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Research Article

RESEARCH ARTICLE

Activity and diversity of methane-oxidizing bacteria along a Norwegian sub-Arctic glacier forefield

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One sentence summary: Methanotrophic activity and diversity in a sub-Arctic glacier forefield.

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ABSTRACT

Methane (CH_4) is one of the most abundant greenhouse gases in the atmosphere and identification of its sources and sinks is crucial for the reliability of climate model outputs. Although CH_4 production and consumption rates have been reported from a broad spectrum of environments, data obtained from glacier forefields are restricted to a few locations. We report the activities of methanotrophic communities and their diversity along a chronosequence in front of a sub-Arctic glacier using high-throughput sequencing and gas flux measurements. CH_4 oxidation rates were measured in the field throughout the growing season during three sampling times at eight different sampling points in combination with laboratory incubation experiments. The overall results showed that the methanotrophic community had similar trends of increased CH_4 consumption and increased abundance as a function of soil development and time of year. Sequencing results revealed that the methanotrophic community was dominated by a few OTUs and that a short-term increase in CH_4 concentration, as performed in the field measurements, altered slightly the relative abundance of the OTUs.

Keywords: Methane; methane-oxidizing bacteria; glacier forefield; high-throughput sequencing; Styggedalsbreen

INTRODUCTION

Methane (CH_4) is one of the most abundant greenhouse gases in the atmosphere. Although its concentration in the atmosphere is considerably lower than that of CO_2, CH_4 represents one of the most powerful greenhouse gases. Its lifetime impact in the atmosphere is 25 times greater than that of CO_2 due to having a higher capacity to absorb infrared radiation and a residence time in the atmosphere of 10 years (Forster *et al.* 2007). The actual global CH_4 budget is estimated at 500–600 terragram CH_4 per year (Wang *et al.* 2004; Conrad 2009) with an annual increase of 4–5 ppb per year (IPCC 2013).

CH_4 can be consumed and converted into CO_2 by aerobic methanotrophs. These methane-oxidizing bacteria (MOB) use CH_4 as the sole source of carbon and energy (Hanson and Hanson 1996; Bourne, McDonald and Murrell 2001) and represents the largest biological sink for atmospheric CH_4 in soils (Pratscher *et al.* 2018). Aerobic methanotrophs oxidize CH_4 into CO_2 by a specific metabolism. CH_4 is first converted into methanol by the enzyme methane monooxygensase (MMO). There are two types of MMO enzymes: (i) the membrane-bound particulate

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MMO that can be found in almost all methanotrophs, except in the genera *Methylocella* and *Methyloferula* (Dedysh and Dunfield 2010; Vorobev *et al.* 2011); and (ii) the soluble cytoplasmatic MMO, which is only present in some methanotrophs (Kolb *et al.* 2003). MMOs differ by their affinity towards CH_4 resulting in high- and low-affinity methanotrophs. High-affinity methanotrophs can oxidize CH_4 at low concentrations, e.g. atmospheric concentrations of about 2 ppm. Low-affinity methanotrophs are adapted to environments with high internal CH_4 concentrations, such as the active layer of permafrost or lacustrine sediments (Liebner and Wagner 2007). Recently, aerobic methanotrophs have been classified within the classes *Alphaproteobacteria* (Type II) and *Gammaproteobacteria* (Type I) in the phylum *Proteobacteria*, as well as in the phylum *Verrucomicrobia* (Type III) (Knief 2015).

Glacier forefields are ideal environments for studying the biochemical cycle of CH_4 along environmental gradients. They represent a natural chronosequence regarding soil development from former subglacial sediments to well-defined soils with distinct soil horizons, and from soils containing mainly allochthonous organic matter to soil containing mainly autochthonous organic material. When glaciers recede, the deglaciated areas become exposed to the atmosphere, with dramatic consequences for the microbial communities that are present in the soils.

Only four studies have hitherto investigated CH_4 oxidation *in situ* in glacier forefields (Bárcena, Yde and Finster 2010; Nauer *et al.* 2012; Chiri *et al.* 2015; Chiri *et al.* 2017), and two studies reported data from laboratory incubations (Bárcena, Finster and Yde 2011; Hofmann, Reitschuler and Illmer 2013). These studies have either focused on determination of the potential CH_4 oxidation rates or on the CH_4 flux rates at a specific point in time, for logistical reasons. So far, only one study has determined the *in situ* temporal variability of CH_4 in a glacier forefield during the growing season (Chiri *et al.* 2017). However, none of those studies has focused on CH_4 oxidation in sub-Arctic glacier forefields.

In this study, samples were collected at eight different sites along the sub-Arctic forefield (total distance of approximately 1 km) of Styggedalsbreen (Norway) covering the entire growing season. We combined field measurements of CH_4 oxidation rates with laboratory incubations and determined the abundance and diversity of methanotrophs using qPCR and a high-throughput sequencing (HTS) approach. We tested the following hypotheses: (i) the *in situ* CH_4 oxidation rates are increasing with increasing distance from the glacier, which is equivalent to soil maturation; (ii) the CH_4 oxidation rates are higher at the moraine-sampling sites (low water content) than in the intermoraine areas (waterlogged); and (iii) the variation in CH_4 oxidation rates is associated with a variation in methanotrophic abundance and diversity. Anaerobic CH_4 oxidation, which has recently been shown to be widespread in surface soils (Gauthier *et al.* 2015) was not addressed in this study. If this process was present at our sampling sites, it would have been an integral part of the determined rates.

MATERIALS AND METHODS

Study area

Styggedalsbreen (61.5°N; 8.3°E) is a small valley glacier located in the Jotunheimen National Park (Norway). Covering a present area of approximately 2 km^2, Styggedalsbreen has receded approximately 800 m since the end of the Little Ice Age (LIA), which terminated in the 1750s. The mean glacier recession rate is 5 m $year^{-1}$ since 1900, when glacier length changes started

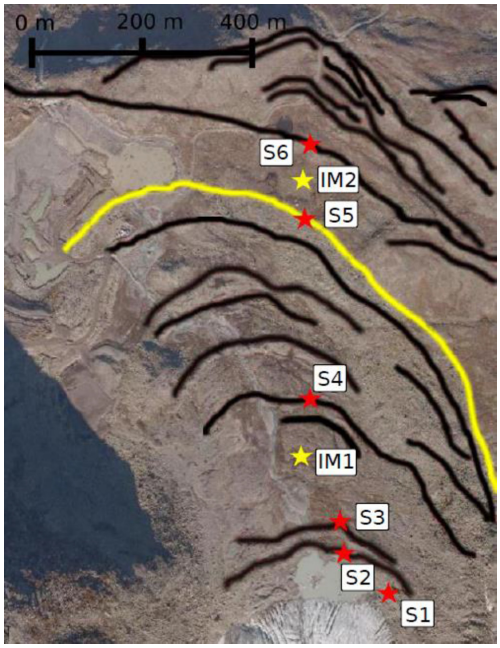

Figure 1. Aerial view of the Styggedalsbreen forefield. Moraines are marked with black color and LIA moraine is shown in yellow. Sampling sites are marked with red (moraine) or yellow (intermoraine) stars (Modified from Mateos-Rivera *et al.* 2016).

to be recorded by the Norwegian Water Resources and Energy Directorate. The glacier forefield is located at an elevation of 1295 m a.s.l. (meters above sea level) in a region with an alpine sub-Arctic climate, a growing season from June/July to September, an average annual precipitation of 860 mm and a local mean air temperature of −3.1°C (Matthews 2005). At the Styggedalsbreen forefield (Fig. 1), there are nine major moraine ridges formed after the LIA, together with numerous smaller moraine ridges (Mateos-Rivera *et al.* 2016). Higher vegetation cover and water content than at the top of the moraines characterize the intermoraine areas. The bedrock consists of pyroxene granulite gneiss with mylonite and peridotite inclusions (Matthews 2005).

Soil sampling

Soil samples were collected at eight different sampling points with increasing distance from the glacier along a chronosequence in the forefield (S1 to S6 and IM1 to IM2; Fig. 1), during the growing season of 2015 (July, August and September) at 3–4 week intervals. The locations were placed: (i) 5 m in front of the glacier front (S1); (ii) the youngest moraines (S2 and S3); (iii) intermoraine areas (IM1 and IM2); (iv) the highest post-LIA moraine (S4); (v) the LIA moraine (S5); and (vi) the moraine formed during the mid-Holocene and placed after the LIA moraine (S6). At each sampling site, the top 10 cm of soil from inside of the chamber and immediately outside the chamber were collected (a total of 16 samples in triplicate per sampling season) with a sterilized spoon and transferred into 15 mL polypropylene tubes. Samples were transported in a cooling box on ice and stored at −80°C until further analysis.

Soil characterization and temperature measurements

Approximately 1 g of the mixed and sieved soil from samples located at the intermoraine areas (i.e. IM1 and IM2) was weighed

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and dried overnight at 80°C for quantification of the Organic Carbon (OC) and the Total Nitrogen (TN) content using a CHN analyzer Flash 2000 EA (Thermo Scientific, USA). A detailed soil characterization of the moraine sampling sites has been reported previously (Mateos-Rivera *et al.* 2016).

Air temperatures during the sampling period were obtained from the meteorological station at Sognefjellhyta (https://www.yr.no/place/Norway/Oppland/Lom/Sognefjellhytta_observation_site/statistics.html), which is located at a similar elevation, 11.4 km away from Styggedalsbreen. Temperatures were confirmed in the field by simultaneous measurements during the sampling campaigns. Temperatures in chambers were measured by placing portable thermometers inside of the chambers that could be read from outside through the transparent Plexiglas walls.

Determination of CH_4 flux analyses in the field

The static chamber method was used to determine CH_4 fluxes (Smith *et al.* 2000). Fifteen polyvinylchloride cylindrical chambers (25 cm long and a diameter of 9.4 cm; Fig. S1, Supporting Information) were placed in the forefield according to the following pattern: (i) two chambers were placed in front of the glacier and at the intermoraine locations (sampling sites S1, IM1 and IM2); (ii) three chambers were placed at the youngest moraine and LIA moraine (sampling sites S2 and S5, respectively); (iii) one chamber was placed on the top of each of the other moraine sampling sites (i.e. sampling sites S3, S4 and S6). To determine the CH_4 oxidation rates, chambers in the field were inserted 10 cm into the soil reaching a final volume inside the chamber of ~1 L. Finally, sampling ports (9 mm in diameter) in each chamber were sealed with a rubber stopper to provide gas-tight conditions.

For each chamber, CH_4 was injected to obtain a chamber concentration of approximately 20 ppm. Gas samples (5 mL) from each chamber were collected in triplicate every 24 h for a period of 72 h and injected into a 5.9 mL evacuated Exetainer vials (Labco Ltd, Buckinghamshire, UK) containing 1 mL of 5M NaCl. After sample injection, the Exetainer vials were turned upside down to prevent gas leakage. In order to maintain pressure conditions during the experiment, 5 mL of ambient air was injected into the chambers after each sample acquisition and accounted for in the calculations.

Incubation experiments in the laboratory

Approximately 3.5 g soil (w/w) collected in triplicate in the field from each of the eight sampling sites was added into 120 mL serum flasks (Glasgerätebau Ochs, Germany) and each sealed with a butyl-rubber stopper and an aluminum cramp to maintain gas tight conditions during the experiment. Following the field procedure, each serum flask CH_4 was injected to a final concentration of 20 ppm. Flasks were then incubated at 5°C (the average temperature during the whole summer campaign) in the dark. As with the *in situ* measurements, CH_4 concentrations were measured every 24 h for 72 h.

Gas measurements

Analyses of the CH_4 concentrations from the incubation experiments were performed using an SRI 310C Gas Chromatograph (SRI, Torrance, California), with helium as the carrier gas, with a pressure of 6 psi and an oven temperature of 40°C. Determination of samples collected in the field were performed with an SRI 8610C Gas Chromatograph (SRI) using a FID detector and a 8600-PK1A silica-gel packed column, where hydrogen was used as the carrier gas, with a pressure of 8 psi and an oven temperature of 40°C. For the incubation experiments, 0.5 mL of the gas samples were injected into the GC, while for the field samples, 1 mL of the gas samples were injected. Both the incubation experiments and the field samples were analyzed using the Peak Simple 2000 software (SRI). Six standards were injected into the GC before sample measurements for system calibration. To estimate CH_4 oxidation rates, first-order kinetics were applied according to previous studies (Ishizuka *et al.* 2009; Bárcena, Yde and Finster 2010).

DNA isolation, PCR amplification and Illumina sequencing

DNA was extracted from 0.25 g of soil using the UltraClean Soil DNA Extraction Kit (Mo Bio, Carlsbad, CA, USA), as per the manufacturer's instructions. The extracted DNA was quantified using a QuBit Fluorometer (Invitrogen, USA). After DNA extraction, PCR amplification was performed following the steps described in Volpi *et al.* (2017), using the primer pair A189f/A682r (Table 1). Briefly, 0.125 μL Takara Taq DNA Polymerase (Takara Bio, Japan) were added to 2.5 μL 10x buffer, 2 μL dNTPs, 18.5 μL dH_2O, 0.5 μL primers (10 mM) and 1.4 μL template. The PCR reaction conditions were as follows: an initial denaturation of 15 min at 95°C, followed by 30 cycles of amplification (denaturation 10 s at 98°C, annealing at 56°C for 30 s and an extension of 1 min at 72°C), and a final extension of 5 min at 72°C. After the 1st PCR, 12.5 μL 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems, South Africa), 9 μL dH_2O and 0.5 μL primers (containing adaptors and Illumina barcodes) were added to 2.5 μL of the PCR-amplified product. The PCR reaction conditions were as follows: initial denaturation 3 min at 95°C followed by 10 cycles of amplification (denaturation 30 s at 95°C, annealing at 56°C for 30 s and extension for 30 s at 72°C), and a final extension of 5 min at 72°C. Non-specific amplification products in samples were removed using the GenElute Gel Extraction kit (Sigma Aldrich, USA), according to the manufacturer's instructions. Finally, samples were indexed with the Illumina Nextera XT Index kit and pooled in equimolar amounts. Paired-end sequencing was performed using the Illumina MiSeq v3 Reagent kit.

Quantitative PCR assays

The isolated DNA from soil samples was used to determine the abundance of (i) *Bacteria* and (ii) the methanotrophs in a Roche LightCycler 480 Real-Time PCR System (Roche, Switzerland). For bacterial quantification, 1 μL of each primer (Bac908F/Bac1075R; Table 1) was added to 10 μL SYBR Green I Master Mix (Roche, Switzerland), 2 μL BSA, 4 μL dsH_2O and 2 μL template DNA. The PCR conditions were, (i) initial denaturation for 5 min at 95°C; (ii) 45 cycles of amplification (denaturation for 30 s at 95°C, annealing 30 s at 60°C and elongation 15 s at 72°C) and (iii) read for 5 min at 80°C. For the methanotrophs, 1 μL of each primer (A189f/A682r; Table 1) was added to 10 μL SYBR Green I Master Mix (Roche), 2 μL BSA, 4 μL dsH_2O and 2 μL of the template. The PCR reaction conditions were (i) initial denaturation for 5 min at 95°C; (ii) 45 cycles of amplification (denaturation for 15 s at 95°C, annealing for 10 s at 60°C and elongation for 20 s at 72°C) and (iii) read for 20 s at 87°C. For both bacterial 16S rRNA genes and the methanotrophs, serial dilutions of a standard (eight in total) in triplicate were performed for each run. 16S rRNA gene and

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Table 1. List of primers used in this study.

Primer	Target	Sequence (5′-3′)	Annealing temp (°C)	Reference
A189f	*MOB*	GGNGACTGGGACTTCTGG	56	Holmes *et al.* (2001)
A682r	*MOB*	GAASGCNGAGAAGAASGC	56	Holmes *et al.* (2001)
908F	Bacterial *16S rRNA* gene	AACTCAAAKGAATTGACGGG	60	Modified from Ohkuma and Kudo (1998)
1075R	Bacterial *16S rRNA* gene	CACGAGCTGACGACARCC	60	Modified from Ohkuma and Kudo (1998)

methanotrophic samples were also run in triplicates. The efficiencies of the qPCR varied between 101% and 109% for the 16S rRNA gene and 81% to 90% for the methanotrophs with $R^2 > 0.99$ in each run. An additional qPCR assay with 10-fold and 100-fold dilutions was performed for the methanotrophs to evaluate the effect of inhibition by e.g. humic substance that can be co-extracted with DNA.

Statistical, multivariate and bioinformatic analyses

All sequences were first checked for chimeras using UCHIME (Edgar *et al.* 2011). HMM-FRAME (Zhang and Sun 2011) was used to detect frameshift errors. Finally, sequences with less than 300 bp were discarded, as reported previously (Lüke and Frenzel 2011; Dumont *et al.* 2014). Reads that passed these quality control steps were merged according to sampling site and growing season and processed using the BBTools software package (https://jgi.doe.gov/data-and-tools/bbtools/) and QIIME (Quantitative Insights into Microbial Ecology, v1.9.0; Caporaso *et al.* 2010) pipeline. OTUs were clustered at 93% sequence similarity, as a distance of 7% of the *pmoA* gene corresponds to a 3% distance for the 16S rRNA gene when discriminating at species level (Degelmann *et al.* 2010, Lüke and Frenzel 2011). Singletons were also removed. Raw data obtained from amplicon sequencing have been submitted to the Sequence Read Archive under the BioProject PRJNA438069.

Relationships between the CH_4 flux rates and CH_4 oxidation rates together with sampling site and time (July, August and September) were tested in a multi-way ANOVA using the statistical environment software R (R Core Team 2013). Additionally, relationships between the qPCR copy numbers from soils collected inside of the chambers after the incubations and CH_4 oxidation rates in the field along the transect were tested using correlation analyses. Data were logarithm- or square root-transformed when necessary.

The relationships between methanotrophic communities at the different sampling sites during the growing season were assessed by Non-Metric Multidimensional Scaling (NMDS) using the R package *vegan* (Oksanen *et al.* 2017) and the function *vegdist*, using the Bray-Curtis coefficient as a dissimilarity measure. Permutational multivariate analyses of variance (PerMANOVA) were used to test the differences in the methanotrophic communities with sampling site and growing season using the function *adonis* with Bray-Curtis distances and 999 permutations. Alpha-diversity of methanotrophic communities was also assessed using the package *vegan* (Oksanen *et al.* 2017) and the function *diversity*. The heatmap was created in R using the package *gplots* and the function *heatmap.2*.

RESULTS

Soil characterization and air temperatures

Data about the OC and TN measurements at the sampling sites located on the top of the moraines were reported in a recent study (Mateos-Rivera *et al.* 2016). The lowest OC and TN content values were measured at sampling site S1, with an OC content of 0.4 mg g^{-1} and a TN content below the detection limit of our method. An increase in the OC and TN content was measured at sampling site S6 (the outermost moraine), with an OC content of 53.1 mg g^{-1} and TN content of 2.98 mg g^{-1} (Mateos-Rivera *et al.* 2016).

The intermoraine sampling sites represents areas with different characteristics than the top of the moraines. Therefore, in this sampling campaign additional data on the intermoraine areas were included. The OC and TN measurements at two intermoraine sampling sites (IM1 and IM2) revealed higher concentrations of both than at the neighboring moraines. The content of OC and TN at sampling site IM1 was 4.62 mg g^{-1} and 0.4 mg g^{-1}, respectively, whereas at sampling site IM2 the OC and TN contents were 77.71 mg g^{-1} and 12.81 mg g^{-1}, respectively (Table S1, Supporting Information).

Average air temperature on the sampling days increased during the growing season (Fig. S2, Supporting Information). The lowest average air temperature was measured in July (6.3°C) and the highest air temperature was measured in September (10.9°C). The same trend in temperatures was measured inside of the flux chambers (Table S2, Supporting Information). Temperatures inside the flux chambers were always higher than the temperatures measured in parallel outside the chambers. Additionally, during the three sampling campaigns, air temperatures increased 5°C (July) and 2°C (August and September) with distance from the glacier between the sampling sites closest to and furthest from the chronosequence.

Determination of CH_4 flux rates at the glacier forefield

Measurements of CH_4 flux rates in samples collected inside the chambers along the forefield showed two general trends (Table 2). Firstly, there was a significant increase in the CH_4 flux rates with increasing distance from the glacier, as a function of soil exposure (from S1 to S6) (ANOVA, $P < 0.005$). CH_4 flux rates varied from −504.6 μmol CH_4 m^{-2} d^{-1} to −1178.9 μmol CH_4 m^{-2} d^{-1} (Table 2). Secondly, CH_4 flux rates increased as a function of season, although this trend was not observed at all sites. In general, CH_4 flux rates measured in July were lower than flux rates measured in August and September (Table 2). Flux rates measured at intermoraine, waterlogged sampling sites (i.e. IM1 and IM2) showed, generally, lower rates than the neighboring moraines throughout the growing season (Table 2).

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Table 2. CH_4 oxidation rates for *in-situ* samples at the end of the chamber incubation.

Samples	μmol CH_4 m^{-2} d^{-1}		
	July	August	September
S1	−504.6 (30.4)	−704.1 (62.5)	−864.7 (38.2)
S2	−600.9 (73.0)	−551.7 (43.3)	−873.8 (40.2)
S3	−824.8 (21.6)	−798.6 (38.2)	−1054.8 (63.8)
IM1	−904.9 (67.1)	−1004.0 (32.3)	−903.9 (58.1)
S4	−841.6 (47.8)	−748.1 (66.7)	−1036.7 (64.0)
S5	−817.7 (86.9)	−980.0 (48.7)	−886.7 (95.7)
IM2	−718.2 (53.8)	−846.3 (60.9)	−595.3 (83.3)
S6	−907.7 (89.7)	−1178.9 (73.6)	−1064.8 (68.9)

Potential CH_4 oxidation rates from laboratory incubation experiments

Potential CH_4 oxidation rates observed from the laboratory incubations also showed a significant increase with soil development (ANOVA, $P < 0.005$). The highest potential CH_4 oxidation rates were measured in soil samples collected on top of the two outermost moraines (S5 and S6), while the lowest CH_4 oxidation rates were determined in samples collected close to the glacier front and at the intermoraine sites, characterized by higher water content (Fig. 2). We did observe a non-significant increase in the potential CH_4 oxidation rates as a function of seasonality (ANOVA, $P = 0.77$; Fig. 2). The CH_4 uptake rates determined in the laboratory incubations and the qPCR based copy numbers were used to estimate the potential CH_4 oxidation rate per cell at the different sampling sites. The highest per cell rates were measured on top of the two outermost moraines, whereas the intermoraine sampling sites showed the lowest per cell rates. The values ranged from 2.71×10^{-12} mol $cell^{-1}$ h^{-1} to 3.18×10^{-15} mol $cell^{-1}$ h^{-1} (Table S3, Supporting Information).

In addition to the potential CH_4 oxidation rates, we performed an experiment to determine the potential CH_4 production rates under anaerobic and water-saturated conditions. After two months of incubation, only a minor production was observed, in fact, atmospheric CH_4 concentrations were only reached in incubations from soil collected at sampling sites that were situated close to the glacier front or in the waterlogged intermoraine areas (data not shown).

qPCR based numbers of Bacteria and the methanotrophs

The results showed a 10- to 100-fold increase in abundance with soil development for both *Bacteria* and the methanotrophs, with the lowest numbers found in samples collected near the glacier front (Table 3). For *Bacteria*, the results ranged from 1.22×10^5 copies (g $soil^{-1}$ w.w.) at sampling site S1, up to 1.37×10^7 copies (g $soil^{-1}$ w.w.) at site S6 (July; Table 3). The qPCR based number of the methanotrophs were lower than the bacterial 16S rRNA gene copies at the same sites, ranging from 1.32×10^2 copies (g $soil^{-1}$ w.w.) at site S1 to 1.25×10^4 copies (g $soil^{-1}$ w.w.) at site S6 (July; Table 3). Furthermore, there was an increase in abundance with time of the year during the growing season for both *Bacteria* and the methanotrophs, with the highest copy numbers found in samples collected in September. The methanotrophic fraction determined in soil samples collected inside the flux chambers (Table S4, Supporting Information) accounted for 0.22% of the total number of bacteria, while the methanotrophic fraction in soils collected outside the chambers accounted for 0.13% of the total number of bacteria. The correlation between CH_4 flux rates and methanotrophic abundance along the forefield increased significantly after removal of the samples from the intermoraine areas (from $R^2 = -0.398$; $P = 0.053$ to $R^2 = -0.561$, $P = 0.01$).

Methanotrophic diversity

In total, 3 085 153 reads were classified into 293 operational taxonomic units (OTUs) after filtering of 35% of the original reads. Twelve OTUs (MOB_OTU1 to MOB_OTU12; Fig. 3) were represented by more than 40 000 reads each, and together they comprised greater than 80% of the total reads. The OTUs were site-specific and none of them was found to be present at all sampling sites along the transect; OTUs present at sampling sites close to the glacier front (such as MOB_OTU1, MOB_OTU9 or MOB_OTU10) were absent at more remote sites that had been exposed for longer time, while OTUs such as MOB_OTU7 and MOB_OTU8 showed a contrasting distribution (Fig. 3).

The methanotrophic community analyses results were compiled and visualized in an NMDS plot, where two different clusters were observed (Fig. 4). The cluster encompassing sampling sites close to the glacier front (S1–S3) was dominated by MOB_OTU7, accounting for ∼22% of all reads, followed by MOB_OTU8, which accounted for 2% of the total reads. MOB_OTU7 was closely related to the uncultured methanotroph Rold 1 (accession number AF148523), which belongs to the USC-alpha clade within the type IIb methanotrophs, while MOB_OTU8 only shared 68% sequence identity with GenBank sequences (Fig. 5). In the cluster made up of the other sampling sites (S4–S6, IM1 and IM2), MOB_OTU1 was the most abundant, accounting for ∼24% of all reads, followed by MOB_OTU2, with ∼13% of all reads and MOB_OTU4 (5% of all reads) (Fig. 3). MOB_OTU1 and MOB_OTU2 were closely related to each other and to methanotrophs isolated from Arctic soils (Pacheco-Oliver *et al.* 2002). MOB_OTU4 and MOB_OTU10 refer to ammonia oxidizers closely related to *Nitrosospira sp.* (accession number AJ298703) (Fig. 5). Ammonia oxidizer-related OTU represented less than 1% of the OTU along the sampled forefield site, with the exception of sites S4, S7 and S8, where ammonium oxidizer-related OTU accounted for 1%–2%, 16% and 18% of all OTU, respectively.

Alpha-diversity was consistently higher in samples collected outside of the chambers (OUT) compared to samples collected inside the chambers (IN) at each sampling site (Table S5, Supporting Information). Although no trend was found with soil development, the lowest values of the Chao 1 estimator were calculated at the youngest sampling site (S1), whereas the highest values were found in samples collected at the outermost moraine (S6). Shannon and Simpson indices revealed the same

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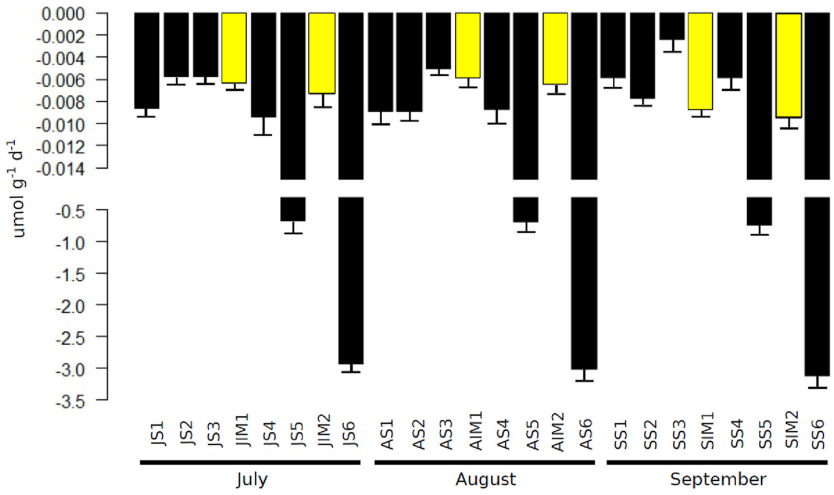

Figure 2. CH_4 oxidation rates for samples incubated in the laboratory after an incubation period of 72 h. Yellow bars indicate samples collected in an intermoraine sampling site.

Table 3. qPCR of the 16S rRNA gene and methanotrophic abundance data from soils collected outside the chamber at the different sampling sites along the growing season. Standard deviations are shown within brackets.

	16S rRNA gene (10^5) g soil^{-1}			Methanotrophs (10^2) g soil^{-1}		
Samples	July	August	September	July	August	September
S1	1.22 (0.42)	1.55 (0.74)	23.99 (13.40)	1.32 (0.31)	9.54 (2.82)	25.86 (9.36)
S2	1.55 (0.95)	13.51 (3.99)	15.31 (0.29)	1.41 (0.92)	13.49 (8.75)	11.03 (0.48)
S3	12.03 (4.44)	31.86 (24.28)	36.76 (6.21)	1.97 (0.43)	18.21(4.44)	29.79 (0.63)
IM1	27.50 (11.16)	38.89 (28.52)	97.08 (38.39)	3.60 (1.25)	60.27 (31.20)	229.33 (62.59)
S4	5.30 (2.13)	4.75 (0.29)	12.89 (6.90)	1.08 (0.61)	1.59 (0.25)	6.17 (2.75)
S5	47.84 (21.23)	85.28 (58.87)	65.70 (32.69)	147.06 (56.63)	21.37 (0.38)	87.53 (9.37)
IM2	90.49 (32.85)	95.60 (8.61)	107.67 (25.72)	48.93 (14.10)	62.37 (2.88)	122.28 (58.46)
S6	137.07 (30.57)	35.00 (4.34)	141.15 (23.01)	125.60 (46.23)	148.51 (56.11)	194.02 (75.80)

trends as the Chao 1 estimator (Table S5, Supporting Information). Higher Shannon and Simpson indices were obtained in samples collected outside of the flux chambers at each sampling site. No significant trend was observed with soil age, although the highest indices were found in samples collected at the oldest sites (S6) and the lowest indices were determined with samples collected at the youngest site near the glacier front (S1).

The methanotrophic communities determined from samples collected both outside and inside of the flux chambers differed significantly with soil development throughout the entire growing season (PerMANOVA, $P < 0.001$; Fig. 3). However, non-significant differences in the methanotrophic communities were observed as a function time of year (July, August and September) in samples collected inside of the flux chambers (Fig. 3), except between the methanotrophic communities in July and September from soil sampled outside of the flux chambers (PerMANOVA, $P = 0.041$; Fig. 3).

DISCUSSION

CH_4 flux and potential oxidation rates

We performed two different types of studies to get comprehensive insights into CH_4 metabolism at Styggedalsbreen forefield: (i) field studies to determine the CH_4 flux rates *in situ* using flux chambers and (ii) laboratory studies to determine the CH_4 oxidation rates under controlled conditions. In addition, we determined both abundance and diversity of the methanotrophic communities, using qPCR-based and HTS approaches.

Despite the overall differences between field and laboratory measurements due to temperature fluctuations, light, moisture and percentage of plant cover observed in the field, both studies revealed the same trends. There was an increase in the CH_4 consumption rates with increasing distance from the glacier throughout the entire growing season. The differences based on distance (and therefore time of deglaciation) from the glacier margin were higher than across the growing season. We also observed reduced CH_4 oxidation rates at the intermoraine sampling sites compared to the closest moraine tops, highlighting the importance of the physical difference between sampling sites on short spatial scales, as the intermoraine areas are waterlogged and oxygen is less accessible.

The increase in CH_4 flux rates in the field with soil maturation has also been observed along glacier forefields from Greenland (Bárcena, Yde and Finster 2010) and the Alps (Chiri *et al.* 2015; Chiri *et al.* 2017), and can be related to the improved access to CH_4 and oxygen because of drier soil conditions with increasing distance from the glacier. Bárcena, Yde and Finster (2010) reported CH_4 flux rates up to -0.047 μmol CH_4 m^{-2} d^{-1} at the LIA moraine, while Chiri *et al.* (2015, 2017) reported CH_4 flux rates up to -137.15 and -230.67 μmol CH_4 m^{-2} d^{-1} along a chronosequence covering the last 120 years. Although Bárcena, Yde and

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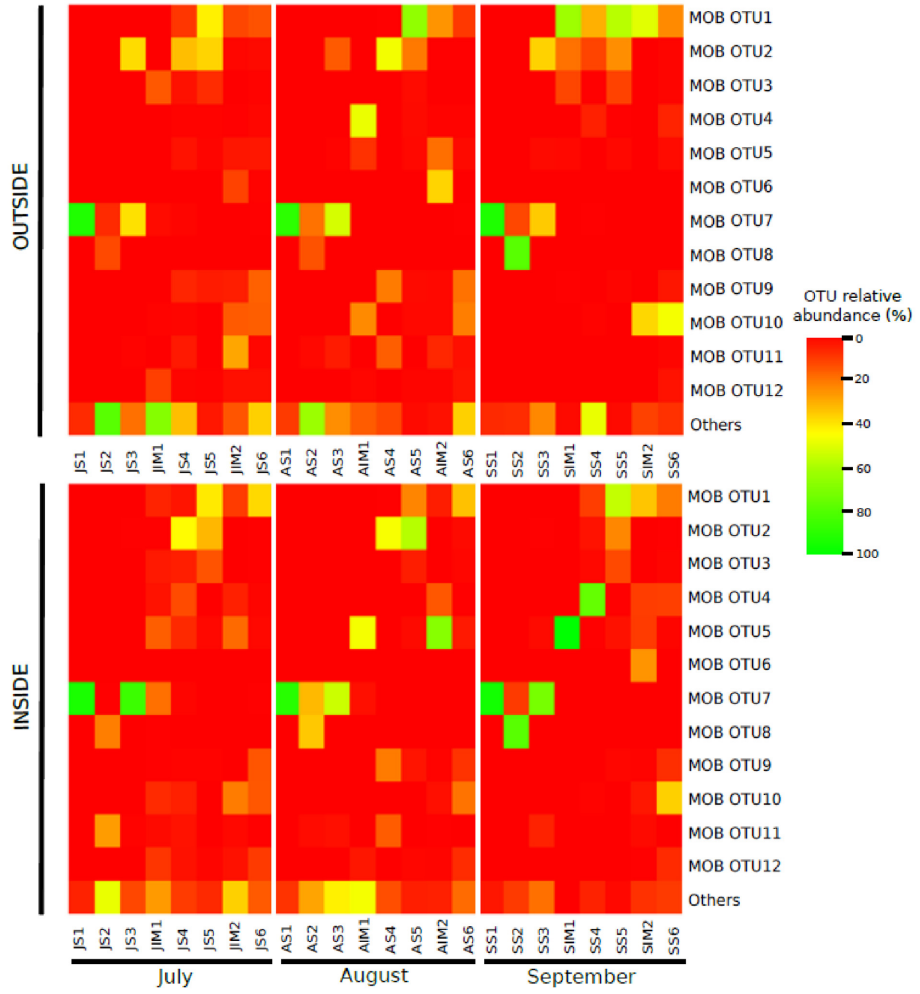

Figure 3. Heatmap showing the percentage relative abundance of the most abundant operational taxonomic units (OTUs) retrieved by targeting the MOB. OTUs retrieved from samples collected outside of the chambers are shown in the upper section and OTUs retrieved from samples collected inside of the chambers are shown in the lower section.

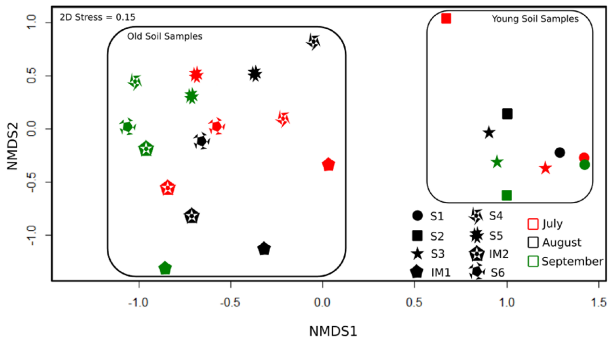

Figure 4. NMDS showing the relationship between the methanotrophic communities at the different sampling sites (characters) during the growing season (colors) in samples collected outside the chambers.

Finster (2010) used the same chambers as in this study, it has to be noted that different chamber types may introduce a bias that only a standardization of chamber size can account for. Therefore, as long as standardized chamber techniques have not been agreed upon the values of methane oxidation rates that are obtained in the different studies only represent relative values that are useful for intracomparison but should be considered with care when used for intercomparison between studies. Furthermore, the different maturation states of the soil cover need to be considered when comparing studies. At the Mittivakkat Gletscher forefield in southeast Greenland, for example, plant cover is very sparse and proglacial soils are less developed than in sub-Arctic regions and in the Alps due to the harsh climate of southeast Greenland. In the present study, we increased the initial concentrations of methane following other studies (Roslev and Iversen 1999; Knief, Lipski and Dunfield 2003; Crossman, Ineson and Evershed 2005), but thereby we also increased the potential for CH_4 oxidation due to the increased availability of the substrate. Therefore, direct comparisons with the studies by Bárcena, Yde and Finster (2010) and Chiri *et al.* (2015, 2017) cannot be established as these studies used atmospheric concentrations of CH_4 as the starting concentration.

In laboratory samples, environmental parameters such as temperature, moisture and light were controlled. Following the procedure described by Bárcena, Finster and Yde (2011), we mixed the top 10 cm to homogenize the soil and removed plants and roots to normalize the amounts of soil in samples. Although the potential for CH_4 oxidation decreases with depth, the top 10 cm soil covers the interval where most of the CH_4 oxidation activity is located (Jørgensen *et al.* 2015). Bárcena, Finster and Yde (2011) reported a CH_4 oxidation rate of 0.00124 μmol g^{-1} d^{-1}

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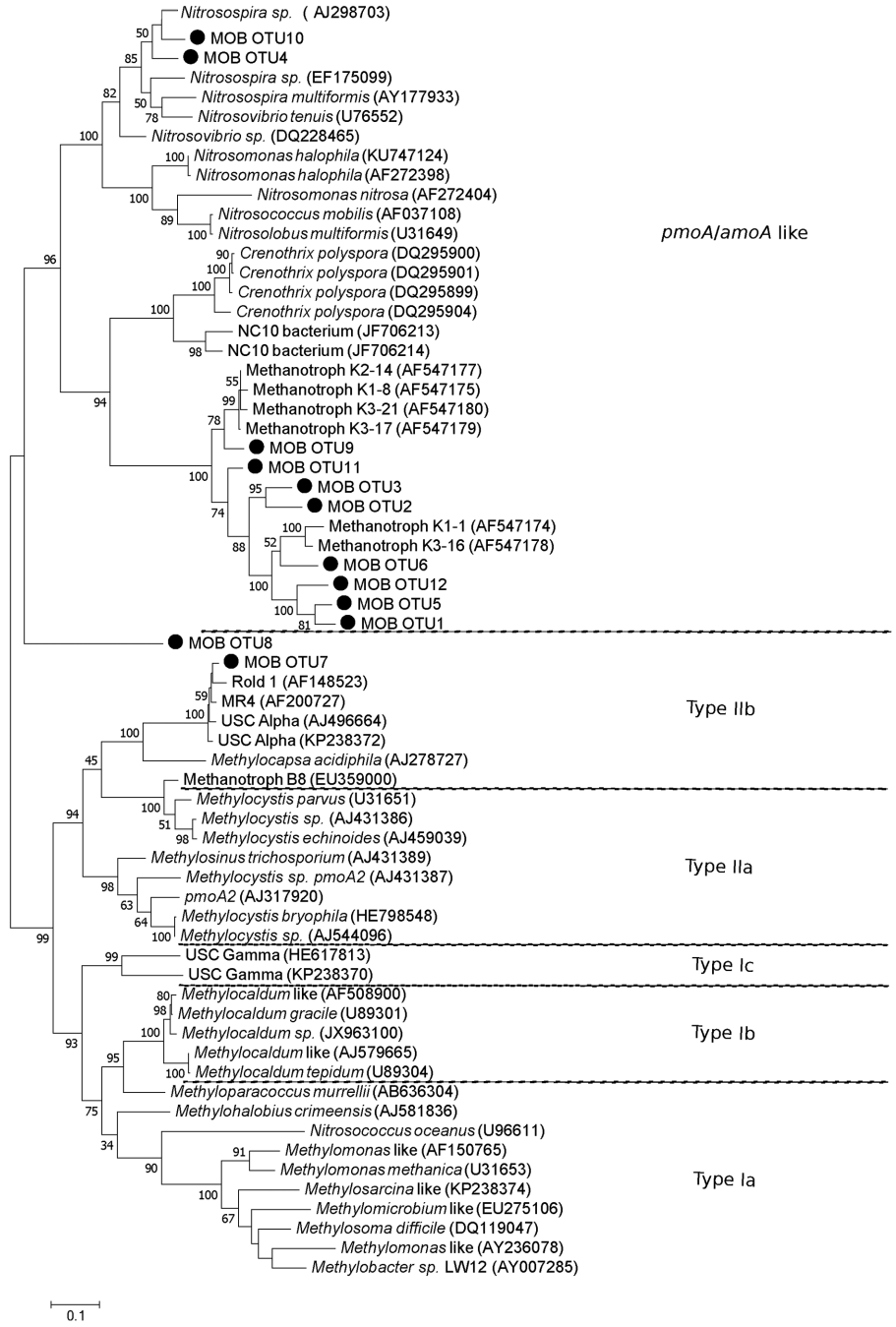

Figure 5. Phylogenetic tree based on the MOB of the 12 most abundant sequences (black dots). The phylogenetic tree was constructed using MEGA 7 (Kumar *et al.* 2016) using the MOB sequences obtained in the present study, together with sequences downloaded from GenBank. Sequences were aligned with MUSCLE (Edgar 2004) in MEGA 7 and the tree was constructed via the Maximum likelihood method based on the Jukes–Cantor model and 100 bootstraps.

only in the very last sample of the transect at the LIA moraine. This rate is in the same order of magnitude as the rate determined at the less-developed soils in our study, but two orders of magnitude lower than the rates obtained at the LIA moraine. This highlights once more the different characteristics of both glacier forefields. The CH_4 oxidation rate per cell obtained in our study was calculated assuming that methanotrophic cells contain two copies of the *pmoA* gene, as previously proposed by Degelmann *et al.* (2010). We found an approximate 20% inhibition of amplification in the undiluted DNA extracts compared to the diluted samples. Taking the inhibition into account, the lowest per cell CH_4 oxidation rates of our study are two orders in magnitude higher than the values reported by Degelmann *et al.* (2010) for atmospheric CH_4 concentrations. However, our values were only twice as high as the per cells CH_4 oxidation rates reported by Kolb *et al.* (2005), where high-affinity methanotrophs such as Cluster I and the USC-alpha clade, which accounted for the largest fraction of sequences found at our study sites, were targeted. The higher per cell CH_4 oxidation rates observed at the outermost moraine (i.e. S6) could be due to: (i) insufficient coverage by the used primer set, for example not targeting anaerobic

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methanotrophs and/or, (ii) the different measurement methods as suggested previously by Kolb *et al.* (2005).

In the field, besides the effect of temperatures on CH_4 flux, which has been observed to enhance CH_4 oxidation in other forefields (Bárcena, Finster and Yde 2011), other characteristics such as soil type and soil depth have also been shown to be important drivers of the activity of the methanotrophic community (Martineau *et al.* 2014). These physical differences are evident when it comes to explaining the different rates measured at the intermoraine sites and sites located on the top of moraines. At the intermoraine sites, the soil OC and TN contents were higher than at sampling sites located on top of the moraines and vegetation was more abundant. The accumulation of OC at the intermoraine sites may result from two main sources: a) a higher primary productivity, and/or b) a reduced degradation of OC due to reduced oxygen availability. Oxygen stimulates organic matter degradation and is required for the degradation of structural plant tissues with high lignin content. Furthermore, the higher water content at the intermoraine sites and the presence of more abundant vegetation can also alter methanotrophic activity (Reay *et al.* 2005), as they limit oxygen availability.

Although pristine soils, exposed at the front of a glacier as the glacier recedes, can have water-saturated areas with reduced accessibility to oxygen favoring methanogenesis, this was not the case in the Styggedalsbreen forefield. Studies addressing CH_4 production in glacier forefields have reported ambiguous results. For example, Bárcena, Yde and Finster (2010) observed CH_4 production only in a sample collected next to the glacier front, whereas Chiri *et al.* (2017) found no evidence for microbial CH_4 production. The production of small amounts of CH_4 after 12 weeks in laboratory incubations and the much higher CH_4 oxidation rates, allow us to conclude that the forefield of Styggedalsbreen acts as a CH_4 sink using atmospheric CH_4.

Abundance and diversity of the methanotrophs in the glacier forefield

Previous studies have reported either low or unsuccessful amplification of *pmoA* genes, when targeting methanotrophic communities in glacier forefields (Bárcena, Finster and Yde 2011; Nauer *et al.* 2012). In our study, amplification with the primer pair A189f/A682r was successful in all samples. Although this primer pair amplifies not only *pmoA* genes of methanotrophs but also *amoA* genes of the ammonia-oxidizers, as observed in this study, it is the most frequently used primer pair to obtain a profile of the methanotrophic community in the environment (Knief 2015). In fact, it has recently been applied not only in glacier forefields (Nauer *et al.* 2012) but also in Arctic and agricultural soils (Kizilova, Yurkov and Kravchenko 2013; Martineau *et al.* 2014).

The increase in *Bacteria* or methanotrophic abundance with increasing distance from the glacier throughout the entire growing season is most likely due to the more favorable forefield conditions, such as: (i) an increase in OC and TN content (Mateos-Rivera *et al.* 2016); (ii) an increase in forefield temperature; (iii) a decrease in water content (Jørgensen *et al.* 2015) due to longer periods without snow cover; together with (iv) an increase in nutrient availability (Hofmann, Reitschuler and Illmer 2013). Although larger numbers of methanotrophs do not *per se* imply that the active methanotrophic community increases, we reported a statistically significant correlation between CH_4 oxidation rates and methanotrophic numbers, except in the intermoraine areas. Furthermore, a small increase in the methanotrophic copy number was observed after CH_4 amendment, suggesting that increased CH_4 availability stimulated methanotrophic growth.

HTS results targeting methanotrophs have been reported from a variety of different environments (Lüke and Frenzel 2011; Dumont *et al.* 2014; Cai *et al.* 2016). However, so far only one other study has applied HTS to target the methanotrophic community in the forefield of an alpine glacier (Chiri *et al.* 2017). Here, we applied HTS to study the diversity of the methanotrophs for the first time in a sub-Arctic glacier forefield. The methanotrophic community at Styggedalsbreen forefield varied based on distance from the glacier throughout the entire growing season. The site-specificity of the OTU composition in our study shows a clear community succession along the chronosequence and also shows that different OTUs are responsible for CH_4 oxidation along the Styggedalsbreen forefield. Interestingly, the variation in the methanotrophic communities along the forefield contrasts with other studies where changes along the transect were not found to be significant (Chiri *et al.* 2017). The differences are most likely due to the deglaciated period, as in the latter study only the last 120 years was encompassed, while we have covered the LIA moraine.

We also observed that increased CH_4 levels, such as those applied in the flux chamber during flux measurement, slightly altered the methanotrophic community composition in the chamber-covered soil. Interestingly, increased CH_4 concentrations stimulated only some of the OTUs, in general those that already were the most abundant ones. This observation indicates that increasing CH_4 levels, which are a likely future scenario, may unfavorably decrease the diversity of the less abundant methanotrophic OTUs. However, according to our results, time after deglaciation, which is equivalent to distance from the glacier, was a more important factor for the methanotrophic community composition than growing season and CH_4 addition as determined by the distances in the NMDS plot.

Despite the coamplification of *pmoA* and *amoA* genes by the primer pair A189f/A682r, the HTS results showed that the methanotrophs represent almost all OTU at sites that were covered by the glacier during the LIA, while the ammonia oxidizer-related OTU increased in abundance only at sites that where located in front of the glacier during the LIA. This may be due to the higher nitrogen content in soils at these sites, which may serve as substrate for the ammonium oxidizers. The majority of the methanotrophic OTUs in our study were closely related to psychrophilic high-affinity methanotroph sequences from cold regions, including soils from the Canadian Arctic (Pacheco-Oliver *et al.* 2002). This suggests that the methanotrophs found in this study are typical for cold climate regions. In addition, the high abundance of MOB_OTU7 in sampling sites close to the glacier front is not surprising as MOB_OTU7 is associated to the USC-alpha clade, whose members have been suggested to be oligotrophic (Pratscher *et al.* 2018). Furthermore, the low sequence identity of MOB_OTU8 to hitherto reported methanotrophic OTUs, suggests that novel, abundant methanotrophic microorganisms are still to be discovered in these areas. More detailed insights into the specific adaptations of the high-affinity methanotrophs to forefield environments would require cultivation. However, efforts to cultivate high-affinity methanotrophs have so far been challenging and little is known about their identity, metabolic characteristics and distribution.

Despite the fact that only a limited number of glacier forefield investigations have focused on CH_4 cycling, the observed

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apparent diversity among the studied forefields and the variation, which is introduced by the different types of chambers that were used to measure the methane fluxes, strongly indicate that the results obtained from one forefield cannot be extrapolated to other forefields. More studies are therefore necessary to identify whether there exist general trends with respect to CH_4 cycling depending on forefield type, history, climate, elevation and/or geographic region.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest

None declared.

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