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Seasonal Dynamics of Methanotrophic Bacteria in a Boreal Oil Sands End Pit Lake

Emad A. Albakistani,[a] Felix C. Nwosu,[a] Chantel Furgason,[a] Evan S. Haupt,[a] Angela V. Smirnova,[a] Tobin J. Verbeke,[a] Eun-Suk Lee,[a] Joong-Jae Kim,[a] Amelia Chan,[a] Ilona A. Ruhl,[a]* Andriy Sheremet,[a] Sarah B. Rudderham,[b] Matthew B. J. Lindsay,[b] Peter F. Dunfield[a]

[a]Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada
[b]Department of Geological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

These authors contributed equally to this work: Emad A. Albakistani, Felix C. Nwosu, and Chantel Furgason. Their order in the byline was determined via coin flipping.

ABSTRACT Base Mine Lake (BML) is the first full-scale demonstration end pit lake for the oil sands mining industry in Canada. We examined aerobic methanotrophic bacteria over all seasons for 5 years in this dimictic lake. Methanotrophs comprised up to 58% of all bacterial reads in 16S rRNA gene amplicon sequencing analyses (median 2.8%), and up to 2.7×10^4 cells mL^{-1} of water (median 0.5×10^3) based on qPCR of *pmoA* genes. Methanotrophic activity and populations in the lake water were highest during fall turnover and remained high through the winter ice-covered period into spring turnover. They declined during summer stratification, especially in the epilimnion. Three methanotroph genera (*Methylobacter*, *Methylovulum*, and *Methyloparacoccus*) cycled seasonally, based on both relative and absolute abundance measurements. *Methylobacter* and *Methylovulum* populations peaked in winter/spring, when methane oxidation activity was psychrophilic. *Methyloparacoccus* populations increased in the water column through summer and fall, when methane oxidation was mesophilic, and also predominated in the underlying tailings sediment. Other, less abundant genera grew primarily during summer, possibly due to distinct CH_4/O_2 microniches created during thermal stratification. These data are consistent with temporal and spatial niche differentiation based on temperature, CH_4 and O_2. This pit lake displays methane cycling and methanotroph population dynamics similar to natural boreal lakes.

IMPORTANCE The study examined methanotrophic bacteria in an industrial end pit lake, combining molecular DNA methods (both quantitative and descriptive) with biogeochemical measurements. The lake was sampled over 5 years, in all four seasons, as often as weekly, and included sub-ice samples. The resulting multiseason and multiyear data set is unique in its size and intensity, and allowed us to document clear and consistent seasonal patterns of growth and decline of three methanotroph genera (*Methylobacter*, *Methylovulum*, and *Methyloparacoccus*). Laboratory experiments suggested that one major control of this succession was niche partitioning based on temperature. The study helps to understand microbial dynamics in engineered end pit lakes, but we propose that the dynamics are typical of boreal stratified lakes and widely applicable in microbial ecology and limnology. Methane-oxidizing bacteria are important model organisms in microbial ecology and have implications for global climate change.

KEYWORDS methane, methanotroph, stratified lake, dimictic lake, land reclamation, end pit lake, methanotrophs

Oil sand is a matrix of inorganic particles, water, and bitumen. Extraction of marketable oil from surface oil sands deposits in AB, Canada is achieved via treatment with hot caustic water and naphtha/paraffin solvents. This process produces fluid fine

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Address correspondence to Peter F. Dunfield, pfdunfie@ucalgary.ca.

*Present address: Ilona A. Ruhl, National Bioenergy Center, National Renewable Energy Laboratory, Golden, Colorado, USA.

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tailings (FFT) comprised of alkaline water (pH = 10), silt, clay, residual bitumen, salts, and unrecovered solvents (1–4). FFT are stored in open tailings ponds, from which water is recycled to the extraction plant. However, mine operators are ultimately required to reclaim mined lands to an "equivalent land capability" of the original landscape (5). Wet landscape approaches to reclamation involve the creation of engineered bogs, fens, or end pit lakes (EPLs) in which FFT are contained and remediated over time (3). EPLs, below-grade bodies of water constructed in surface mining pits, are common in other sectors of the mining industry. However, proposed oil sands EPLs are shallower and larger in area than most EPLs, and their environmental issues arise from organic pollutants and dissolved salts rather than acidification and metal toxicity (6). Only one full-scale demonstration EPL presently exists in the Alberta oil sands region, Base Mine Lake (BML). BML is an approximately 780-ha lake (Fig. S1) created from an exhausted mining pit filled with about 45-m-depth of FFT and capped with fresh water in 2012 (2). The water cap at the time of the present study was 10 m to 12 m deep (7). There is a clear boundary between the sedimented tailings material (called sediment in this text) and the water cap. BML behaves as a typical boreal dimictic lake with summer and winter stratification periods separated by spring and fall turnover events (Fig. S2) (8, 9).

Solvents used in bitumen extraction, and to a lesser extent bitumen itself, are substrates for methanogenesis (10–12). Active oil sands tailings ponds can therefore be strongly methanogenic, emitting 30 to 26,000 kg CH_4 ha^{-1} annually (13). Although tailings are no longer being deposited into BML, methanogenesis of the deposited solvents has continued. Methane emissions from BML averaged 610 kg ha^{-1} y^{-1} between 2014 and 2019, with a gradual decline over time (14). Emissions are highest during spring thaw, when methane that accumulates under the ice cover is released (14). Dissolved methane concentrations in the sediment are very high, and increase rapidly with depth. In 2016 and 2017, concentrations ranged from $<$ 0.1 mM at the sediment-water interface to $>$ 2 mM at a depth of 1.0 m to 1.5 m in the FFT layer (15), and reached saturation ($\sim$4 mM) at 1.5 m to 3.5 m depth in the FFT (16). During summer stratification, a methane gradient is also evident in the water column, ranging from 20 to 40 μM in the hypolimnion (subsurface zone, 6 to 9 m) to 0.38 to 1.2 μM in the epilimnion (surface zone, 0 to 4 m) (7). Dissolved O_2 is present throughout the water column, from 70% to 85% of saturation at the top of the metalimnion (4 to 6 m) to 1% to 5% saturation at the sediment interface (7). The hypolimnion remains somewhat oxic due to physical mixing processes from the epilimnion (16, 17). These methane/O_2 counter gradients indicate that active aerobic methane oxidation occurs in the metalimnion, hypolimnion, and sediment-water interface of BML. In fact, O_2 consumption in the lake was shown in 2016 to be largely driven by methane and NH_4^+ oxidation (7).

Although methanogenic substrates in natural lakes are primarily plant or algal polysaccharides rather than petroleum hydrocarbons, an active methane cycle is common in temperate and boreal lakes, which are major sources of atmospheric methane globally (18). In seasonally ice-covered lakes methane is emitted via diffusive and ebullitive fluxes as well as an episodic spring thaw flux that accounts for an average of 27% of total annual emissions (19, 20). However, much of the methane produced in natural lakes (30% to 99%) is not emitted to the atmosphere, but rather oxidized by aerobic methanotrophic bacteria in oxic water or sediment layers (21, 22). Aerobic methanotrophs comprise a few taxonomic clusters of bacteria that use methane monooxygenase (MMO) enzymes to oxidize methane to methanol, which is then metabolized for energy and carbon. In temperate and boreal areas, *Alphaproteobacteria* methanotrophs (families *Methylocystaceae* and *Beijerinckiaceae*) predominate under acidic and oligotrophic conditions, such as in *Sphagnum* bogs and bog-lakes, while *Gammaproteobacteria* methanotrophs (family *Methylococcaceae*) are predominant in pH-neutral minerotrophic environments, including most lake waters and sediments (23, 24). Typical gammaproteobacterial genera found in seasonally stratified lakes include *Methylobacter*, *Methyloparacoccus*, *Methylomonas*, *Methylosoma*, and *Crenothrix* (24–28). Methanotrophs

are usually present throughout the water column but their activity may peak in the oxycline, which is located in the metalimnion when the hypolimnion is anoxic (e.g., in eutrophic lakes [29–31]), or at the sediment interface when the hypolimnion is oxic (32, 33). Methane oxidation rates can peak during fall turnover, when methane stored in the hypolimnion mixes with well-oxygenated surface water (21, 29, 34, 35). Methanotrophic species assemblages in these lakes may change with depth and season (27, 28, 35).

We have previously documented the activity of *Methylococcaceae* methanotrophs in the surface layers of active oil sands tailings ponds (36). In the present study we examined the dynamics of methanotrophic bacteria in a reclaimed end pit lake, BML. Qualitative and quantitative methods were used to determine community compositions and populations sizes, and assess how these changed with season and depth.

RESULTS

Potential methane oxidation rates. Methane oxidation rates estimated via an O_2 depletion assay were similar to rates estimated via CH_4 depletion, verifying the method. Potential methane oxidation rates in the water column from 2015–2017 are presented in Fig. 1 and Table 1. Winter samples were extremely variable, but a consistent seasonal pattern for the rest of the year was observed. Methane oxidation rates were high during spring and fall. Rates declined during summer stratification, but only in the epilimnion. This effect was significant: methane oxidation rates in summer were significantly higher in the hypolimnion (1 m from the sediment) than in the epilimnion (0.5 m from the surface), based on a paired-sample ANOVA with Bonferroni correction for two comparisons ($P = 0.014$), but no significant depth or seasonal patterns were detected when the lake was unstratified (Table 1). Examination of samples taken at 1-m intervals verified that methane oxidation rates were similar throughout the water column in fall, winter and spring, but increased with depth during summer stratification (Fig. S3).

Seasonal dynamics of methanotrophic bacteria in the water column. Putative methanotrophs made up a large portion of the bacterial community in the water, averaging 9.0% of 16S rRNA gene reads (median 2.8%) in all samples but reaching as high as 58.5% in particular samples. Most (>99%) of the identifiable methanotroph reads belonged to the *Methylococcaceae* family of the class *Gammaproteobacteria*. Methanotroph amplicon sequence variants (ASVs) present in at least 20 samples and with an average relative abundance >0.1% are listed in Table S1, and Fig. 2 shows their respective phylogenetic positions. The most abundant ASVs belonged to the genera *Methylobacter*, *Methyloparacoccus*, and *Methylovulum/Methylosoma* (closely related to both genera, but hereinafter referred to only as *Methylovulum*). Less abundant ASVs were related to the genera *Methyloprofundus*, *Methylococcus*, *Crenothrix*, and a few other *Methylococcaceae* that could not be classified to the genus level. *Methylocella* and *Methylocystis* were the most abundant alphaproteobacterial methanotrophs, but comprised only 0.012% and 0.0058%, respectively, of all reads. ASVs belonging to the family *Methylomirabilaceae* were not detected in any BML sample, although they were sometimes detected in a neighboring freshwater reservoir (data not shown).

There was a U-shape in the relative abundance of methanotrophs in 16S rRNA gene amplicon sequencing sets from 2017 to 2019, with the highest relative abundances observed during fall and winter, and the lowest during summer stratification (Fig. 3). This pattern was evident in both surface and bottom waters, although summer abundances in the hypolimnion were higher than in the epilimnion (Fig. S4). There was also a regular seasonal succession of the three main genera, evident qualitatively in time courses of ASVs pooled into genus-level groups (Fig. 3), and quantitatively in an analysis of composition of microbiomes (ANCOM) (75) (Fig. 4). *Methyloparacoccus* predominated during late summer and fall turnover, increasing dramatically as fall turnover progressed, while *Methylobacter* and *Methylovulum* dominated in winter and spring, usually declining as summer progressed (Fig. 3 and 4). The *Methyloparacoccus* was predominantly (>95%) a single ASV. There were several *Methylovulum* ASVs. Sometimes different ASVs of this genus predominated in different years, but all showed a similar

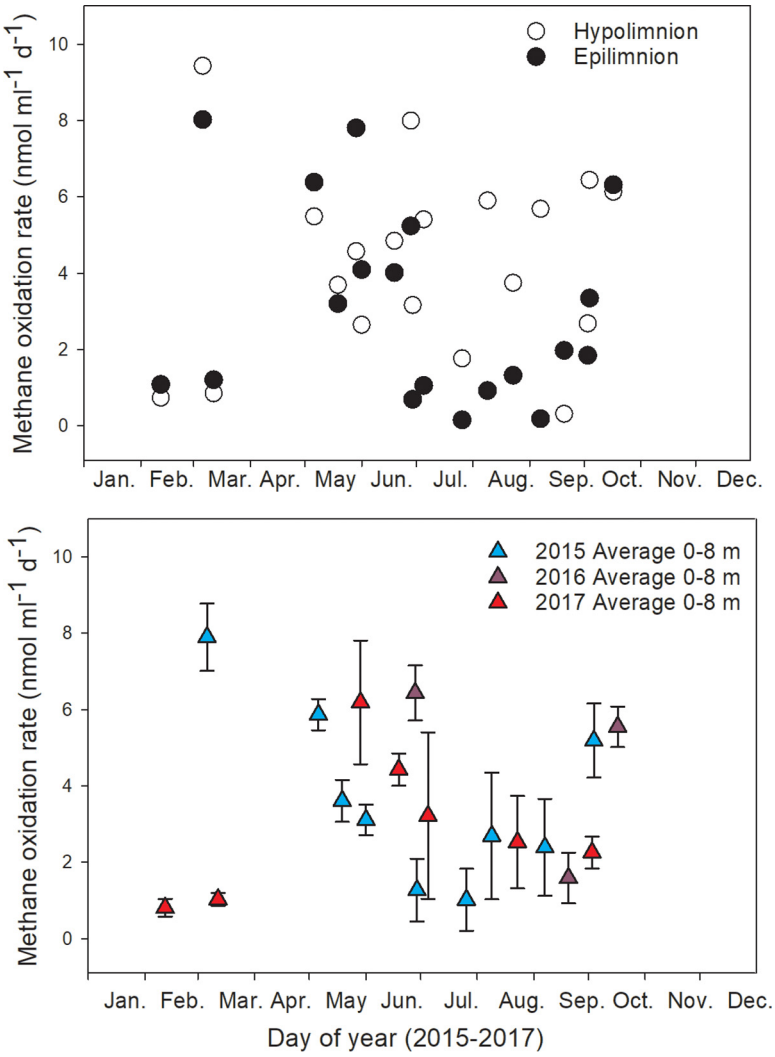

FIG 1 Potential methane oxidation rates in BML water samples with time of year, demonstrating the lower epilimnetic rates during summer stratification. All samples were incubated at 20°C except winter samples, which were incubated at 5°C. Top panel: samples at 0.5 m (epilimnion) and 8 m (hypolimnion). Values are averages of samples from the three sampling platforms. Bottom panel: depth-averaged rates for each of three sampling years, based on averages of 3 platforms × 5 depths for 2015–2016 (0.5, 2, 4, 6, and 8 m) or 3 platforms × 2 depths for 2017 (0.5 and 8 m), ± 1 SEM. There is a significant difference between the surface and bottom in summer only, based on the ANOVA results shown in Table 1.

pattern of peaking in winter/spring (Fig. 4; Fig. S5). *Methylobacter* ASVs were more variable. The two most abundant *Methylobacter* ASVs (ASV4 and ASV8) peaked in winter, but others peaked in both winter and mid-summer (Fig. 4; Fig. S5). Several less abundant ASVs, including a *Methylococcus* and *Methyloprofundus*, were evident primarily in summer and fall (Fig. 4). The overall alpha diversity of methanotrophs usually peaked in summer (Fig. S6), perhaps because summer stratification increased the number of microniches selecting for particular species. A gradual but significant increase in methanotroph diversity was also observed over the 5-year course of the study (Fig. S6).

Analyses of *pmoA* genes reinforced the conclusions of 16S rRNA gene sequencing. Complete *pmoA* genes from *Methyloparacoccus* and *Methylobacter* were recovered from metagenomes (Fig. S7), supporting the predominance of these genera. The seasonal patterns in *pmoA* gene counts via qPCR closely mirrored the patterns of relative 16S rRNA gene abundances described above. The lowest counts in the universal *pmoA* gene assay were measured in midsummer (in both the epilimnion and the hypolimnion), and the

TABLE 1 Potential methane oxidation rates in BML surface water (0.5 m from surface), bottom water (8 m to 11 m), and average throughout the water column in different seasons from 2015–2017[a]

Season	No. of sampling dates	Methane oxidation rate, mean ± 1 SEM (nmol mL^{-1} d^{-1})		
		Surface	Bottom	Avg water column
Winter (sub-ice)	3	3.09 ± 1.90	3.88 ± 2.75	3.25 ± 2.32
Spring turnover	6	5.01 ± 0.88	4.61 ± 1.11	4.94 ± 0.70
Summer stratification	6	0.23 ± 0.49*	5.44 ± 0.92*	2.19 ± 0.34
Fall turnover	4	3.43 ± 1.07	2.81 ± 1.07	3.65 ± 1.01

[a]Raw data are means of measurements at three platforms (surface and bottom) or at three platforms × 2 to 9 depths (average water column). These were then averaged over multiple sampling dates to give date-averaged means ± 1 SEM for each season. A significant difference between the surface and bottom in summer based on an ANOVA is indicated with an asterisk.

highest counts in fall and winter (Fig. 5). The *Methylococcus*-group-specific *pmoA* assay (encompassing primarily *Methyloparacoccus* in BML) showed a similar pattern, with the lowest counts in early summer, gradually increasing in late summer and peaking in late fall or early winter (Fig. 5). *Methylobacter*-group-specific *pmoA* counts (encompassing primarily *Methylobacter* and *Methylovulum* ASVs) were highest in winter and spring, then declined gradually throughout the calendar year (Fig. 5). All three patterns match the amplicon-based analyses (Fig. 3 and 4).

Methylotrophic bacteria in the order *Methylophilales* are known to grow in concert with methanotrophs in oilsands tailings ponds (36). These were also abundant in the water, particularly in winter and spring (Fig. S8).

Patterns of methanotrophic bacteria with depth. Similar to the pattern noted for methane oxidation rates, higher methanotroph relative abundances were measured in the hypolimnion than in the epilimnion during summer stratification of each year (Fig. S4). In all three *pmoA* qPCR assays, summer hypolimnetic waters also generally had higher *pmoA* gene counts than epilimnetic waters (Fig. 5).

Counts of *pmoA* genes in the surface tailings sediment (to 50-cm depth below the sediment interface) were compared with counts throughout the water column on four dates. In each case, regardless of season, two to four orders of magnitude higher gene copies were evident in the sediment (Fig. S9). Copy numbers of the *pmoA* gene in the sediment were consistently high, hence the methanotrophs in sediment probably respond to shifting conditions by altering their activity rather than via growth. Based on relative abundances from 16S rRNA gene amplicon sequencing, a zonation of methanotroph genera was observed across the sediment-water interface, with *Methylobacter* dominating just above the sediment interface and *Methyloparacoccus* just below it (Fig. 6). The water column above the sediment interface showed little vertical zonation of the methanotroph genera, but instead displayed the seasonal pattern of *Methyloparacoccus* predominating in summer and fall, and *Methylobacter* and *Methylovulum* in winter (Fig. 6).

Temperature response of methane oxidation. The seasonal pattern of methanotroph succession could be controlled by temperature. To test this, temperature responses of representative samples from winter (March 12 to 14, 2018) and summer (August 26, 2019) were compared. The winter sample showed a psychrophilic response of methane oxidation activity, with maximal oxidation rates at 15°C, while the summer sample showed a mesophilic response, with peak activity at >30°C (Fig. 7). Incubation of water samples under a methane-enriched headspace at ≤4°C resulted primarily in the growth of *Methylobacter*, while enrichment at ≥8°C resulted primarily in the growth of *Methyloparacoccus* (Fig. 7). Enrichment at intermediate temperatures of 8°C to 10°C resulted in the growth of both genera, but slightly more *Methyloparacoccus*. These growth data, although not quantitative, demonstrate that the *Methylobacter* are more psychrophilic than *Methyloparacocccus*, but that the *Methyloparacocccus* also grow at temperatures as low as 8°C and may be psychrotolerant. The genus *Methylovulum* did not enrich in this experiment, although the community analyses suggested it is also psychrophilic.

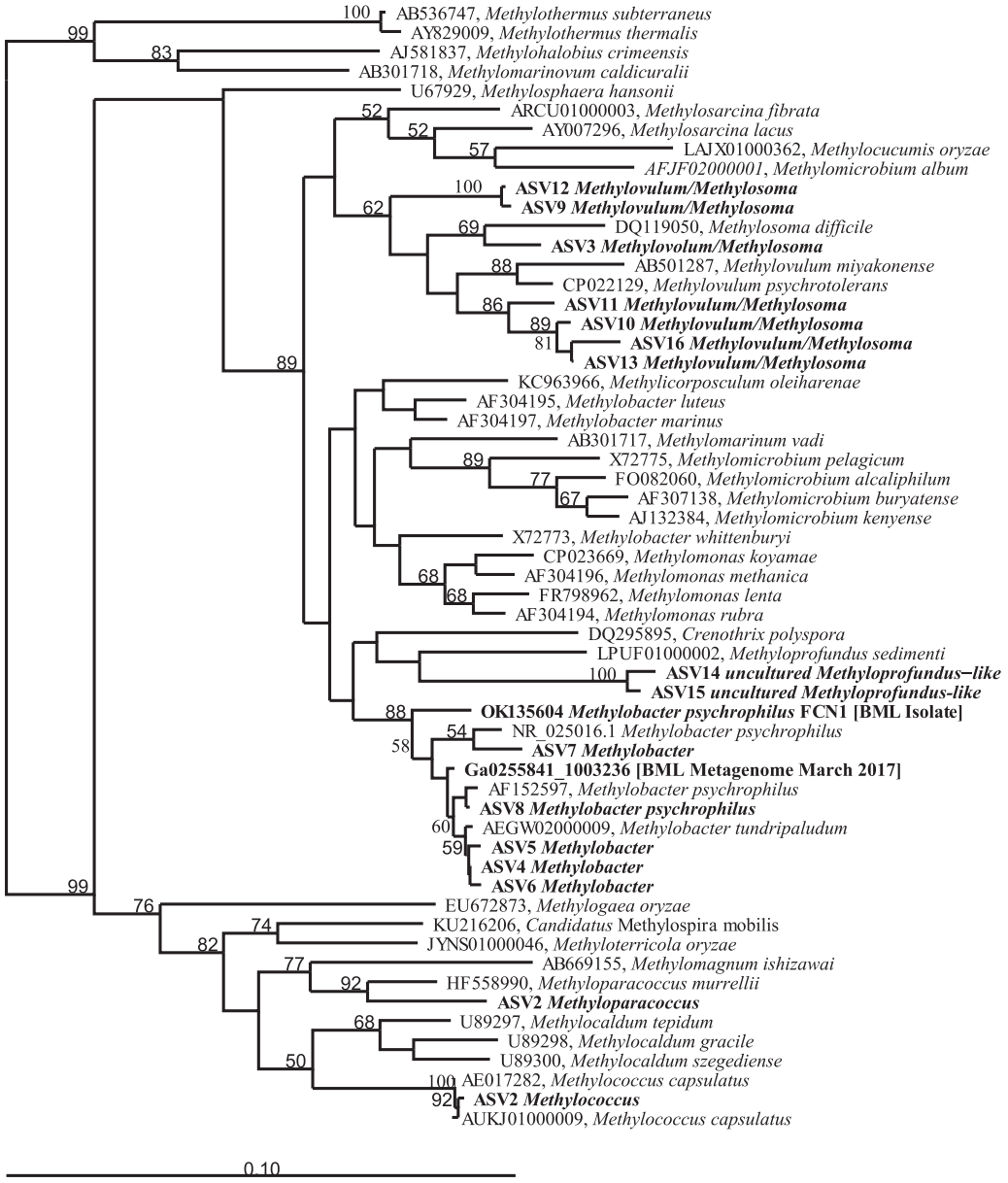

FIG 2 Phylogenetic tree based on partial 16S rRNA gene sequences, showing the major methanotroph ASVs detected in BML (in bold, from Supplementary Table S1), in relationship to other methanotophic bacteria of the class *Gammaproteobacteria*. The tree was constructed using a Neighbor-Joining algorithm with a Jukes-Cantor correction. Bootstrap values >50% based on 1,000 constructions are shown at the nodes. The scale bar represents 0.1 change per nucleotide position.

These results suggest that the methanotrophic communities in BML are temperature-adapted, with psychrophilic *Methylobacter* dominating in winter/spring versus more mesophilic *Methyloparacoccus* in summer/fall. This assumption is consistent with measured water and sediment temperatures (Fig. 3C). Water temperatures in winter range from 0°C at the lake surface to 4°C at the sediment interface. At the peak of summer stratification these increase to roughly 20°C and 15°C, respectively. The FFT deposits in the sediment are a source of heat that is slowly declining over time, and are warmer than the overlying water in the winter, possibly accounting for the consistent prevalence of more mesophilic *Methyloparacoccus* in sediment throughout the year. Temperatures immediately below (~0.25 m) the sediment-water interface ranged from 8°C to 12°C in 2013 to 2014 and from 5°C to 11°C in 2018 to 2019. The magnitude of seasonal

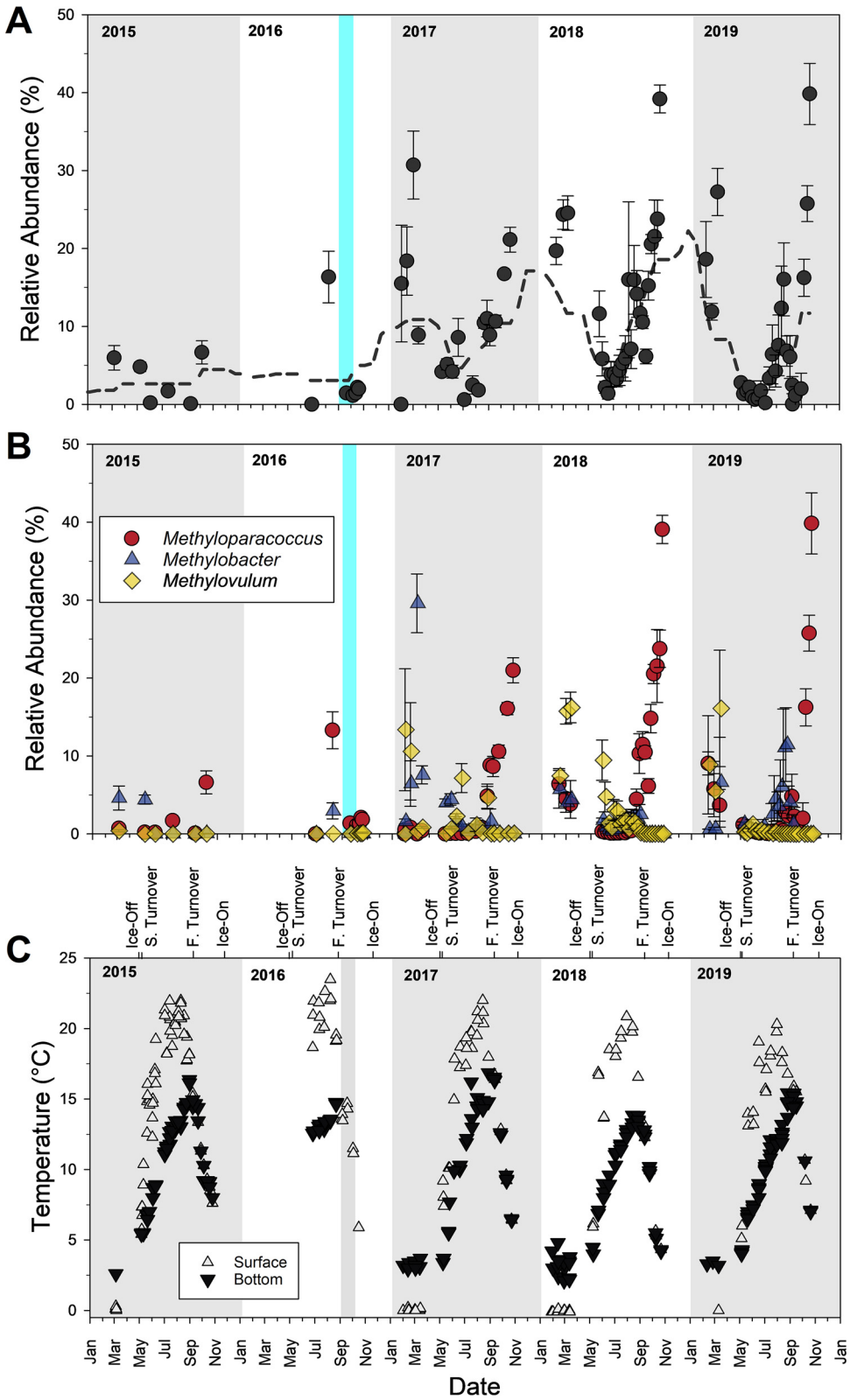

FIG 3 Relative abundances of methanotrophs in BML water as a percent of all bacteria, based on 16S rRNA gene amplicon sequencing. Data are depth-averaged values throughout the water column (0 m to 12 m) for each platform, given as the mean ± 1 SEM of three platforms. Separated epilimnetic and hypolimnetic data are presented in Fig. S4. Panel A: Relative abundance of all methanotrophs. The dashed line indicates a running average of three successive sampling dates. Panel B: Relative abundances of the three main methanotroph genera. Each genus is the sum of several pooled ASVs. Panel C: Temperatures measured at 0 m to 2 m (surface) and 8 m to 12 m (bottom), showing the timing of thermal stratification and turnover events. The blue strip represents the addition of alum to the lake in 2016.

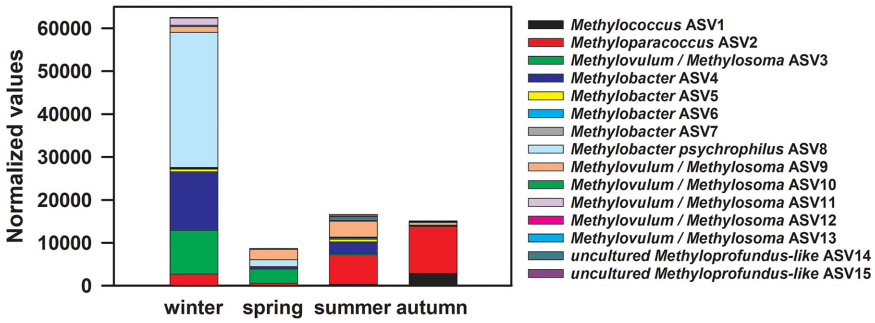

FIG 4 Analysis of composition of microbiomes (ANCOM) (75) of methanotroph ASVs in BML, showing the predominance of *Methyloparacoccus* and *Methylococcus* in summer and autumn, versus *Methylobacter* and *Methylovulum* in winter in spring. The ASV numbers correspond to Supplementary Table S1. The four seasons were delineated based on turnover events (see Fig. 3C). Normalized values are results of two-step transformations: first, Aitchison log-ratio of abundance of each taxon relative to the abundance of all remaining taxa is determined one at a time, then, the Mann-Whitney U is calculated on each log ratio to compare groups.

variations decreases with depth and are minimal below 5.5 m, where the average temperature is ~11°C and seasonal fluctuations are < 0.5°C (15, 16).

DISCUSSION

BML is intensively sampled via an adaptive management program intended to determine whether EPLs are a viable strategy for land reclamation in the oil sands industry. Piggybacking on this program allowed us to assemble a data set on methanotroph dynamics in a seasonally stratified lake that, to our knowledge, is unique in the field in its sampling intensity and duration. Most previous studies on methanotrophs in lakes sampled only once, or a few times in a single year. Here we sampled over 5 years, in all four seasons, as often as weekly, and were therefore able to document consistent annual community dynamics.

The species composition of the methanotrophic communities and their population sizes appear to be similar to other seasonally stratified temperate and boreal lakes. The predominance of *Gammaproteobacteria* over *Alphaproteobacteria* methanotrophs is a common property of temperate, boreal, alpine, and other freshwater lakes, especially when these are nutrient- and methane-rich (24–28, 37–40). *Alphaproteobacteria* methanotrophs were always minor components of BML, including in the methane-poor epilimnion where they thrive in some other lakes (41), and in the surface sediment where they are more important in some eutrophic lakes (42). It is likely that the alphaproteobacterial *Methylocystaceae* and *Beijerinckiaceae* methanotrophs are more affected by the salts, alkaline pH and organic pollutants in BML, as they are proposed to be less tolerant of these stresses than *Gammaproteobacteria* methanotrophs (23, 43, 44). However, the high methanotroph populations and the similar species composition compared to natural lakes suggest that toxicity is not a major constraint on methanotrophy. The three major methanotroph genera in BML (*Methyloparacoccus*, *Methylobacter*, and *Methylovulum*) have all been identified in other freshwater lakes, with either *Methyloparacoccus* or *Methylobacter* often the most prevalent genus (27, 35, 39, 40, 42, 45). *Methylovulum* and *Methylobacter* species include psychrophilic isolates (46, 47), and are frequently detected in low-temperature habitats (28, 36, 39, 44, 45).

Methanotrophs were frequently a predominant part of the bacterial community in the BML water column, comprising from 0% up to 58% (median 2.8%) of reads in 16S rRNA gene amplicon analysis of different samples. The dominance of methanotrophs within the overall microbial community is similar to other seasonally stratified lakes, where methanotroph relative abundance has been reported to peak as high as 11% to 57% (28, 35, 38, 39). Up to 5.4×10^4 (median 1.0×10^3) *pmoA* gene copies mL^{-1} were detected based on qPCR. Counts of *pmoA* genes likely overestimate cell counts, as

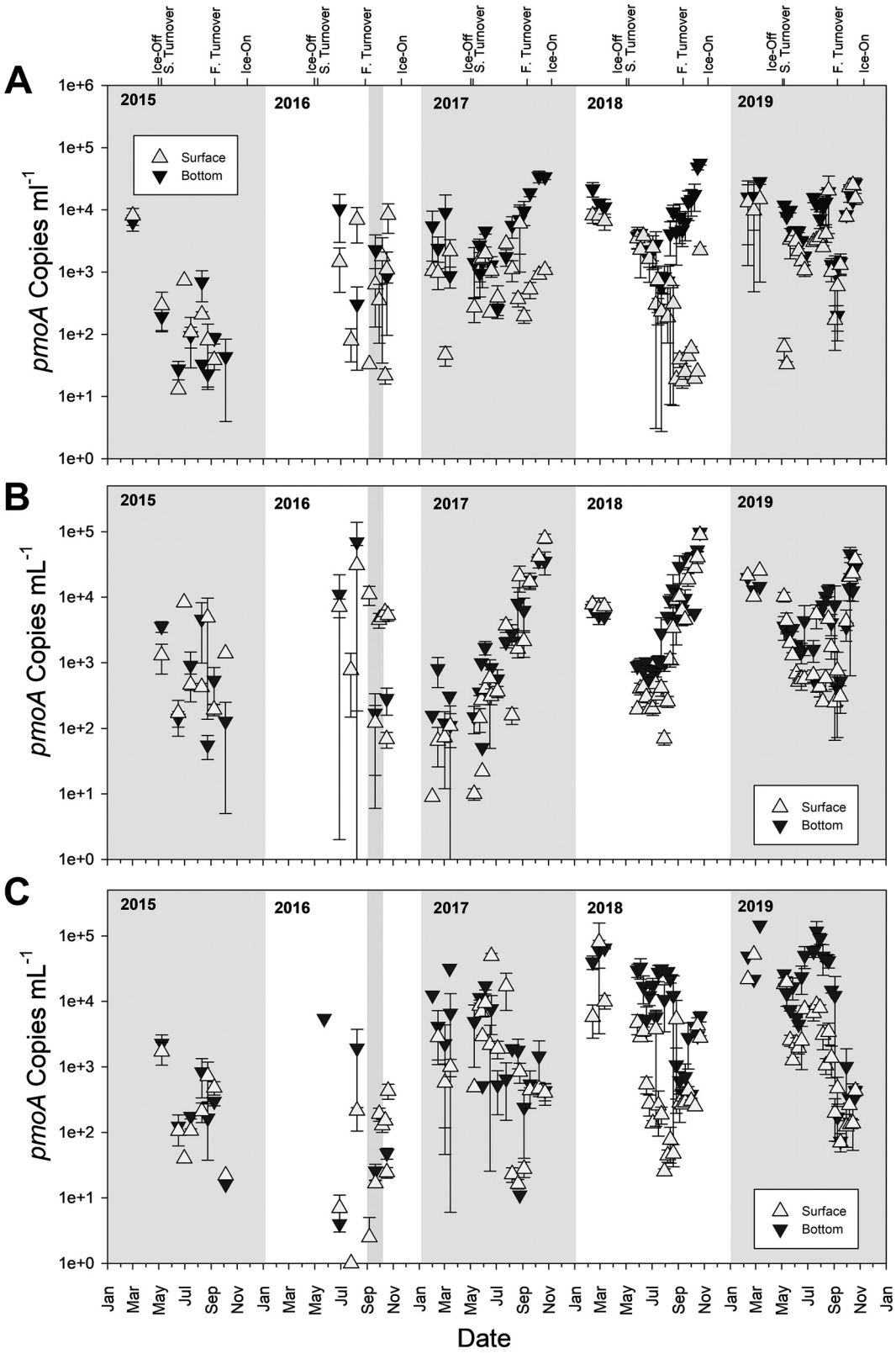

Fig. 5 qPCR quantification of *pmoA* genes over time as a proxy for methanotrophic bacterial populations in surface (0.5 m) and bottom (0 m to 1 m above the sediment) waters of BML from 2015–2019. Panel A: Universal *pmoA* assay for total methanotrophs. Panel B: Assay for *Methyloparacoccus* and closely related genera. Panel C: Assay specific for *Methylobacter*, *Methylovulum* and closely related genera. Data are means of three platforms ± 1 SEM. The vertical strip in 2016 represents the addition of alum to the lake.

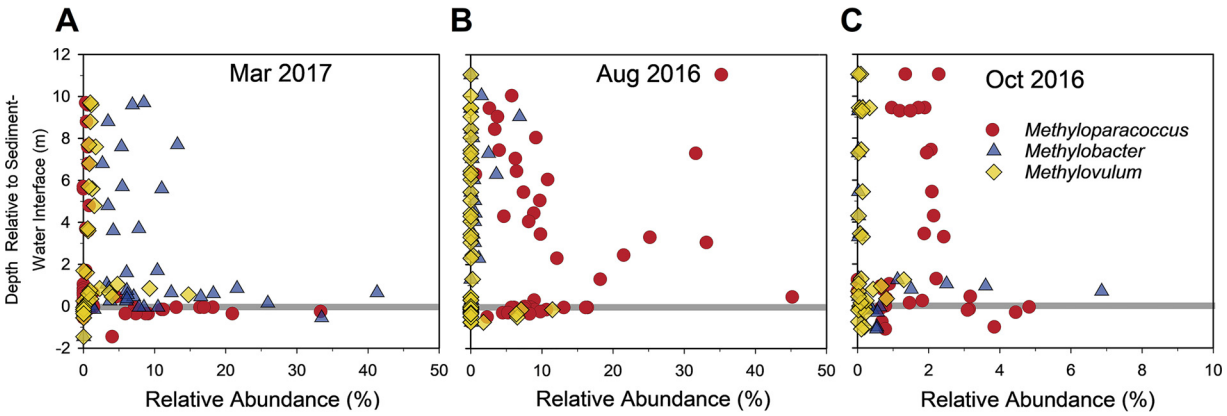

FIG 6 Relative abundances of different methanotroph genera with depth in the water column and across the water-sediment interface on three sampling dates (in March under ice cover, in August during summer stratification, and in October during fall turnover), based on 16S rRNA gene amplicon sequencing. Depths are relative to the sediment interface. The depth of the water column at the three sample platforms varies from 9 m to 12 m. Points represent individual samples.

most methanotrophs have two to three *pmoA* copies (48, 49). Therefore, we estimate maximum water populations as 2.7×10^4 cells mL^{-1} (median 0.5×10^3). The maximum *pmoA* gene counts in the BML water column are slightly lower than detected in several other natural lake waters (peaking at 6×10^5 to 7×10^6 copies mL^{-1}) (27), and slightly lower than maximum populations of methanotrophs estimated in seasonally stratified Lake Rotsee using FISH (4×10^5 cells mL^{-1}) (35, 37). However, the population ranges in different samples of BML and these other lakes overlap, indicating a general similarity in methanotroph abundance. Methanotrophs were much more abundant in the BML sediment than in the water column, reaching up to 9×10^7 *pmoA* copies g^{-1} (wet weight [w.w.]). This quantity closely resembles that detected in natural lake sediments (40, 42, 45, 50). For example, in stratified perialpine Lake Bourget, sediment *pmoA* counts averaged about 2.5×10^7 copies g^{-1} (w.w.) (40). Greater abundances in surface sediment compared with water are typical of lakes without an anoxic water zone, such as Lake Bourget and Lake Constance (40, 50). Surface sediment should present ideal conditions for methanotrophs, where O_2 and CH_4 diffusion gradients cross.

Methanotrophs were most abundant in the water column during fall turnover and under winter ice-cover, and least abundant during summer stratification. This trend was evident in relative abundances (16S rRNA gene amplicon sequencing), absolute abundances (qPCR of *pmoA* genes), and potential methane oxidation rates. It is likely that during

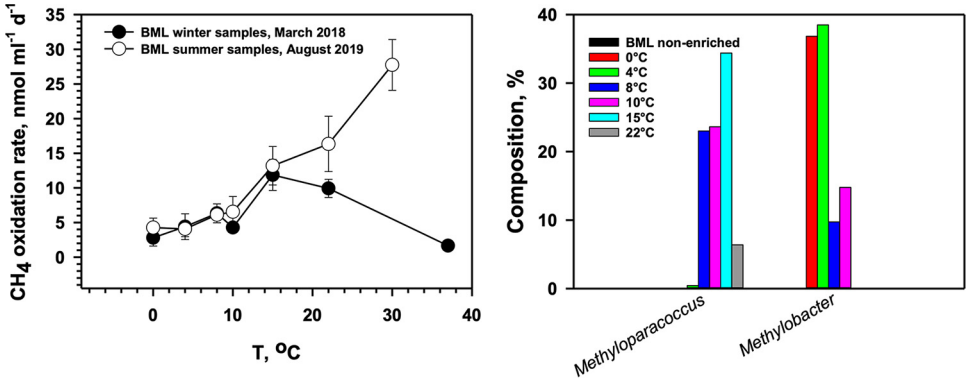

FIG 7 Temperature response of methanotrophy. Left panel: Temperature response of methane oxidation rates in samples taken in winter or summer. Data are means of three replicates ± 1 SEM. Right panel: Methanotrophs as a percent of the total bacterial communities based on 16S rRNA gene sequencing, after 30 d of incubation of a water sample taken on August 26, 2019 under 10% vol/vol methane in air at different temperatures, showing preferential enrichment of *Methylobacter* at low T (≤4°C) and *Methyloparacoccus* at higher T (≥8°C). Where bars are not shown they are below 1% of the total community.

summer, methane oxidation occurs predominantly at the sediment-water interface and deep in the hypolimnion. As the hypolimnion is hypoxic but not anoxic (7), the methanotrophic layer should occur as close to the sediment methane source as possible. ASVs of three main genera (*Methylobacter*, *Methylovulum*, and *Methyloparacoccus*) segregated seasonally and spatially. In the water column, abundances of *Methylobacter* and *Methylovulum* strains peaked in winter/spring, when the methane oxidation activity was psychrophilic, while abundances of *Methyloparacoccus* peaked in summer/fall, when the methane oxidation activity was mesophilic. Vertically, *Methyloparacoccus* sp. was also predominant below the water-sediment interface, and populations of *Methylobacter* sometimes peaked just above the interface. These data are all consistent with niche differentiation of the different methanotrophs based on temperature and on concentrations of CH_4 and O_2, although O_2 and CH_4 co-vary and are difficult to disentangle. Principally, the *Methylobacter* and *Methylovulum* spp. in BML appear to be psychrophilic and adapted to high O_2/low CH_4 conditions while the *Methyloparacoccus* sp. is more mesophilic and adapted to high CH_4/low O_2 concentrations. Methane oxidation rates in boreal lakes and sediments usually show a weak dependence on temperature, with Q_{10} values of only 1.4 to 2.3 (51–53). Our results suggest that one reason for this weak response may be the presence of multiple methanotroph populations with different temperature optima.

The methanotrophic population and activity in the BML water column increased during fall turnover, when methane rich hypolimnetic water mixed with O_2 rich epilimnetic water. Although some of the population increase may have been due to suspension of *Methyloparacoccus* cells from the sediment, the relative abundance of this genus became much higher in the water than in the sediment, which would not be expected simply from suspension of sediment bacteria. Similar observations of maximum methane oxidation during fall turnover have been made in other lakes (21, 29, 34, 35, 54). In the seasonally-stratified temperate Lake Rotsee, methanotrophs reached a maximum relative abundance of 28% during fall turnover based on 16S rRNA gene amplicon analysis, a similar dominance to BML. Methane oxidation rates and *pmoA* transcripts also increased in this site during turnover (35), similar to our observations in BML. Unfortunately, we were unable to sample BML during the early ice-over period, but sometime between fall turnover and midwinter the predominant *Methyloparacoccus* strain was replaced by *Methylobacter* and *Methylovulum* strains in the water, and the overall methanotroph populations remained high. A higher abundance of methanotrophs under ice cover compared to summer was also observed in multiple Swedish lakes (27). Ice cover traps methane bubbles, allowing waters to become rich in dissolved methane.

Although the present data set is more extensive than previous studies, the discussion above suggests that the temporal and spatial dynamics of methanotrophic species in BML are typical of seasonally stratified northern-latitude lakes. Niche differentiation of different species, distinct seasonal communities, and rapid growth during fall turnover have all been noted before, albeit with less resolution. Differences between summer and winter (sub-ice) communities were observed in several Swedish lakes, where different *Methylobacter* strains dominated in different seasons (27). In temperate Lake Rotsee, methanotroph succession was observed during the transition from summer to fall, with rapid growth of several strains including a *Methylosoma* sp. during fall turnover (35). Vertical partitioning of strains was also evident in two temperate stratified lakes, where *Methylobacter* and *Crenothrix* preferred the low O_2/high CH_4 hypolimnion, while a *Methylovulum* preferred the oxycline area with higher O_2 (28).

The similarity of BML to natural lakes is perhaps surprising, as BML is physically and chemically atypical in several ways. The pH falls within the typical range of boreal lakes in Canada (average 7.7 [55]), but the salinity is much greater. Conductivity in BML is 2.6 mS cm^{-1}, or about 5% of seawater, while other boreal lakes in Canada average only 0.166 mS cm^{-1} (55). Salinity is a major factor shaping methanotroph communities (56). Furthermore, methane is produced in BML from sediment-entrained hydrocarbon pollutants rather than recent organic matter. Despite this, methane production rates of BML are not atypical of lakes within this climatic region. Annual methane emission

rates in active tailings ponds range from 30 to 26,000 kg ha^{-1} y^{-1}, and the rate from the active pond west-in pit (WIP), immediately before its conversion to BML, was estimated as 3,300 kg ha^{-1} y^{-1} (13). The average emission rate in the 6 years after conversion of this pond to the end pit lake BML gradually decreased to 610 kg ha^{-1} y^{-1} (14). This falls well within the range measured in natural boreal, temperate, and arctic lakes (8 to 3,000 kg ha^{-1} y^{-1} [19, 20]). It is similar to emission rates from eutrophic lakes (17 to 290 kg ha^{-1} y^{-1} [29, 54 and references cited therein]) and other constructed reservoirs (14). The methane concentrations measured in BML water (up to 150 μM in the hypolimnion [7]) are also within the range of values reported for other boreal and temperate lakes, which range from $<$1 μM to over 1 mM in the hypolimnion (20, 22, 34, 39, 54, 57, 58). Therefore, despite the altered chemistry compared to natural lakes, indicators of the methane cycle are similar.

When lakes are eutrophic, methanotrophy has been shown to be largely responsible for O_2 demand, accounting for as much as 60% of the total biological oxygen demand (BOD) (29, 31, 52). We noted that potential methane oxidation rates in BML were similar to BOD rates (data not shown), suggesting that when present, methane can account for a large amount of the oxygen demand. This assessment is consistent with previous calculations of *in situ* methane oxidation rates (7). Trapping of methane-saturated water under ice in eutrophic lakes can even result in complete lake anoxia due to methane oxidation, resulting in a "winter kill" of fish and invertebrates (29, 54). However, while winter anoxia was observed just before ice-off in 2013, it has not been noted subsequently in BML (9). The dependence of methane emissions from BML on atmospheric pressure changes suggests that ebullition plays a major role in methane efflux (14), and bubbling is commonly observed (8). However, as methane efflux has been declining over time (14), a higher proportion of the flux is likely to become diffusive in the future, and methanotrophs may become relatively more important for limiting methane emissions.

In summary, in this study we present one of the most extensive data sets ever assembled for lake methanotroph populations, and document consistent seasonal patterns of species dominance and turnover, likely controlled by recurring niches of particular temperatures and O_2/CH_4 concentrations created by seasonal turnover cycles. Methanotroph populations were most predominant in the water column in fall and winter. Psychrophilic *Methylobacter* and *Methylovulum* dominated methanotroph communities in winter. Conversely, more mesophilic *Methyloparacoccus* dominated in the hypolimnion during summer and in the sediment year-round, and accounted for most of the rapid overall increase in the methanotroph population throughout the water column in fall. Besides the three main genera, a variety of other species also appeared in summer, when methanotroph diversity was maximal, and may take advantage of microniches created by counter-gradients of O_2 and CH_4 during stratification. Finally, there appears to have been a gradual increase in methanotroph diversity over time as this end pit lake has aged. This trend could be related to gradual changes in lake chemistry such as declining turbidity and salinity, or to the gradual decrease in the intensity of the methane cycle as the end pit lake has aged (14). This trend may point to a changing ecosystem able to support higher biodiversity with time. Although comparison with previous studies is somewhat speculative because few studies have sampled as intensively at this one, biotic factors such as methanotroph community composition, potential activity, population sizes, and seasonal turnover all indicate that the methane cycle of BML is in some ways similar to that of natural seasonally stratified lakes.

MATERIALS AND METHODS

Sampling. A map of BML (56.7267° N, 111.3790° W) showing the positions of three fixed sampling platforms is provided in Fig. S1. A detailed description of BML has been provided in previous studies (2, 9). Briefly, the original mining pit located on this site (WIP) was begun in 1978 and converted into a tailings pond in 1994. From 1994 to 2012 it was gradually filled with FFT to a depth of 45 m to 50 m, and in 2012 capped with an additional 5 m of fresh water, forming the present end pit lake, renamed Base Mine Lake. There is no outflow from BML to the local watershed, but the water level is maintained at 308.7 m above sea level via pump-in of freshwater or pump-out of excess water to the extraction plant

(2). Due to gradual FFT settling and dewatering, the depth of the water cap increased to approximately 10 m to 12 m by 2019. BML is dimictic, with ice-on in November, ice-off in April to May, onset of summer stratification in May to June, and fall turnover in September (9). Water temperatures vary from 0 to 24°C depending on season, and the maximum summer thermal gradient spans about 12°C (8, 9). A schematic describing the seasonal lake dynamics is shown in Fig. S2.

Water quality in BML has gradually improved over time. Concentrations of heavy metal and most hydrocarbon contaminants are similar to undisturbed freshwater sites in the region (59). The primary organic contaminants in BML, a class of carboxylic acid compounds known as naphthenic acids, have concentrations near 30 mg L^{-1} (59), whereas freshwater sources in the area contain 1 to 2 mg L^{-1} (60). The pH of FFT is alkaline (pH = 10), but the FFT porewater has been diluted by freshwater inputs, and the BML water cap has ranged from pH 7.8 to 8.6 (59). Due to clay suspension from FFT, water turbidity from 2012 to 2016 (50 to 350 NTU) was about an order of magnitude higher than that of freshwater bodies in the region (9). In an attempt to manage the turbidity, a chemical coagulant (aluminum potassium sulfate or alum) was added to BML in September 2016 to a final concentration of 1.12 mg of aluminum per liter of BML water (59). Turbidity decreased following the alum addition: in 2015, mean turbidity in the water column (0 to 8 m) ranged from 35 to 130 NTU and from 2017 to 2019 it ranged from 2.6 to 48 NTU (59). Although aluminum inhibits atmospheric methane oxidation in forest soils at concentrations as low as 0.63 μg/g soil (61, 62), high methane oxidation rates in BML (see Results) suggest that there was no adverse effect of alum on methane oxidation. Unfortunately because of the scarcity of pre-alum samples, firm conclusions about the effect of alum addition cannot be made.

At 1- to 4-week intervals from 2015 to 2019, 500-mL water samples were taken using Van Dorn water samplers from the surface (0 to 50 cm) and from c. 1 m above the sediment (tailings) interface, from each of the three fixed sampling platforms in BML (Fig. S1). Once every month, samples were also taken from 1-m intervals through the entire water column. Below-ice winter samples were taken after ice-coring. The only major interruption in the sampling program was due to a wildfire in early 2016. A fixed interval sampler was also deployed irregularly to collect samples at 10-cm intervals for 1 m across the sediment-water interface (63). All samples were stored in polypropylene bottles and shipped on ice to the University of Calgary (c. 2 d shipping time). On arrival, samples were stored at 5 to 8°C, generally for 1 to 2 d until processing.

Biogeochemical rate measurements. For samples taken in 2015 to 2017, 5-d biological oxygen demand (BOD5) was estimated. For each sample, 400 mL (3 platforms $\times$ 2-10 depths on each date) were shaken in 1-L serum bottles at 120 rpm for 15 min to equilibrate with ambient O_2. Dissolved oxygen (DO) was then measured on an Orion Star A323 Dissolved Oxygen/Portable Meter (Thermo Scientific, Waltham, MA, USA) or a Hach HQ440 Portable LDO Meter (Hach, London, Ontario, Canada) using a DO probe. Each sample was then filled to the brim of a 100-mL Pyrex bottle and capped with a butyl rubber stopper pierced by two needles that allowed excess sample and air bubbles to be removed during capping. Bottles were incubated for 5 days at 22°C, then DO was again measured. BOD5 values were calculated from the loss between 0 and 5 d.

For each sample, a second BOD5 incubation was performed with methane added to the water to estimate the potential methane oxidation rate. The procedure was exactly as described for BOD, except that in the initial step, 10% vol/vol methane was added to the headspaces of the 1-L serum bottles to equilibrate the water with elevated CH_4 as well as O_2. Subtracting the BOD5 from the BOD5+CH_4 value gave the estimated O_2 demand by methanotrophs, and also the potential methane consumption rate using a stoichiometry of $2O_2$:$1CH_4$. Direct measurement of methane depletion resulted in similar methane oxidation rates as this O_2-based method (see "Temperature response" section).

Molecular community analyses via 16S rRNA gene amplicon sequencing. Samples were centrifuged and processed as described in Table S2. The pellet of clays + cells was resuspended in about 0.5 mL of sample water, transferred into 2-mL tubes, and frozen at −80°C. DNA was extracted using the FastDNA Extraction Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) with an additional purification step using 5.5 M guanidine thiocyanate (64), and eluted in Qiagen Elution Buffer (Qiagen, Toronto, Ontario, Canada). DNA extracts quantified with a Qubit HS kit (Invitrogen, Carlsbad, CA, USA) ranged from 5 to 100 ng μL^{-1}. The V3-V4 region of the 16S rRNA gene was amplified using universal bacterial primers 341fw (5′-CCTACGGGNGGCWGCAG-3′) and 785r (5′-GACTACHVGGGTATCTAATCC-3′) (65). Amplicons for barcoded Illumina MiSeq analyses were produced as described in Sheremet et al. (66) using a MiSeq reagent kit v3 with 600 cycles (Illumina part number MS-102-3003). A maximum of 400 amplicon samples were sequenced per MiSeq lane, and a total 759 samples and 10,313,858 reads were processed.

Sequence data were analyzed using Quantitative Insights Into Microbial Ecology version 2 (QIIME2) (67). Primers and adaptor sequences were trimmed from all fastq files using cutadapt software (68), and DADA2 was applied to denoise, pair reads, and remove chimerae (69). A quality score was analyzed for 10 random samples per run to adjust denoising parameters. Reads were truncated at position 230 (forward reads) or 210 (reverse reads) from the 3′-end, and at position 20 from the 5′-end. Feature tables from various lanes were merged into a single amplicon sequence variant (ASV) feature table. Taxonomy was assigned using SILVA132 (70). All ASVs attributed to *Eukarya* (504,950 reads and 2,124 ASVs) were removed, leaving a total of 54,848 ASVs. Of these, 29 ASVs were classified to taxonomic genera or families known to contain only methanotrophs (Table S1). These 29 ASVs were used in most downstream analyses (GenBank accession numbers: OK217110–OK217124).

Phylogenetic construction. Phylogenetic constructions were performed using the ARB software (71). Sequences from 16S rRNA gene amplicon analysis were aligned using the ACT aligner of the SILVA website (https://www.arb-silva.de/aligner/) and added to the SILVA 138 SSU reference database (70). Some SILVA-based assignments of methanotrophic genera were corrected after phylogenetic construction.

For phylogenetic construction based on the methanotroph-specific *pmoA* gene, a reference database was used (48), and *pmoA* genes from BML added to this. The *pmoA* genes from BML were obtained from exploratory metagenomes sequenced and assembled as described in Table S3. Briefly, DNA extracts from BML surface water samples taken from Platform 1 on May 9, 2015, August 8, 2016, October 17, 2016, and March 15, 2017. DNA libraries were prepared using NexteraXT DNA Library Prep Kits (Illumina, Inc., San Diego, CA, USA), sequenced on partial Illumina MiSeq lanes using MiSeq reagent kit v3 (600 cycles) (Illumina, Inc., San Diego, CA, USA), and assembled using 4.SPAdes-3.8 metagenomic assembler software (72). BML metagenomes were queried for *pmoA* genes via HMMER searches (HMMER 3.3.2, http://hmmer.org/) on Prodigal gene prediction software.

***pmoA*-gene based quantitative PCR.** All aerobic methanotrophic genera detected in the 16S rRNA gene amplicon analyses are known to encode a particulate methane monooxygenase (pMMO). *Alphaproteobacteria* genera like *Methyloferula* and *Methylocella* (73) that use only a soluble methane monooxygenase (sMMO) enzyme for methane oxidation were found in only small numbers in BML (<0.5% for 99% of samples based on 16S rRNA relative abundance data). This suggests that methanotrophs in BML can be effectively quantified by targeting the *pmoA* gene, which encodes a subunit of pMMO. qPCR quantification of this gene has been extensively used to identify and quantify methanotrophs in diverse environments (43).

First, three qPCR assays designed by Kolb et al. (49) were chosen to target the major genera found in our amplicon analysis. These targeted (i) total proteobacterial methanotrophs (A189F: 5′-GGNGACT GGGACTTCTGG-3′; Mb661R: 5′-GGTAARGACGTTGCNCCGG-3′); (ii) *Methylobacter/Methylovulum/Methyloprofundus/Methylosoma* (mbac189F: 5′-GGNGACTGGGATTTCTGG-3′; Mb601R: 5′-ACRTAGT GGTAACCTTGYAA-3′); and (iii) *Methylococcus/Methyloparacoccus/Methylocaldum* (A189F: 5′-GGN GACTGGGACTTCTGG-3′; Mc468R: 5′-GCSGTGAACAGGTAGCTGCC-3′), as shown in Table S4. To verify that these primers designed in 2003 were still specific, they were compared with a *pmoA* database assembled from all methanotroph genomes available to 2019 (48), and to the *pmoA* genes retrieved from the BML metagenomes. This proved that these primers (49) were effective, with only one minor modification needed in the forward primer targeting the *Methylobacter* group (Table S4).

qPCR was performed in reactions containing 1 μL of sample gDNA, 5.0 μL of SYBR GreenPCR Mix (Qiagen, Venlo, the Netherlands), 1.25 μM each forward/reverse primer, and DNase-free water to make up to 10-μL (Qiagen, Venlo, the Netherlands), on a Rotor-Gene 6000 (Qiagen, Venlo, the Netherlands). Standards for the universal assay and the *Methylococcus/Methyloparacoccus/Methylocaldum* assay were constructed from the *pmoA* gene of *Methylococcus capsulatus* Bath, PCR-amplified with A189f/Mb661R as described above and cloned into a PJET 1.2 plasmid vector as per the CloneJET PCR Cloning Kit protocol (Thermo Scientific). The plasmid was extracted with a plasmid DNA mini-prep kit as per manufacturer instructions (Bio Basic Inc., Markham, Ontario, Canada). The extracted plasmids were quantified using a Qubit HS kit, and serially diluted over 6 orders of magnitude. Standards for the *Methylobacter/Methylovulum/Methyloprofundus/Methylosoma* assay were made in the same way, from *pmoA* genes PCR amplified from a methanotrophic bacterium designated *Methylobacter psychrophilus* strain FCN1. This strain was enriched from BML on medium 10 incubated at 8°C under a headspace containing 10% vol/vol CH_4 and 5% vol/vol CO_2 in air, as described previously (74).

Temperature response. To estimate the temperature response of methane oxidation, 75-mL aliquots of water from winter (March 12 to 14, 2018) and summer (August 26, 2019) were added to sterile 120-mL serum bottles. Bottles were capped with butyl rubber stoppers, supplemented with 10% (vol/vol) of CH_4, vigorously shaken to equilibrate the liquid with methane, and filled to the brim with more sample water, taking care to avoid air bubbles. The bottles were incubated at 0, 4, 8, 10, 15, 22, and 30°C. Sterile water was used as a control. After 24 h, 3 mL of water was removed from each bottle with a syringe and injected into an 11-mL capped serum bottle for estimation of dissolved CH_4. Vials were well-shaken and headspace CH_4 determined via gas chromatography on a Model 8610C GC (SRI Instruments, Torrance, CA, USA) equipped with a flame ionization detector (Column 190°C, detector 300°C, and N_2 as carrier gas) and a 0.1-mL sampling loop. The sampled 3 mL of water was replaced. Measurements were carried out at 2- or 3-d intervals until the methane was exhausted (14 to 33 d). Calculated methane oxidation rates were based on linear regression of initial, linear rates (generally 24 d). At the end of the incubation, the water was centrifuged at 8,000 g for 10 min and the pellet was used to isolate and process DNA as described above.

Data availability. The partial 16S rRNA genes representing the 29 putative methanotroph ASVs used in these analyses are available in GenBank as accession numbers OK217110–OK217124. The GenBank accession numbers of the partial 16S rRNA gene and *pmoA* gene of enriched *Methylobacter psychrophilus* strain FCN1 are OK135604 and OK157433, respectively. The metagenomes used to recover *pmoA* genes are available in the Joint Genome Institute Integrated Microbial Genomes (JGI IMG) database under the following JGI Sequencing Project IDs (May 2015, Gp0053501; October 2016, Gp0266534; March 2017, Gp0294137).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 6.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

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We declare no conflict of interest.

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