclastic pathway when operating at low temperatures (9.7 ± 2.4 °C). Similarly, Singh et al.,(2019) reported dominant acetoclastic pathway at low temperatures (10 and 20 °C) in batch reactors, highlighting the role of *Methanosaeta*.

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4.5. A putative hydrogenotrophic detour. Interestingly, at 30 °C, we measured a change in the main methanogenic group, with hydrogenotrophic methanogens, such as *Methanocorpusculum*, representing 53.3 % of the archaea community (Figure 2). This suggests a change in the CH_4 generation pathway that could be linked to the presence of acetate-consuming bacteria that can produce hydrogen (e.g. *Clostridium* and other syntrophic acetate-oxidizing bacteria (SAOB)) (Schink, 1997). The shift from acetoclastic-dominated methanogenesis to SAO-coupled hydrogenotrophic methanogenesis has been previously observed in anaerobic digestion microbiome exposed to different environmental disturbances (Hardy et al., 2021). The genus *Candidatus Cloacimonas* presents a maximal relative abundance at 30 °C with 14.1% of total sequences. The *in silico* proteome analysis of *Candidatus Cloacimonas acidaminovorans* (not cultivated) indicated that this bacterium, present in many anaerobic digesters, is a syntrophic producer of H_2 and CO_2 from formate (Pelletier et al., 2008; Solli et al., 2014). Also, *Clostridium* was present over the whole temperature range (representing 1.7% at 5 °C and 5.1% of total sequences at 37 °C). This group is able to produce H_2 and volatile fatty acids through a syntrophic pathway (Ghasimi et al., 2015). Psychrophilic species of *Clostridium* have been reported in arctic permafrost growing between 5 and 6 °C (Shcherbakova et al., 2004). Finally, the genus *Geobacter* (representing between 0.3% and 4.9% of total sequences) is an anaerobic microorganism that completely oxidizes acetate, among other fatty acids, to CO_2 with Fe (III) as an electron acceptor (Lovley et al., 1993).

The increase of temperature can also favor other members of the community that could compete with methanogens and CH_4-associated microorganisms. Among them, the most abundant genus within Proteobacteria was *Desulfobacterium* (representing between 0.9% and 4.9% of total sequences), which is a strict anaerobic bacteria reducing sulfate, able to use acetate among other fatty acids as carbon source, as reported in Siberian permafrost (Rivkina et al., 2016).

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4.6. Main bacterial members affected by temperature. Our results of alpha-diversity corroborate the hypothesis that, in an Antarctic inoculum, the diversity decreases with increasing temperature, selecting only the microorganisms capable of growing at mesophilic temperatures. In addition, the temperature has also an effect on bacterial community composition and relative abundances, especially on taxa known to be involved directly or indirectly in methane-cycling, as shown below.

The most abundant bacterial phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes* exhibited different patterns in response to temperature. While *Proteobacteria* remained constant whatever the temperature (mean relative abundance of 27.1 ± 5.3% of total sequences), the relative abundance of the phylum Bacteroidetes (that represented between 61.03% at 5 °C and 35.62% at 37 °C of total sequences) decreased with increasing temperature. Among this phylum, several genera followed this pattern. *Parabacteroides* (constituting 19.9% at 5 °C and 0.5% of total sequences at 37 °C) and *Anaerophaga* (representing 8.3% at 5 °C and 0.1% of total sequences at 37 °C) produce volatile fatty acids, such as acetate and succinate (Denger et al., 2002; Sakamoto and Benno, 2006). Also, *Flavobacterium* (representing between 5.4% at 5 °C and 0.8% of total sequences at 37 °C) are facultative anaerobes able to ferment polysaccharides, as evidenced in permafrost (Zhang et al., 2007). This genus is widely studied in cold environments and is present in Antarctic lakes (Humphry et al., 2001). The negative impact of increasing temperature on these bacteria suggests that, in the studied environment, they might be adapted and specific to low temperatures. In contrast, the abundance of genus *Bacteroides* increased with temperature above 30 °C (constituting 1.8% at 5 °C and 26.8% of total sequences at 37 °C). This genus is recognized to be anaerobic and fermentative of sugar and peptone, and was reported in Alaskan tundra for example (Lipson et al., 2013). It seems that this genus can tolerate low temperatures, but its optimal temperature would be closer to the mesophilic range. Finally, the relative abundance of

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the phylum *Firmicutes* increased with temperature (Figure 3). A representative genus is *Clostridiisalibacter*, which never exceeded 0.04% of total sequences from 5 to 25 °C and represented up to 12% at 37 °C. This genus is recognized as a moderate halophilic, thermotolerant, chemoorganotrophic anaerobe (Liebgott et al., 2008). A second representative genus is *Thermaerobacter* which is present in the range from 5-30 °C in average relative abundance of 0.05% ± 0.02% of total sequences, while at 37 °C it represented 4.70% of total sequences. *Thermaerobacter* has characteristics of aerobic thermophile, neutrophil heterotroph, capable of using organic substrates (Takai et al., 1999).

5. Conclusion

Under psychrophilic conditions, the newly generated Antarctic inoculum successfully maintained relatively high CH_4 production rate at low temperature, more efficiently than a mesophilic sludge, and had higher specific methanogenic activity than a mesophilic sludge. Temperature affected the CH_4 generation as well as methanogenic and total bacterial and archaeal community. The microbial community was similar between 5 and 25 °C, but the diversity decreased above 25 °C, and our data suggest a shift of methanogenic pathway from acetoclasty to SAO-coupled hydrogenotrophy. The generated Antarctic inoculum has a biotechnological potential and could be considered as a future solution, after further enrichment and immobilization, for the treatment of wastes at low temperatures in cold mid- and high-latitude regions of the planet.

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Author contributions

Polette Aguilar-Muñoz: Conceptualization, Methodology, Investigation, Writing; **Céline Lavergne:** Investigation, Supervision, Writing, review & editing; **Rolando Chamy:** Resources; **Léa Cabrol:** Conceptualization, Methodology, Investigation, Supervision, Writing, review & editing

Conflict of interest

Authors declare no conflict of interest

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Table 1: Kinetic parameters of the Antarctic inoculum obtained from incubations along a temperature gradient. MPR stands for methane (CH_4) production rate. R1 and R2 indicate the batch replicates.

Temperature (°C)	MPR (nmol CH_4 g^{-1} DW d^{-1})		MPR (mL CH_4 g^{-1} VS d^{-1})		Yield (mmol CH_4 g^{-1} COD)		Latency period (days)	
	R1	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
5	4275	4314	2.7	2.7	8.6	8.2	37	37
10	4391	4005	2.8	2.5	8.5	7.9	24	24
15	5720	5658	3.6	3.6	8.4	8.3	17	17
20	6262	6553	3.9	4.1	8.2	8.3	10	10
25	8891	9218	5.6	5.8	10.9	11.4	10	10
30	8540	8185	5.4	5.2	11.4	10.6	10	10
37	6489	6343	4.1	4.0	10.7	10.9	22	22

Table 2: Published data reporting CH_4 production rates (MPR) in different soil/sediment samples from low temperature ecosystems.

Latitude	Origin	Sample type	Acetate amendment concentration (mM)	Incubation temperature (°C)	MPR (nmol CH_4 d^{-1} g^{-1} DW)	Reference
Northern hemisphere						
72° N	**Siberia**	Peat	20	0.4 - 7.5	170 - 1 560	(Wagner et al., 2005)
72° N	**Siberia**	Permafrost	10	5	1 - 120	(Wagner et al., 2007)
71° N	**Alaska**	Lake sediments	2	4 - 10	3 400 - 4 700	(de Jong et al., 2018)
68° N	**Alaska**	Lake sediments	1	10	1 000 - 9 000	(Lofton et al., 2015)
50° N	**Germany**	Peat swamp	5	20	8	(Horn et al., 2003)
46° N	**Swiss Alps**	Lake sediments	5-10	6 - 30	370 - 2 940	(Nozhevnikova et al., 1997)
37° N	**Tibetan Plateau**	Swamp wetland	10	10	60.9 - 552.7	(Yang et al., 2017)
Southern hemisphere						
53-54° S	**Sub-Antarctic**	Lake and reservoir sediments	30	5 - 20	590 - 50 280	(Lavergne et al., 2021)
62-63° S	**Antarctica**	Soil and sediments	30	5 - 25	4 295 - 9 055	This study
69° S	**Antarctica**	Subglacial sediments	10	1 - 12	0.04 - 0.2	(Ma et al., 2018)

Table 3: Bibliographic survey of methanogenic activity of low temperature anaerobic digesters (LTAD), obtained in batch tests with acetate amendment. UASB= Upflow Anaerobic Sludge Bed; EGSB-AF = Expanded Granular Sludge Bed- Attached Film; EGSB = Expanded Granular Sludge Bed; UASB-GAC = Upflow Anaerobic Sludge Bed- Granular Activated Carbon; AnMBR = Anaerobic Membrane Bioreactor. Seed = unadapted inoculum, initial time in reactor, Adapted = inoculum with previous adaptation period inside the reactor at low temperature.

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			Operation characteristics of the reactor		Batch Tests			
Reactor type	**Waste type**	**Sample type**	**Operating period (days)**	**Operating temperature (°C)**	**Acetate amendment concentration (mM)**	**Temperature for MPR (°C)***	**MPR (mL CH_4 g^{-1} VS d^{-1})**	**Reference**
Batch	Acetate	Soil and Sediment	10-37	5-25	30	5-25	2.71 – 5.71	This study
UASB	Sewage	Adapted	178	13	33.3	13	28.0	(Uemura and Haradа, 2000)
UASB	Synthetic Waste water	Seed	0	37	30	22	0.9	(McHugh et al., 2004)
		Adapted	165 - 300	16 - 25	30	15 - 22	14.7 -31.0	
EGSB-AF	Synthetic waste water	Seed	0	20	30	15	24.8	(Collins et al., 2006)
		Adapted	111 - 250	18-20	30	15	117.0 - 516.0	
UASB	Sewage	Adapted	236	9.7-27.1	33.3	10	1.8	(Sumino et al., 2007)
UASB	Synthetic waste water	Seed	0	37	12	15	36.0	(Akila and Chandra, 2007)
		Adapted	630	15	12	15	134.4	
HUSB/ UASB	Domestic waste water	Seed	0	NR	16.7	20	9.1 – 17.5	(Álvarez et al., 2008)
		Adapted	76 - 97	13.1 - 17	16.7	13.1 - 17	8.8 – 18.9	
EGSB–AF	Synthetic waste water	Seed	0	37	30	15	31.0	(McKeown et al., 2009b)
		Adapted	673 - 1243	4 - 9.5	30	4 - 15	17.0 – 83.0	
EGSB-AF	Synthetic waste water	Adapted	218 - 313	9 - 12	30	15	145.0 - 342.0	(Enright et al., 2009)
EGSB	Synthetic waste water	Seed	0	NR	30	15	0.0	(O'Reilly et al., 2009)
		Adapted	66 - 194	15 - 37	30	15	11.0 – 44.0	
EGSB-AF	Synthetic waste water	Seed	0	30	30	15	21.5	(Madden et al., 2010)
		Adapted	202 - 361	15	30	15	12.9 - 66.5	
AnMBR	Synthetic waste water	Adapted	15 - 75	15 - 25	10	15 - 25	7.0 – 66.5	(Ho and Sung, 2010)
EGSB	Waste water	Adapted	355	10	30	10	38.0	(Bialek et al., 2013)
Hybrid sludge bed/fixed-film	Synthetic sewage	Seed	0	35	30	12	7.0	(Keating et al., 2018)
		Adapted	105 - 889	12	30	12	0 - 51.0	

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Reactor								
UASB-EGSB	Synthetic waste water	Seed	0	37	30	15	4.3	(McAteer et al., 2020)
		Adapted	110 - 443	15 - 37	30	15	16.6 – 65.8	
UASB/ UASB-GAC	Municipal waste water	Adapted	30 - 120	20	16.7	20	13.8 – 93.9	(Zhang et al., 2020)

* For the MPR determination, a subsample from the reactor is incubated in sealed vials. Temperature for the MPR determination sometimes vary from the operating temperature of the reactors.

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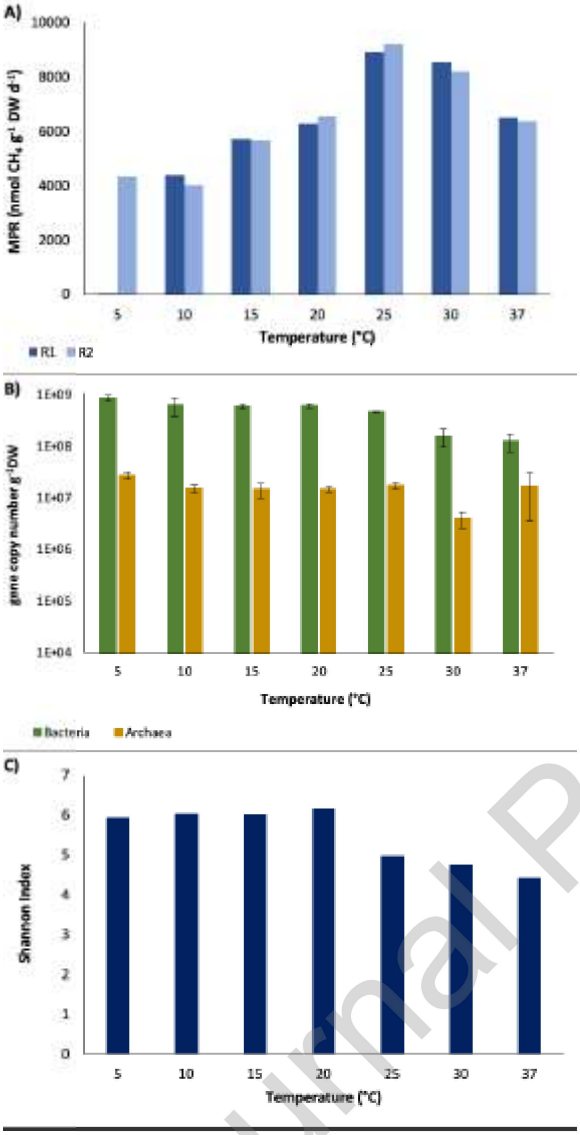

Figure 1: A) Methane production rate (MPR) of Antarctic inoculum, R1 and R2 indicate the batch replicates; B) Absolute abundance of bacteria and archaea from the Antarctic inoculum incubated at different temperatures (Bacteria= bacterial 16S rRNA gene, Archaea= archaeal 16S rRNA gene); C) Shannon index of Antarctic inoculum. In the panel B), errors bars represented the standard deviation (n = 4).

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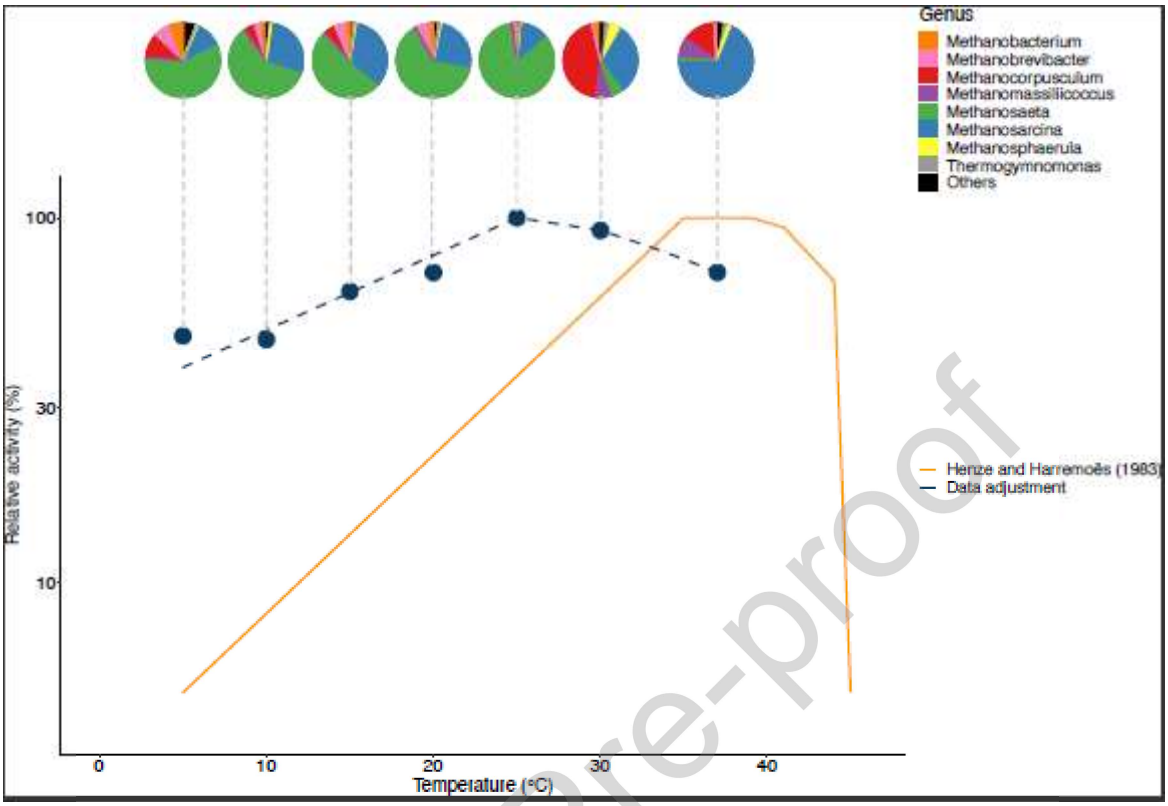

Figure 2: Relative MPR (expressed as a percentage of maximal MPR) in function of temperature for the Antarctic inoculum (in blue) and 32 conventional mesophilic reactors (in orange; Henze and Harremoës, 1983). Note that the y-axis represented the log-transformed percentage of maximal MPR. The pie charts represented the methanogen composition in the Antarctic inoculum at the genus level.

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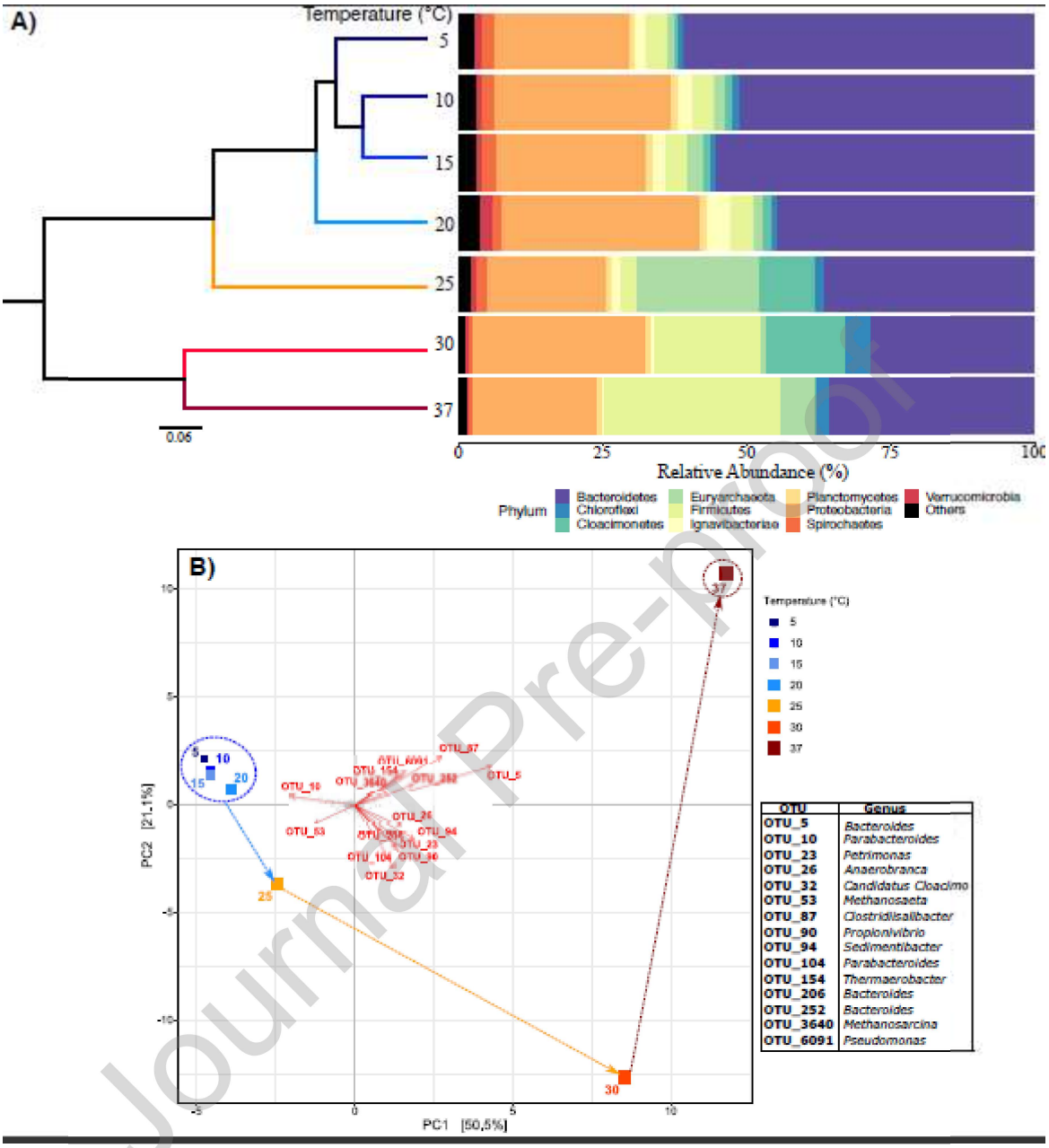

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Figure 3: Microbial community structure of Antarctic inoculum incubated at different temperatures. A) Taxonomic composition at the phylum level (ten most abundant phyla). The clustering is based on Bray Curtis distance and calculated with the UPGMA method. B) Principal component analysis (PCA) based on Bray Curtis distance from 16S rRNA gene diversity of the Antarctic inoculum incubated at different temperatures. The 15 most discriminant OTUs are

plotted in red (identified as described in the Material and Method section). Taxonomic affiliation of the discriminant OTUs is indicated in the table on the right.

CRediT authorship contribution statement

Polette Aguilar-Muñoz: Conceptualization, Methodology, Investigation, Writing; **Céline Lavergne:** Investigation, Supervision, Writing, review & editing; **Rolando Chamy:** Resources; **Léa Cabrol:** Conceptualization, Methodology, Investigation, Supervision, Writing, review & editing

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.

Highlights

- Antarctic sediments are suitable for low-organic load residue treatment in cold regions
- Methanogenic activity and microbial community are affected by temperature (5-37 °C)
- Maximum methane production rate at 25 °C
- *Methanosaeta* was the predominant archaea between 5 and 25 °C
- At low temperature Antarctic inoculum was 40% more efficient that mesophilic sludge

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