PEATLANDS

Methane Cycling Microbial Community Characteristics: Comparing Natural, Actively Extracted, Restored and Unrestored Boreal Peatlands

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Abstract
The microbial community in peat is responsible for organic matter degradation and greenhouse gas emissions, yet its response to peat extraction and peatland restoration remains poorly understood. We investigated how different physicochemical conditions in natural, restored, unrestored, and actively extracted peatlands influenced the methanogenic and methanotrophic community characteristics. 16S rRNA amplicon sequencing allowed for the comparison of the abundance of both groups. The communities were similar at sites restored in 1991 and 2009 (25 and 7 years prior to our research) that had high water table and were dominated by sedges. A different, shared pattern of microbial membership was observed at natural, unrestored, and actively extracted sites, and a site restored in 2012 (4 years prior to the research). These similarities were reflected in peat chemistry and hydrology in canonical correspondence analysis (CCA) ordination that was used to investigate how these factors affected the abundances of methane cycling taxa. Based on the CCA, methanotrophs reached their highest abundances close to the water table, at high and moderate concentrations of phosphate and propionate, and low concentrations of formate. Methanogens exhibited a more distributed pattern, with organisms responding to opposite conditions along these environmental gradients. Methane-cycling community in older restored sites departed from the reference community at the natural bog likely due to the fen-like conditions that developed at these sites.

Introduction

Wetlands are the largest natural methane (CH_4) emitters, contributing at least 20% of global CH_4 emission from all sources (Saunois et al. 2020). The amount of CH_4 released from these ecosystems is determined mainly by the balance between CH_4 production by methanogenic *Archaea* in waterlogged anoxic conditions and CH_4 oxidation by methanotrophic *Bacteria* in oxic conditions (Horn et al. 2003; Andersen et al. 2013a; Esson et al. 2016).

Among wetlands, peatlands represent a globally important store of carbon, estimated at 600 Gt while occupying only 3% of land area (Yu et al. 2011). Most peatlands (~67.5%) occur at high and mid latitudes in the northern hemisphere (Xu et al. 2018). These store almost one third of global soil carbon (455 – 547 Gt, Gorham 1991; Yu et al. 2010; Yu 2012) and confer a net cooling effect due to their removal of carbon from the atmosphere (Frolking et al. 2006). Based on process-based models, CH_4 emissions from

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the northern peatlands will reach 42 Tg C yr^{-1} by the end of this century (Zhao and Zhuang 2023), which is ~ 8.5% of total global emission of CH_4 from all sources as estimated with atmospheric inversion models (Saunois et al. 2020).

Peat is commercially extracted for a variety of reasons, including for use as a fuel for combustion (Vasander et al 2003). In Canada, the majority of peat is vacuum-harvested for use in horticulture (Environment Canada 2023). About 37 kha of Canadian peatlands and ~ 5.7 Mha of European peatlands have been disturbed by peat extraction (Vasander et al. 2003; Environment Canada 2023).

Peat extraction disturbs the stability of the oxic and anoxic zones and promotes accelerated mineralization of organic matter, which results in CO_2 emission, while CH_4 production either ceases or decreases (Sundh et al. 2000; Turetsky et al. 2002; Glatzel et al. 2004; Bonn et al. 2014; Esson et al. 2016). However, drainage ditches installed to lower the water table (WT) remain a source of CH_4 at peat extraction sites (Sundh et al. 2000; Waddington and Day 2007; Cooper et al. 2014; Nugent et al. 2018). During extraction, the top layers of peat are removed (Quinty and Rochefort 2003) and the remaining lower quality peat is poorly colonized by CH_4 producers and oxidizers (Basiliko et al. 2013).

Post-extraction peatland restoration aims to re-establish the natural hydrological conditions and peatland vegetation, with the primary goal of recovering a fully self-sustaining, functional ecosystem able to accumulate peat (Rochefort et al. 2003). Currently, the monitoring of post-restoration outcome is based mainly on plant community composition, diversity and richness (e.g., González et al. 2013; Boudreau and Rochefort 2008; Poulin et al. 2013; Grman et al. 2013; Ruiz-Jaen and Aide 2005). The microbial community is typically not considered as it falls beyond the scope of regular site monitoring or definition of restoration outcome. However, the microbial community can provide additional information about functional recovery (Andersen et al. 2006). Broad evaluation of the entire microbial community shows that restoration efforts encourage a shift in microbial characteristics of post-extracted peatlands towards pre-disturbance conditions (Basiliko et al. 2003, 2013; Andersen et al. 2006, 2010; 2013a, b; Bossio et al 2006; Artz et al. 2008; Reumer et al. 2018). Peatland vegetation, a source of fresh substrate at restored peatlands, is a strong determinant for the microbial community composition together with peat chemistry and the microhabitats present (Jaatinen et al. 2007; Lin et al. 2014; Robroek et al. 2015; Putkinen et al. 2018; Chroňáková et al. 2019).

Research focused on CH_4-cycling microbial communities in managed peatlands is very limited (Juottonen et al. 2012; Putkinen et al. 2018; Reumer et al. 2018). However, the hydrological and biogeochemical factors that generally influence CH_4 production and consumption are more thoroughly understood. Large WT fluctuations at extracted peatlands promote the regeneration of non-oxygen terminal electron acceptors (TEAs) (Lovley et al. 1996; Küsel et al. 2008; Gao et al. 2019). Since methanogenesis is thermodynamically less favourable compared to many other decomposition pathways (e.g., sulphate and nitrate reduction; Conrad 1999; Hausmann et al. 2016; Smeaton and Van Cappellen 2018), it can be suppressed if sufficient TEAs and organisms capable of their reduction are present.

Once stable redox conditions are reestablished during peatland restoration, the availability of carbon substrate is the most important factor controlling CH_4 production and oxidation (Couwenberg 2009; Ho et al. 2013; Reumer et al. 2018) coupled to the abundance of methanogens (Sun et al. 2012) and methanotrophs (Juottonen et al. 2012). The availability of carbon substrate for methanogenesis also depends on syntrophic bacteria that ferment complex molecules of organic matter into short chain fatty acid ions (e.g., lactate, acetate, succinate, butyrate, pyruvate, and propionate) that serve as electron donors in anaerobic metabolic reactions that further mineralize organic carbon (Min and Zinder 1990). Methanogenesis is the final stage of organic matter decomposition (Conrad 1999; Bridgham et al. 2013). Acetate is used as an electron donor in the acetoclastic methanogenesis pathway by members of the *Methanosaeta* and *Methanosarcina* (Schmidt et al. 2016) but most known methanogens use the hydrogenotrophic methanogenesis pathway (Galand et al. 2002; Horn et al. 2003; Bridgham et al. 2013). Some methanogens consume formate (Dedysh et al. 2004; Pan et al. 2016) but little is known about the role of formate in CH_4 production in peatlands (Hunger et al. 2011, 2016; Zalman et al. 2018).

Here, we investigate how hydrological conditions in the peat depth profiles, the presence/absence and type of peatland vegetation, the chemistry of peat, especially the presence of potential TEAs, and the availability of carbon substrate affect methanogenic and methanotrophic community characteristics in the upper 1 m and at the base of the peat deposit. We examine microbial community responses across post-extraction peatlands at different stages of management from current extraction (the Active site), through a post-extraction unrestored site (the Unrestored site), to sites restored at different times in the past: 2012, 2009, and 1991 (5, 7, and 25 years prior to our study, respectively) and a natural bog (hereafter referred to as the Natural site). We assess phosphate as a potential driver since the addition of phosphate can stimulate the abundance of methanogens and methanotrophs in soil (Gao et al 2020). Phosphate rock is used in peatland restoration as a fertilizer to initiate the growth of *Polytrichum strictum* that nurses *Sphagnum* moss (Quinty and Rochefort 2003; Pouliot et al. 2015). Additional variables such as pH, the concentration of DOC and carbon to nitrogen ratio (C:N) have been previously reported as

important factors driving microbial community structures in natural, extracted and restored peatlands, and are included in our investigation (e.g., Juottonen et al. 2012; Lin et al. 2012; Basiliko et al. 2013).

Our specific objectives were: 1) To characterize CH_4-cycling communities in peat columns based on their composition, abundance, and alpha and beta diversity considering the dominating vegetation type/bare peat and hydrological conditions, 2) To assess if there is a development of methanogenic and methanotrophic communities with time post restoration and if there are any similarities in CH_4-cycling communities among the sites, and 3) To determine how peat chemistry and hydrology drive the abundance of methanogenic and methanotrophic taxa.

Material and Methods

Study Site

The study was conducted at a horticultural peat extraction complex of peatlands near Seba Beach, Alberta, Canada (53° 28' 24.67" N, 114° 51' 43.33" W) in the boreal climate zone

(Köppen et al. 2011). Prior to peat extraction, the sites were a continuous boreal bog. Six sites were included in the research: an actively extracted site (the Active site), a site left unrestored for research purpose after extraction (the Unrestored site), three sites restored at different times in the past with moss layer transfer technique (Quinty and Rochefort 2003): in 1991 (RES-1991), 2009 (RES-2009), and 2012 (RES-2012) and an undisturbed bog (the Natural site) (Supplementary Fig. 1). Photographs of the sites are shown in Fig. 1 and the vegetation descriptions are provided in Supplementary Data. The depth of each peatland was assessed by ground penetrating radar (GPR) surveys (unpublished results).

The Natural site was a treed bog with 5.0 – 5.4 m peat deposit at the study location, characteristic hummocks and hollows and poorly decomposed *Sphagnum* peat. RES-1991 was one of the oldest peatlands restored in Canada. The peat deposit was 3.0 – 4.0 m deep. The site was inundated with a partially floating mat. Even in an extremely dry year, the WT remained at or slightly above the peat surface. The drainage ditches were blocked but not filled in. A mosaic of dense hummocks of *Sphagnum* and sedges dominated the peatland vegetation. Peat at RES-2009 was 1.5 – 2.5 m deep, largely covered with sedges, grasses and shrubs with overgrowing

Fig. 1 Pictures of the study sites in Seba Beach horticulture peatland complex, Alberta, Canada: the Natural site (**A**), restored in 1991 (RES-1991; **B**), restored in 2009 (RES-2009; **C**), restored in 2012 (RES-2012; **D**), Unrestored (**E**), and currently extracted site (Active; **F**)
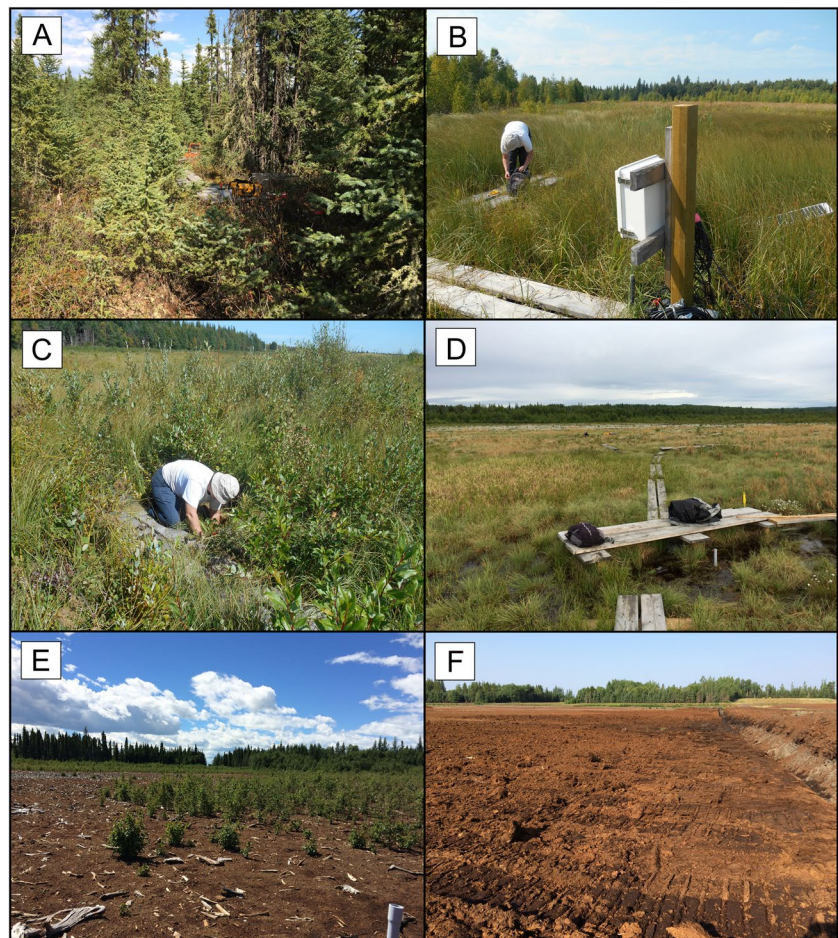
Sphagnum and other bryophytes. At RES-2012, the shallow (~ 1.5 m) and dry eastern part of the peatland gradually transitioned to deeper (> 3.0 m) peat with wet conditions in the western section. The wet part was severely flooded and inaccessible following heavy rain events. The Unrestored site was a section of a larger extracted site left unrestored in 2012 at which time the drainage ditches were filled up with peat and the surface levelled. No other restoration effort was undertaken. The perimeter ditch around the site remained functional, and natural peatland vegetation had not recovered at the time of this study. The great majority of the site remained bare, though birch and sedges progressively colonized the western side of the site and peatland margins. The peat deposit exceeded 2.5 m depth. Peat at the Active site was < 2 m deep at the sampling location, with active drainage ditches and poorly decomposed *Sphagnum* peat (H3 – H4 in Von Post scale) compacted by heavy machinery. The peat surface was stripped of vegetation.

Sampling

Peat samples for microbial community analysis were collected with a Russian corer in August 2016 during a period of high WT levels. At each site except the Active site, two cores were sampled targeting major peat surface cover types: sedge dominated (sedgy) and moss dominated (mossy) at the restored sites, bare peat and sedgy at the Unrestored site, and hummocks and hollows that were both covered with dense moss at the Natural site. Since a part of RES-2012 was flooded and a part of it dry, we sampled at a moderately wet location between these two extremes where single tussocks of cotton grass were scattered between patches of moss and bare peat. One core representing bare peat was sampled from the Active site. These sampling locations were selected to be representative of the dominant hydrological conditions and vegetation cover.

A total of 11 cores were collected for microbial community analysis using the Russian corer. Eleven cores for paired physicochemical analyses were taken within 20 cm of the cores obtained for microbial community analysis. Peat was sampled to a depth of 1 m. Additionally, one 10 cm long sample per core was taken from the greatest depth possible to sample, given the conditions at each site. Core extraction and sectioning into 10 cm long segments for microbial community analyses was conducted rapidly on site to ensure minimal exposure to ambient air. Accounting for sectioning with depth, a total of 119 samples were collected for microbial analysis. A further 119 samples at corresponding depths were collected to determine peat chemistry and physical properties. Microbial samples were packed into sterile polyethylene bags, immediately flash frozen in liquid nitrogen, transported the same day in dry ice and stored at -80 °C. All equipment was thoroughly washed with 70% ethanol before and between sampling. Corresponding peat samples for physicochemical analyses were packed in re-sealable zipper bags, immediately placed in coolers with ice, transported the same day and stored at -20 °C.

Peat Chemistry and Environmental Conditions

Over 1,300 WT measurements were collected in years 2016 and 2017. The WT was measured biweekly in 2016 and weekly during the 2017 growing season in water wells installed near each sampling location. Year 2017 was wet compared to 2016. The water table fluctuation zone (WTFZ) was calculated from the lowest and highest levels of the WT which provided information on the stability of the redox conditions for microbes at a given depth and site. Peat samples were assigned to one of three groups: below, within, or above the WT level measured at the time of sampling, as well as to one of the two groups: within or below the WTFZ (Supplementary Table 1).

The Von Post scale of peat humification was used to assess the degree of decomposition of peat (Government of Canada 2013). Peat porosity and particle density were calculated following Hao et al. (2008). The ratio of carbon to nitrogen (C:N) was calculated from total carbon (C) and total nitrogen (N) percentage concentration measured on ground, sieved through 500 μm mesh, and freeze-dried peat. The analysis was performed at the Agriculture and Food Laboratory at the University of Guelph, Ontario using the Elementar Vario Macro Cube. EC (μS cm^{-1}) and pH of peat were measured using a Hanna conductivity/pH meter in dried, ground samples inundated in deionized water in 1:15 ratio (w/w).

DOC (mg g^{-1} of dry peat) was measured using the non-purgeable organic carbon method on a Shimadzu TOC-LCPH/CPN equipped with a non-dispersive infrared (NDIR) gas analyzer. Details on sample preparation are given in the Supplementary Data. For the analysis of organic acid anions and inorganic anions (expressed in μg g^{-1} of dry peat), a part of the initial supernatant was filtered through a 0.2 μm polypropylene filter and stored at -20 °C prior to the analyses. We targeted formate (CHO_2^-), acetate ($CH_3CO_2^-$), lactate ($C_3H_5O_3^-$), succinate ($C_4H_4O_4^{2-}$), butyrate ($C_4H_7O_2^-$), pyruvate ($C_3H_3O_3^-$), propionate ($C_3H_5O_2^-$), citrate ($C_6H_5O_7^{3-}$), nitrite (NO_2^-), nitrate (NO_3^-), sulphate (SO_4^{2-}), and phosphate (PO_4^{3-}). Samples were analyzed with a Dionex (Thermo Fisher) ICS-5000 Capillary Ion Chromatograph. The calibration was made in external mode using triplicates of at least five different concentrations prepared gravimetrically from National Institute of Standards and Technology certified standards. The concentration of total oxidizable iron (Fe) in peat (hereafter referred to as Fe^{3+}) was determined using

the ferrozine method (Stookey 1970 modified according to Lovley and Phillips 1986; Viollier et al. 2000). Samples were exposed to ambient air to measure the Fe pool which is potentially available as an electron acceptor for micro-organisms due to the WT fluctuations and oxygen supply via roots (Metje and Frenzel 2005). Specific details of the method are provided in the Supplementary Data.

Samples for dissolved organic carbon (DOC), pH and electrical conductivity (EC) from sites restored in 1991 and 2009 were accidentally mislabelled and discarded after sampling.

Microbial Community

Deoxyribonucleic acid (DNA) was extracted from all peat samples using the MoBio PowerSoil DNA Isolation Kit. A total of 119 samples (all samples collected from the study sites) passed quality control measures and underwent ampli-con sequencing of the V4 region of the 16S ribosomal ribo-nucleic acid (16S rRNA) gene (U.S. Department of Energy Joint Genome Institute). See Supplemental Data for full extraction, quality control, and sequencing methods. We did not target the functional methanogenic and methanotrophic genes because the sequencing was a part of a larger 16S rRNA and internal transcribed spacer (ITS) project. Also, targeting the 16S rRNA gene enabled comparison of the size of the putative methanogenic and methanotrophic com-munities. Predictions of methanogenic and methanotrophic populations were based on the known taxonomic distribution of these traits.

The raw reads are available in the NCBI SRA database under BioProject number: PRJNA640052, BioSample num-bers: SAMN15297727—SAMN15297845. Microbial com-munity analyses were carried out in QIIME 2 v. 2018.11 and 2019.11 (Bolyen et al. 2019), including read quality process-ing, taxonomic assignment, and alpha diversity metrics (see Supplementary Data for detailed parameters and workflow).

We rarefied the amplicon sequence variants (ASV) tables to the depth of 28,000. A total of 56 samples were filtered based on this threshold, many of which showed poor ampli-fication and extremely low coverage. A total of 63 samples remained in the database (Supplementary Table 1). Poor amplification resulting in low number of sequence counts can be caused by inhibitors in the peat matrix, e.g., humic substances (Matheson et al. 2010). The methanogenic and methanotrophic communities were analysed based on the rarefied tables.

Heatmaps of the absolute abundance (frequency) of methanogens and methanotrophs on the rarefied dataset were made in QIIME 2 with taxa collapsed at the genus level. Abundances were normalized by adding a pseudocount 1 followed by a log10 transformation in QIIME 2.

Diversity and Statistical Analysis

Since the rarefaction of the ASV tables applied only to the molecular data, we could still use all samples including 56 samples that were filtered based on the ASV rarefaction threshold, to compare general physicochemical conditions at the sites. Shapiro–Wilk test was performed in R package ggpubr (Kassambara 2020) to determine if physicochemical parameters were normally distributed. Kruskal–Wallis one-way analysis of variance was used to determine if environ-mental factors varied significantly between sites. Descriptive statistics (n, mean, std) of physicochemical data were cal-culated for the rarefied samples using the R package Rmisc (Hope 2013). Limits of detection (LOD) and quantification (LOQ), accuracy and precision for the chemical analyses are provided in Bieniada et al. (2020). Citrate, butyrate, pyru-vate, lactate, and nitrite were excluded from further analyses due to $\geq 50\%$ of values below LOD.

Alpha diversity (the diversity within a sample) was cal-culated in QIIME 2. Three metrics were applied; two qual-itative richness metrics that are based on the presence or absence of taxa, which are the number of amplicon sequence variants (ASVs), and Faith's index that incorporates phylo-genetic relations between taxa (Faith 1992). Additionally, one quantitative metric was calculated, the Shannon's index, that accounts for taxa abundance and evenness (i.e., how similar the abundances of different taxa are in the commu-nity; Shannon and Weaver 1949). We determined if these indices were significantly different depending on the sam-ple location within or below the WTFZ using the non-par-ametric Kruskal–Wallis test on samples from all sites col-lectively. We could not determine the differences at each site separately due to low number of samples below the WTFZ ($n < 5$ in a rank causes Kruskal–Wallis H not to follow chi-squared distribution well; McDonald 2014).

The principal coordinates analysis (PCoA) of weighted and unweighted UniFrac (Lozupone and Knight 2005; Lozupone et al. 2007) was used to determine the methano-genic and methanotrophic communities' similarity between samples and sites (beta diversity). The weighted UniFrac is a quantitative phylogenetic measure of community beta diversity, while unweighted UniFrac is a qualitative measure. Both were calculated and visualized in R using the phyloseq package (McMurdie and Holmes 2013) with the tidyverse package (Wickham 2017) and qiime2R to read QIIME 2 output files (Bisanz 2018).

Canonical correspondence analysis (CCA) was per-formed using the vegan R package (Oksanen et al. 2019) to determine the relationship between environmental fac-tors and the recovery of CH_4-cycling microorganisms. Since the recovery of methanogenic and methanotrophic activity is reflected in the CH_4-cycling community abun-dance (Reumer et al. 2018), we used the rarefied sequence

count of microorganisms in the analysis. CCA models were validated according to Oksanen (2012) using an ordistep function. CCA graphs were made using ggplot2 (Wickham 2016), ggvegan (Simpson 2019), and ggrepel (Slowikowski 2019). The WT variable used for CCA analysis was the depth of peat in relation to the WT at the time of sampling, e.g., when the actual WT level was at 10 cm below the ground, the sample from 10 – 20 cm depth was assigned 0 cm, and the sample from 0 – 10 cm depth was assigned + 10 cm.

Results

Physicochemical and Hydrological Conditions

The WT fluctuated from -5.5 cm to -79 cm at the Natural site (with larger fluctuations at the hummock than at the hollow site), from + 20.5 cm to -60 cm at RES-1991, from + 10 cm to -87 cm at the RES-2009, from + 20 cm to -79 cm at RES-2012, from + 6.5 cm to -79 cm at the Unrestored, and from -3 cm to -67 cm at the Active site (Fig. 2). The Unrestored site had the lowest, while RES-1991 and the Natural site the highest peat porosities (Fig. 2, Table 1).

All physicochemical factors varied significantly between sites (Supplementary Table 2). The depth profiles of peat physicochemical properties had individual patterns for
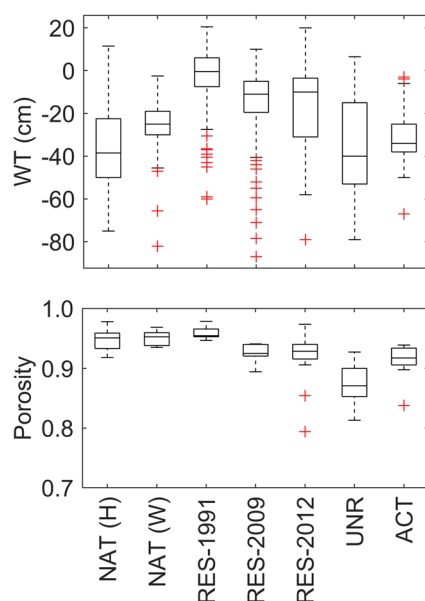

Fig. 2 Water table fluctuation and peat porosity at Seba Beach peatlands, Alberta, Canada. Natural site (NAT) is an undisturbed treed bog, RES-1991, RES-2009, and RES-2012 are peatlands restored post-extraction in the given years, UNR is a site left unrestored post-extraction, and ACT is an actively extracted site. H and W indicate microforms at the Natural site: hummocks and hollows, respectively

each site in most cases, with the highest $[PO_4^{3-}]$ in the surface peat of the Natural and all restored sites but not at the Unrestored and Active sites (Supplementary Table 1, see Bieniada et al. (2020) for the Active site). Among the 63 samples with adequate sequence counts for inclusion in the molecular analysis, the only sample from the Natural hummock (20 – 30 cm depth) had the highest [DOC] and pH (Supplementary Table 1).

The mean [DOC] was comparable at the remaining sites and surface cover types (moss or sedge dominated or bare peat) aside from a relatively low values found at the Active site and Unrestored sedgy location (Table 1). The lowest mean C:N ratios (<30) were found in sedgy cores of the Unrestored, RES-2009, and RES-1991 sites and in the only sample from the Active site (the depth of 70 – 80 cm). Both restored sites had fen-like conditions with shallow WT and dominance of sedges. These two sites were also the only ones where pH exceeded 5.0, while the remaining sites had lower pH (Supplementary Table 1). Higher C:N values (>30) were found in the Unrestored bare peat, Natural hollow, and RES-2012 sites, as well as in RES-2009 at 0 - 30 cm depth (Table 1 and Supplementary Table 1). The mean EC in most cases exceeded 200 µS cm^{-1}.

Total oxidizable iron was most abundant among all studied potential TEAs. Concentrations exceeding 1000 µg g^{-1} of dry peat were measured in the sedgy core from RES-2009 (Table 1, Supplementary Table 1). All Seba Beach restored sites had higher $[Fe^{3+}]$ than the Natural site. The highest $[Fe^{3+}]$ was found at RES-2009 (3063 µg g^{-1} of dry peat) in the bottom peat that was mixed with clay. High Fe^{3+} (~ 2000 µg g^{-1} of dry peat) was also found at 70 - 80 cm depth at the Unrestored site. Both sites also had relatively high concentrations of SO_4^{2-} (Table 1, Supplementary Table 1).

The Unrestored and Active sites were relatively poor in short chain fatty acid ions, while the restored sites were comparatively rich. Across all samples, acetate and formate were more abundant than propionate.

The Characteristics of CH_4-Cycling Microorganisms and their Development with Time Post Restoration

We obtained a total of 25,517 reads taxonomically classified to canonically methanogenic archaea, and 14,605 reads classified to known methanotrophic lineages, accounting for the total of 264 methanogen-associated and 127 methanotroph-associated ASVs. Methanogens were found in 60 of 63 samples, missing only from surface peat (0 – 10 cm) of the Unrestored bare peat core, the RES-2009 sedgy core (0 – 10 cm), and the sample from Natural hummock (20 – 30 cm, Supplementary Table 3). Methanotrophs were found in 61 of 63 samples, missing from Unrestored bare peat at

Table 1 Mean values and standard deviations of peat chemistry and physical properties in 63 peat samples from Seba Beach peatland complex (Alberta, Canada) with 16S rRNA sequence count above the rarefaction threshold (method: Illumina Tag sequencing)

Site		Natural Hollow	Natural Hummock	Restored 1991		Restored 2009		Restored 2012		Unrestored		Active
Surface		mossy	mossy	mossy	sedgy	mossy	sedgy	mossy	sedgy	bare peat	sedgy	bare peat
DOC (mg g⁻¹)	n	4	1	6	5	10	9	8	5	5	3	1
	mean	2.2	4.4	2.5	2.6	2.3	1.2	2.0	1.9	1.9	1.2	1.4
	std	0.6	NA*	0.9	0.7	1.0	0.4	0.4	0.2	0.5	0.3	NA*
C:N	n	4	1	0	5	0	9	8	5	5	3	1
	mean	34	52	NA	22	NA	19	37	31	32	15	20
	std	5	NA*	NA	3	NA	9	12	14	9	1	NA*
pH	n	4	1	0	5	0	8	8	5	5	3	1
	mean	4.1	3.7	NA	5.1	NA	5.1	4.4	4.4	4.0	4.6	4.6
	std	0.2	NA*	NA	0.1	NA	0.4	0.3	0.3	0.2	0.3	NA
EC (µS cm⁻¹)	n	4	1	0	5	0	8	8	5	5	3	1
	mean	184	286	NA	298	NA	248	233	220	244	182	140
	std	47	NA*	NA	72	NA	56	53	18	36	14	NA*
Von Post	n	4	1	7	5	10	10	9	6	6	4	1
	mean	4	3	4	3	4	4	3	4	4	5	3
	std	0	NA*	2	1	1	1	0	1	1	0	NA*
Porosity	n	15		11		11		17		12		10
	mean	0.95		0.96		0.92		0.92		0.85		0.91
	std	0.02		0.01		0.02		0.04		0.10		0.03
WT (cm)	n	139	132	242		269		218		292		35
	mean	−26	−36	−3		−14		−16		−36		−31
	std	12	18	13		14		19		22		16
WT at the time of sampling (cm)		−30	−17	0	0	0	0	−7	−7	−30	−30	−3
Acetate (µg g⁻¹ of dry peat)	n	4	1	7	5	10	10	9	6	6	4	1
	mean	66	41	30	29	20	23	24	28	12	17	36
	std	48	NA*	22	19	10	8	7	7	8	3	NA*
Formate (µg g-1 of dry peat)	n	4	1	7	5	10	10	9	6	6	4	1
	mean	8	6	13	13	22	18	44	39	11	50	31
	std	3	NA*	8	7	13	20	24	19	13	17	NA*
Propionate (µg g⁻¹ of dry peat)	n	4	1	7	5	10	10	9	6	6	4	1
	mean	5	7	7	9	2	6	5	12	4	3	44
	std	3	NA*	3	6	2	4	7	8	5	2	NA*
NO₃⁻ (µg g⁻¹ of dry peat)	n	4	1	7	5	10	10	9	6	6	4	1
	mean	4	4	3	3	3	6	14	3	6	2	8
	std	1	NA*	1	2	1	5	22	0	4	0	NA*

Table 1 (continued)

Site		Natural Hollow	Natural Hummock	Restored 1991	Restored 2009	Restored 2012	Unrestored	Active
SO_4^{2-} (µg g^{-1} of dry peat)	n	4	1	7	10	9	6	1
	mean	42	101	55	58	40	322	38
	std	10	NA*	23	48	33	84	NA*
PO_4^{3-} (µg g^{-1} of dry peat)	n	4	1	7	10	9	6	1
	mean	22	170	24	2	36	9	1
	std	20	NA*	30	2	83	7	NA*
Fe^{3+} (µg g^{-1} of dry peat)	n	4	1	7	10	9	6	1
	mean	44	33	444	799	271	257	8
	std	44	NA*	336	620	303	92	NA*

DOC dissolved organic carbon, *C:N* total carbon to nitrogen ratio, *EC* electrical conductivity, *WT* water table. The Natural site is an undisturbed treed bog, Restored 1991, 2009, and 2012, are post-extraction restored sites with restoration completed in given years, Unrestored is a site left unrestored post-extraction, and Active is an actively extracted site. Porosity is calculated at the site level.

*NA – one sample collected

30 – 40 cm depth and from the Natural hummock sample (20 – 30 cm, Supplementary Table 4). Due to poor amplification, the Natural profiles were limited to five depths and the Active profile to one depth. Based on 16S rRNA gene amplicon sequencing, the sum of both methanotrophs and methanogens accounted for up to 8.4% of the rarefied bacterial and archaeal community in individual samples (mean 2.3%; 100% = 28,000; Supplementary Fig. 2).

Methanogens and methanotrophs were distributed along depth patterns characteristic for each site and for the taxa (Fig. 3, Supplementary Tables 3 and 4). The rarefied sequence counts of methanogens generally increased below the WTFZ or at the lower boundary of the WTFZ, where the WT was relatively stable, while that of methanotrophs were less dependent on the WTFZ.

The dominating vegetation type or bare peat had a limited (site-specific) effect on methanogenic and methanotrophic abundances and diversities. For example, an abrupt large increase in the sequence count of both methanogens and methanotrophs at 10–20 cm depth at RES-1991 was observed only at the sedge-dominated location (Fig. 3, Supplementary Table 3 and 4). The differences between diversity indices at mossy and sedgy locations were more pronounced for methanogens than methanotrophs (Figs. 4 and 5). Alpha diversity was consistently higher at sedgy than at mossy locations at RES-1991 (Faith's index of methanogenic community; richness) and at the Unrestored site where bare peat was contrasted with spontaneous revegetation (all indices for methanogens and most for methanotrophs) (Figs. 4 and 5). The methanogenic community at moss dominated location was generally more diverse than at the sedgy location at RES-2009 (Fig. 4), while no clear patterns were found among methanotrophs at that site (Fig. 5).

The Characteristics of Predicted Methanogenic Communities

A fairly large portion of the methanogenic community (12 of 21 identified organisms) in Seba Beach peatlands had no cultured relatives. The majority of methanogens (17,534 methanogen-associated sequence counts in all samples; 19 taxa of 21 identified methanogenic lineages) belonged to the classes *Methanobacteria*, *Methanomicrobia*, *Thermococci*, and *Thermoplasmata* within the phylum *Euryarchaeota* (see Table 2 for taxon code names in alphabetical order). *Methanomicrobiales* dominated the methanogenic communities in Seba Beach sites. *Methanoregula* (MG7) in the order of *Methanomicrobiales* and *Bathyarchaeia* (MG1) were the most abundant methanogens found across all sites (Fig. 3 and Supplementary Table 3). RES-1991 site was most abundant in methanogens, followed by RES-2009, and contained almost all methanogenic taxa identified in our study including
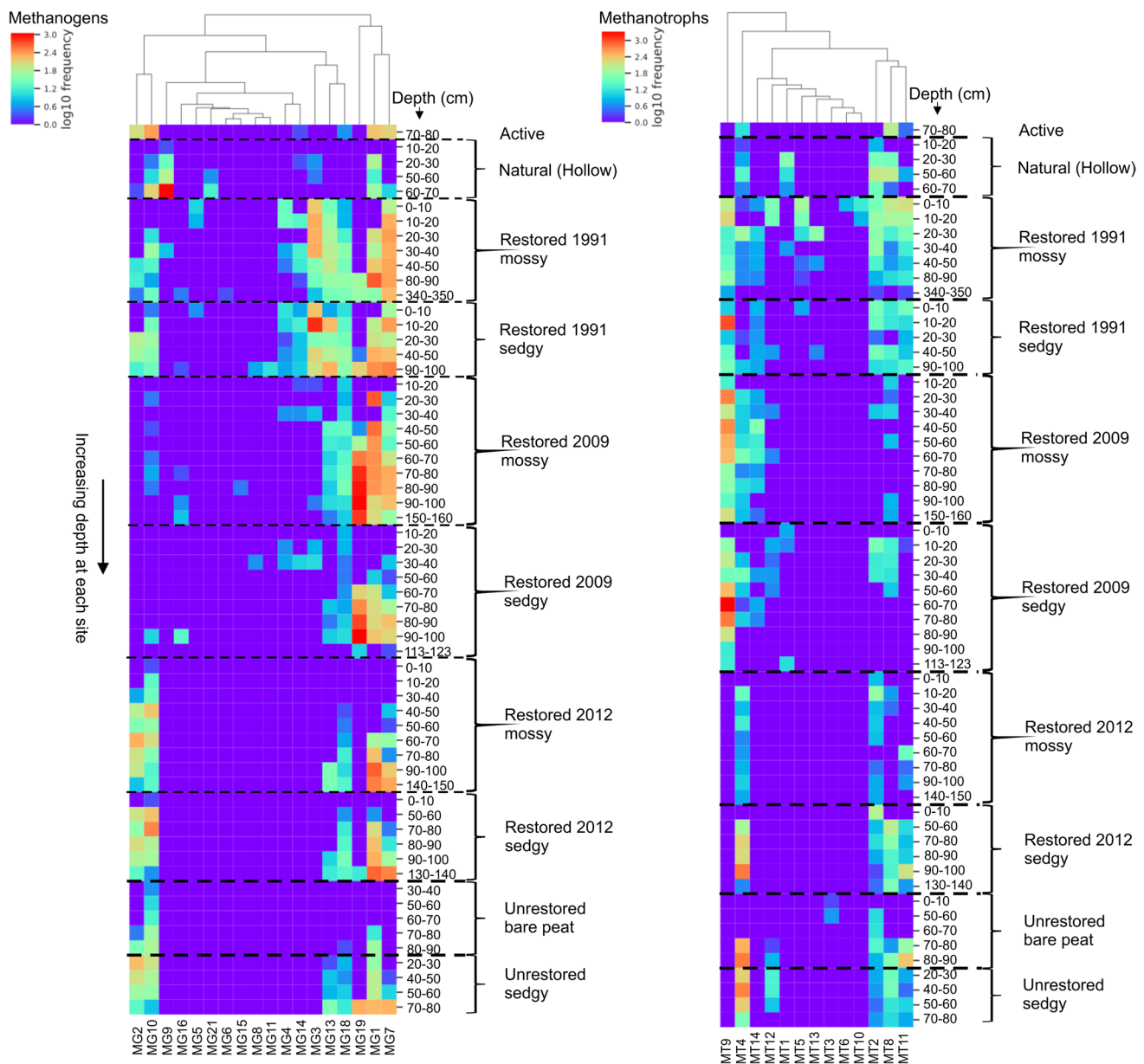
Fig. 3 Heatmaps of predicted methanogen and methanotroph log-transformed frequencies in the normalized dataset from peat collected from Seba Beach peatlands, Alberta, Canada. Three taxa (uncultured Methanomicrobiales, Methanofastidiosales, and Methanomassiliicoccles), identified only to the level of order, are not shown. See Table 2 for the explanation of codes for methanogens and methanotrophs

acetoclastic genera *Methanosaeta* and *Methanosarcina* that were not observed at the Natural site. The abundance of methanogenic taxa was substantially lower at the Natural bog than at these two restored sites. The community at the Natural peatland was dominated by *Methanosarcina sp.* of Rice Cluster II and an uncultured archeon from Rice Cluster II. With data from only one depth (70 – 80 cm) at the Active site, we were unable to characterize methanogen abundance and composition at that site or to compare it with the remaining sites. The Unrestored sedgy location had similar methanogenic community

characteristics as RES-2012. The core collected at the bare peat of the Unrestored site contained only some of the putative methanogens found at the sedgy location (Fig. 3, Supplementary Table 3).

The alpha richness and diversity metrics were similar at all sites aside from RES-1991 where their values were notably higher (Fig. 4). Thus, we did not observe a clear increase in the diversity within the samples with the age post-restoration. Alpha diversity was similar at the youngest restored site, the Unrestored site, and the Natural site.

Table 2 Methanogenic Archaea and methanotrophic Bacteria identified in peat samples from Seba Beach peatland complex, Alberta, Canada with sequence count above the rarefaction threshold and their codes. Method: Illumina Tag amplicon sequencing of 16S rRNA gene

Taxon	Code
Archaea; Crenarchaeota; Bathyarchaeia; uncultured methanogenic archaeon	MG1
Archaea; Crenarchaeota; Verstraetearchaeia; Methanomethyliales; Methanomethyliaceae; Candidatus Methanomethylicus	MG2
Archaea; Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobacterium	MG3
Archaea; Euryarchaeota; Methanomicrobia; Methanocellales; Methanocellaceae; Methanocella	MG4
Archaea; *Euryarchaeota*; *Methanomicrobia*; *Methanocellales*; *Methanocellaceae*; Rice Cluster I	MG5
Archaea; *Euryarchaeota*; *Methanomicrobia*; *Methanocellales*; uncultured; uncultured archaeon	MG6
Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanoregulaceae; Methanoregula	*MG7*
Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanospirillaceae; Methanospirillum	MG8
Archaea; *Euryarchaeota*; *Methanomicrobia*; *Methanomicrobiales*; Rice Cluster II; *Methanosarcina sp.*	MG9
Archaea; *Euryarchaeota*; *Methanomicrobia*; *Methanomicrobiales*; Rice Cluster II; uncultured archaeon	MG10
Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; uncultured; uncultured *Methanomicrobiales* archaeon	MG11
Archaea; Euryarchaeota; Methanomicrobia; Methanomicrobiales; uncultured	MG12
Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosaetaceae; Methanosaeta	MG13
Archaea; Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanosarcina	MG14
Archaea; Euryarchaeota; Thermococci; Methanofastidiosales; uncultured; uncultured archaeon	MG15
Archaea; Euryarchaeota; Thermococci; Methanofastidiosales; uncultured; uncultured bacterium	MG16
Archaea; Euryarchaeota; Thermococci; Methanofastidiosales; uncultured	MG17
Archaea; Euryarchaeota; Thermoplasmata; Methanomassiliicoccales; Methanomassiliicoccaceae; uncultured	MG18
Archaea; Euryarchaeota; Thermoplasmata; Methanomassiliicoccales; uncultured; uncultured *Thermoplasmatales* archaeon	MG19
Archaea; Euryarchaeota; Thermoplasmata; Methanomassiliicoccales; uncultured	MG20
Archaea; Euryarchaeota; Thermoplasmata; uncultured; uncultured methanogenic archaeon	MG21
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Beijerinckiaceae*; *Methylocella*	MT1
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Beijerinckiaceae*; *Methylocystis*	MT2
Bacteria; *Proteobacteria*; *Alphaproteobacteria*; *Rhizobiales*; *Beijerinckiaceae*; *Methyloferula*	MT3
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylococcaceae*; *Candidatus Methylospira*	MT4
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylococcaceae*; *Methylocaldum*	MT5
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylococcaceae*; *Methylomagnum*	MT6
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylococcaceae*	MT7
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; *Crenothrix*	MT8
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; *Methylobacter*	MT9
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; *Methyloglobulus*	MT10
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; *Methylomonas*	MT11
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; *Methylovulum*	MT12
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; pLW-20	MT13
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*; uncultured	MT14
Bacteria; *Proteobacteria*; *Gammaproteobacteria*; *Methylococcales*; *Methylomonaceae*	MT15

The richness (observed ASV and Faith's index) of methanogens generally increased with depth (Fig. 4), which was expected, as methanogens prefer stable waterlogged conditions. The Kruskal–Wallis pairwise comparison showed that observed ASV ($n_{Below} = 14$, $n_{Within} = 46$, $p = 0.004$, $H = 8.05$), Faith's index ($p = 0.004$, $H = 7.92$), and Shannon's index ($p = 0.021$, $H = 5.34$) were significantly higher in samples from below than from within the WTFZ. This analysis includes all 63 samples from all sites and it should be interpreted with caution, since the number of samples is representative for both above and below WTFZ only at the restored sites (Fig. 4).

Beta diversity showed some similarities between sites. The weighted UniFrac beta diversity PCoA (Fig. 6A), which takes into account the relative abundance of taxa, clustered RES-1991 and RES-2009 next to each other in the ordination. The clustering by depth of peat or its location in relation to the WTFZ was not observed and is not shown in figures. In the unweighted UniFrac PCoA (Fig. 6B), which is based only on the presence or absence of taxa, the majority of the samples formed a cluster with site transition from the oldest to the youngest restored (RES-1991 through RES-2009 to RES-2012). Sites RES-2009 and RES-1991
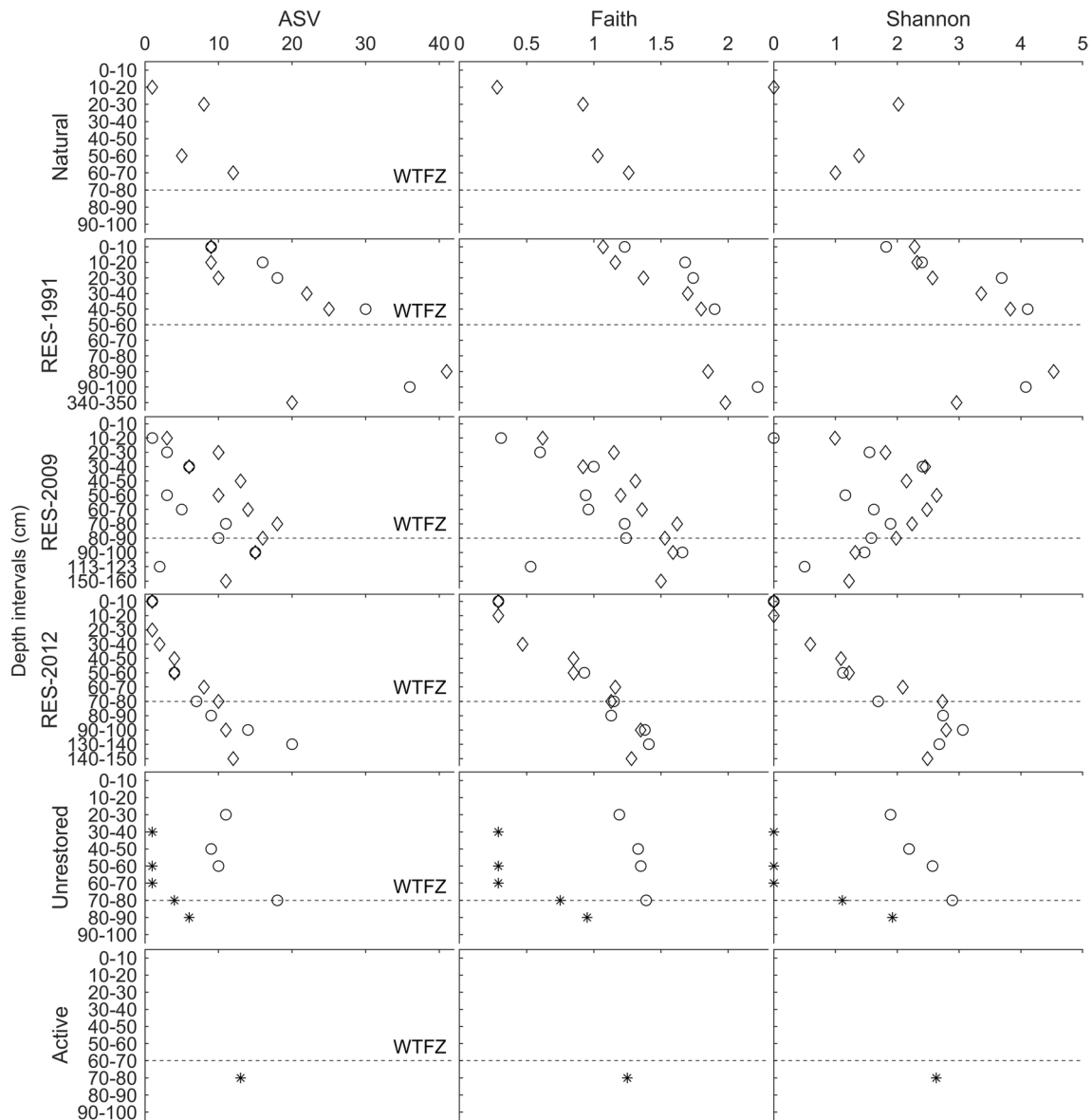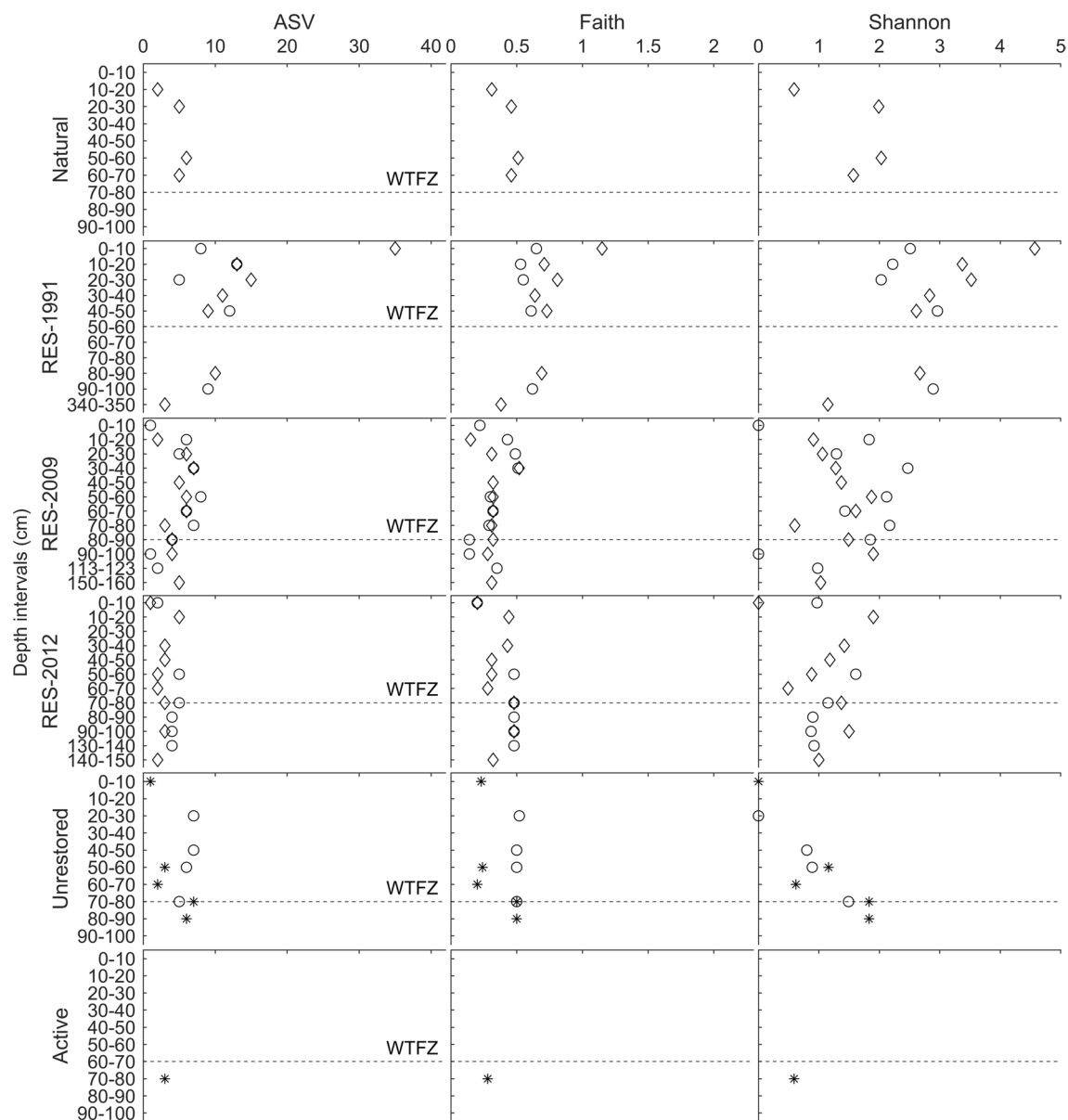
Fig. 4 Alpha richness and diversity metrics of predicted methanogens at the peat extraction Active site, post-extraction Unrestored, restored in 1991 (RES-1991), in 2009 (RES-2009), in 2012 (RES-2012), and the Natural sites in Seba Beach horticulture peat complex in Alberta, Canada. Diamonds are moss-dominated locations, circles are sedge-dominated locations, stars are bare peat locations. ASV - amplicon sequence variants; WTFZ - the lower boundary of the water table fluctuation zone

had some similarities among shallow peat samples (down to 30 – 40 cm depth) that created a separate cluster in the lower parts of the ordination figure (Fig. 6B, depths not shown). This transition in taxa composition from the oldest to the youngest restored sites could be an indicator of methanogenic community development with time post-restoration especially in deep peat layers. Both weighted and unweighted UniFrac showed similarities in the methanogenic communities at RES-2012, Unrestored, Active and Natural sites; these sites were clustered together, but not entirely separately from RES-2009 and RES-1991.

The Characteristics of Predicted Methanotrophic Communities

All identified methanotrophs were members of the phylum *Proteobacteria* (see Table 2 for taxon code names in alphabetical order). The methanotroph-associated sequence count was the highest at RES-1991, which contained 14 of 15 identified methanotrophs, followed by RES-2009. The remaining sites (RES-2012, Unrestored, Natural, and peat from the Active site at 70 – 80 cm) had smaller methanotrophic communities. The methanotrophic community across

Fig. 5 Alpha richness and diversity metrics of predicted methanotrophs at the peat extraction Active site, post-extraction Unrestored, restored in 1991 (RES-1991), in 2009 (RES-2009), in 2012 (RES-2012), and the Natural sites in Seba Beach horticulture peat complex in Alberta, Canada. Diamonds are moss-dominated locations, circles are sedge-dominated locations, stars are bare peat locations. ASV - amplicon sequence variants; WTFZ - the lower boundary of the water table fluctuation zone

all samples was dominated by *Gammaproteobacteria*. The largest abundance of *Alphaproteobacteria* was found at the Natural site.

Each site had its unique composition of methanotrophs that were distributed more uniformly than methanogens; however, similarities between sites in methanotrophic abundances and composition of taxa were observed between RES-2012 and the Unrestored site, as well as between RES-1991 and RES-2009 (Fig. 3, Supplementary Table 4). The values of alpha diversity metrics were more uniform across the depths in peat profiles than those of

methanogens (Fig. 5). The highest values were observed at RES-1991, while the values for other sites were lower and similar to each other. Only at the Unrestored site the evenness of methanotrophic community increased with depth (Shannon's index; Fig. 5). Alpha diversity indices did not indicate microbial community development with time post-restoration.

The Kruskal–Wallis pairwise comparison of observed ASV, Faith's and Shannon's indices for the methanotrophic community indicated no significant differences ($p > 0.05$) between samples from within and below the WTFZ. We found
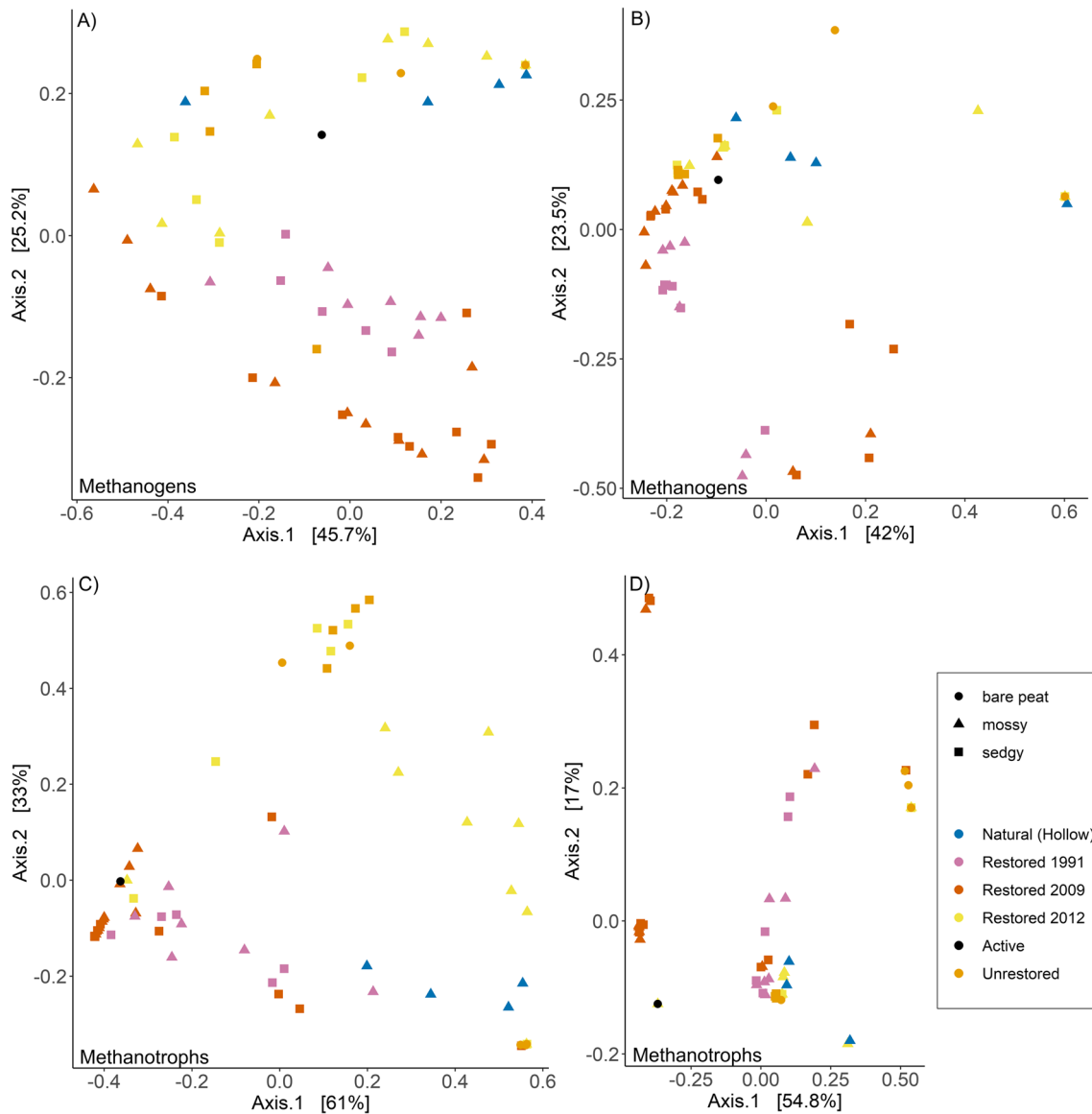
Fig. 6 Weighted (**A** and **C**) and Unweighted (**B** and **D**) UniFrac beta diversity measures for methane cycling microorganisms in the actively extracted (Active), post-extraction unrestored (Unrestored), restored at different times (in 1991, 2009, and 2012), and undisturbed (Natural) peatlands at Seba Beach horticulture peatland complex, Alberta, Canada

methanotrophs even at depths where peat was waterlogged, e.g., *Methylobacter* (MT9) that was abundant in deep peat at RES-1991 and RES-2009 and dominated methanotrophic communities at these sites.

The weighted UniFrac PCoA of methanotroph-related communities showed similarities between RES-2009 and RES-1991, while the majority of RES-2012 samples clustered separately together with the Unrestored site (Fig. 6C). The communities at the Natural site had highest similarity to each other but were not separated from communities from other sites in the ordination. The unweighted UniFrac (Fig. 6D) did not show any meaningful similarities in methanotrophic communities between sites. The weighted Unifrac

(the relative abundances of CH_4-cycling taxa) was a better measure of similarities in microbial communities among sites than the unweighted UniFrac (presence or absence of taxa). Consistently, RES-2009 and RES-1991 were similar while the youngest restored RES-2012 site clustered closer to the Unrestored site.

The Relationships Between the Environmental Conditions and the Rarefied Sequence Count of CH$_4$-Cycling Microorganisms

The CCA model validation excluded some explanatory variables (DOC, Von Post index, pH, [acetate], [SO_4^{2-}],

and [NO_3^-]) as redundant leaving C:N, the distance of the sample to the WT, [formate], [propionate], [PO_4^{3-}] and [Fe^{3+}] in the model (Fig. 7A). The concentrations of PO_4^{3-}, Fe^{3+}, and formate, as well as C:N were the dominant factors controlling the sequence count of identified taxa. Total oxidizable iron divided the ordination into two groups: one with high [Fe^{3+}] at RES-2009 and one with low [Fe^{3+}] but high values of all other factors at the remaining sites (Fig. 7B).

Methanogens tolerated a variety of chemical conditions in peat, with individual preferences depending on the methanogenic taxa. Some methanogens, e.g. *Cand*. Methanomethylicus (MG2), and uncultured methanogens MG10 and MG15 were likely to reach their highest abundance at conditions prevailing at the youngest restored, the Unrestored, and the

Active sites at depths greater than 20 – 30 cm (Fig. 7A, B, C). A great majority of methanogens and methanotrophs would not reach their highest abundances in conditions prevailing at RES-2009 that were dominated by high concentrations of Fe^{3+} (Fig. 7A, B). One methanogen, *Methanospirillum* (MG8) and one methanotroph, *Methylobacter* (MT9) would tolerate moderately high concentrations of this ion in exchange of relatively fresh, good quality organic matter (low C:N ratio). A group of uncultured methanogens (MG6, MG16, MG17, MG19, MG20) created a separate cluster and were likely to reach their highest abundances in conditions observed at RES-2009 at depths greater than 60 – 70 cm (Fig. 7A, C). They were only weakly associated with total oxidizable iron and formate but relied on stable hydrological conditions.
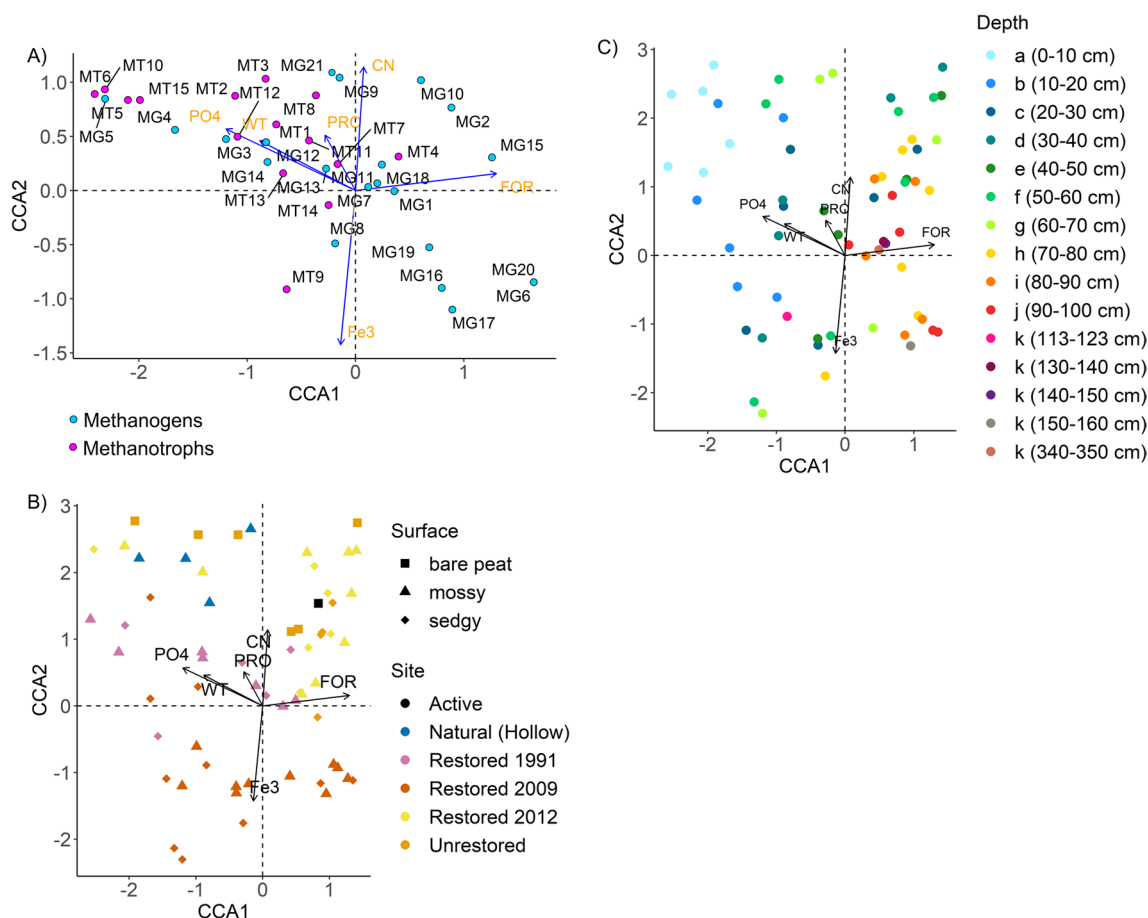
Fig. 7 Canonical correspondence analysis (CCA) of physicochemical peat properties and their relationship with the normalized sequence count of predicted methanogens and methanotrophs. Samples were collected at three post-extraction sites restored in 1991, 2009, and 2012, a site left unrestored post-extraction (Unrestored), an actively extracted site (Active), and an undisturbed treed bog (Natural) at Seba Beach horticulture peatland complex in Alberta, Canada. Fe3 – oxidizable iron, PO4 – phosphate, PRO – propionate, FOR – formate, CN – total C:N ratio, WT – depth of peat in relation to the water table

level at the time of sampling. See Table 2 for the explanation of codes for methanogens and methanotrophs. Depths 0–100 cm are marked with letters a – j and are common for all sites. Depths marked with letter k are the greatest depth possible to sample at individual sites: 113–123 cm at the site restored in 2009 at the sedgy location, and 150–160 cm at the mossy location; 130–140 cm at the site restored in 2012, sedgy location, 140–150 at the mossy location; 340–350 cm at the site restored in 1991, mossy location

Many methanogens, e.g., those most closely related to *Methanobacterium*, *Methanocella* and Rice Cluster I (MG3, MG4, MG5, respectively), as well as acetoclastic methanogens (MG13, MG14) are likely to reach their highest abundances at high concentrations of PO_4^{2-} and close to the WT in shallow peat (<50 cm) at RES-1991. Acetoclastic methanogens also prefer high concentrations of propionate. Most methanotrophs would reach their highest abundances at similar conditions at all peatlands (except RES-2009) even at lower quality organic matter (high C:N).

The similarities in geochemical conditions at the Natural, Unrestored, and RES-2012 sites (Fig. 7B) were aligned with the similarities in their CH_4-cycling communities (Fig. 6). Generally, the weighted UniFrac for methanogens (relative abundance of taxa; Fig. 6A) and the CCA analysis (Fig. 7B) that includes the sequence count of each methanogenic and methanotrophic taxa, had similar site transition in the ordination plots with RES-2009 and RES-1991 close together and slightly apart from the remaining sites that were not separated from each other.

Discussion

General Characteristics of Putative Methanogens and Methanotrophs in Peat Profiles

Methanogens identified in Seba Beach sites have been previously found in other bogs and fens (Galand et al. 2005; Metje and Frenzel 2005; Godin et al. 2012; Söllinger et al. 2015) and in restored and unrestored post-extraction peatlands (Basiliko et al. 2013). The prevalence of *Methanomicrobiales* is common in peatlands (Horn et al. 2003; Cadillo-Quiroz et al. 2006; Dettling et al. 2007; Høj et al. 2008; Godin et al. 2012). *Methanoregula,* known for its abundance in acidic bogs (Sun et al. 2012; Reumer et al. 2018), forms the majority of the archaeal community in terrestrial ecosystems together with potentially symbiotic *Bathyarchaeia* (Xiang et al. 2017), also present in Seba Beach peatlands. Rice Cluster II methanogens (MG9 and MG10), that dominated at the Natural site, have been identified in northern bogs (e.g., Cadillo-Quiroz et al. 2006) and fens (e.g., Godin et al. 2012). Acetoclastic *Methanosaeta* present in a fen-like RES-1991 is a dominant fen taxa (Dettling et al. 2007).

The composition of the methanotrophic communities at Seba Beach sites was similar to those reported by Narrowe et al. (2017) in wetland soils. Type II methanotrophs (*Alphaproteobacteria*) abundant at Seba Beach Natural site were identified as tolerant to the acidic environment in natural peatlands and to low temperatures, but prefer stable conditions (Ho et al. 2013; Putkinen et al. 2018). They have been found in boreal fens and acidic bogs (Dedysh 2009; Yrjälä

et al. 2011; Juottonen et al. 2012; Esson et al. 2016). At higher pH (5.0 – 6.0), which was measured at RES-1991 and RES-2009 sites, type I methanotrophs (*Gammaproteobacteria*) become active and typically both type I and II metabolize CH_4 (Dedysh 2009; Ho et al. 2013). *Methylobacter* was abundant in deep waterlogged peat at RES-1991 and RES-2009. It is a versatile genus and the most abundant key taxon for CH_4 oxidation in wetlands, able to survive anoxic conditions (Smith et al. 2018).

We did not identify any microorganisms phylogenetically close to archaeal anaerobic CH_4 oxidizers (ANME). They have been sporadically found in peatlands (Raghoebarsing et al. 2006; Etto et al. 2012). Although quantitative studies on anaerobic CH_4 oxidation (AOM) show its importance as a CH_4 sink in wetlands, the processes are not yet completely understood (Smemo and Yavitt 2007; Zhu et al. 2012; Gupta et al. 2013; Hu et al. 2014; Segarra et al. 2015; Miller et al. 2019).

The presence of methanotrophs in waterlogged peat can be explained with oxic microsites in the bulk anoxic peat, related to WT fluctuations and trapped air bubbles (Baird et al. 2004) or to oxygen supplied by the root system of vascular plants (Ström et al. 2005; Strack et al. 2006; Bridgham et al. 2013; Popp et al. 2000). Also, water table fluctuation at disturbed peatlands can shift the location of microorganisms in a peat profile (Andersen et al. 2013a, b). For example, at the Unrestored site, four out of six methanotrophic taxa present in the bare peat core, *Cand.* Methylospira, *Crenothrix*, *Methylomonas*, and *Methylovulum*, were not found in the top 70 cm of the core. Their largest abundances were detected below the WTFZ where they could have been forced by intensively fluctuating WT (Fig. 2). Some microorganisms identified with Illumina Tag can exist in an inactive state, since the method does not distinguish between active and inactive cells. However, methanotrophs are quite resilient. They can survive changes from oxic to anoxic conditions (Roslev and King 1996) and are able to respond to changes in WT fluctuations (resume their activities) within days, while methanogens require months (Blodau and Moore 2003). Hence, a relatively uniform distribution of methanotrophs in peat profiles was reflected in their abundance and diversity compared to that of methanogens at Seba Beach sites, even though methanotrophs preferred shallow peat (Fig. 7). Previous studies indicate that their activity has been predominantly observed around the oxic-anoxic boundary where oxygen and CH_4 are readily available (Sundh et al. 1995; Segers 1998; Clymo and Bryant 2008).

Methanogens preferred waterlogged conditions at Seba Beach sites, but they were also present in shallow peat. Post-extraction peat has high water retention due to its small pore size (Waddington and Price 2000), meaning that it can form anoxic microsites above the WT (Estop-Aragonés et al. 2013) that could potentially sustain

anaerobic microbial metabolisms. There is, to the best of our knowledge, no published research on methanogenic activity specifically in these microsites, but redox zonation leading to anaerobic metabolism within anoxic microsites is well established (Pallud et al. 2010; Prietzel et al. 2010; Rezanezhad et al. 2017).

Relatively stable hydrology at the oldest restored site (RES-1991) allowed for colonization by vascular plants (sedges) that supply labile carbon for methanogens in a form of root exudates and litter (Tuittila et al. 2000a; Bridgham et al. 2013; Markelova et al. 2018) and can affect CH_4-cycling microorganisms once appropriate redox conditions were established. Indeed, we observed an abrupt increase in methanogenic and methanotrophic sequence counts in shallow peat (rhizosphere) in the presence of sedges. Sedges were also likely associated with better development of methanogenic community at the Unrestored site when compared to the bare peat. Still, the potential rate of CH_4 production in the spontaneously revegetated part of the Unrestored site was close to zero (Bieniada et al. 2021). Without the restoration effort, the recovery of the CH_4-cycling microorganisms and their activity appears to be slow regardless of some evidence of microbial community development. At the restored sites RES-1991 and RES-2009, well developed *Sphagnum* in the absence of sedges was associated with higher abundance of methanogenic and methanotrophic communities (Supplementary Table 3). Putkinen et al. (2018) found a link between *Sphagnum* recovery at restored peatlands and the methanogenic and methanotrophic abundance, community structure, and activity, depending on the age of the restored sites.

Notably higher diversity at a fen-like RES-1991 than at other sites was in agreement with previous studies where methanogenic communities were more diverse in fens than in bogs, but the diversity reported in other studies was site specific and depended on peat chemistry (e.g.,Galand et al. 2005; Juottonen et al. 2005; Dettling et al. 2007; Juottonen 2020). Shannon diversity values at RES-1991 were twice as high as reported for a range of peatlands in North America (Yavitt et al. 2012). Although, it should be noted that alpha diversity indices have their limitations and should be compared rather within one dataset, e.g., they are sensitive to technical aspects of molecular analyses such as sequencing depth and rarefication depth.

The Development of CH_4-Cycling Communities with Time Post Restoration

The inclusion of sites restored at different times in the past prompted us to investigate if the development of CH_4-cycling communities progressed with time post restoration. Beta diversity clustering indicated possible progression in the relative abundance and the composition of the methanogenic communities (Fig. 6). Lack of clear separation of the sites in the UniFrac ordination of putative methanotrophic and methanogenic communities was likely linked to the history of the sites being one continuous peatland prior to the disturbance (Yavitt et al. 2012).

Older restored sites at the Seba Beach complex had different composition and diversity of CH_4-cycling communities than at the natural site, which was also observed by Reumer et al. (2018), but these patterns are likely site-specific or dependent on the restoration method. For example, Juottonen et al. (2012) found only moderate differences in methanogenic community composition and no difference in methanotrophic community composition between natural and 10–12 year old sites restored by filling in the drainage ditches with peat.

Recent studies in restoration ecology are dedicated to developing methods to predict time to full recovery and monitor progress at restored sites (Waldén and Lindborg 2016; Brudvig 2017; Brudvig et al. 2017; Laughlin et al. 2017). A new method, the ordination-regression based approach (ORBA), emerged to track ecosystem recovery post-restoration and to predict a time to recovery (Rydgren et al. 2019, 2020; Auestad et al. 2020). Initial tests showed that our sites were not a good fit for ORBA due to limited number of cores analyzed and lack of an appropriate reference site. The CH_4-cycling communities at older restored Seba Beach peatlands appeared to be diverging from that at the reference bog. Restored peatlands in Canada, but also in Europe, tend to resemble fens more than bogs at the early stage of restoration in their chemistry, vegetation, hydrology, and general functioning (e.g., Wind-Mulder et al. 1996; Tuittila et al. 2000b; Wind-Mulder and Vitt 2000; González and Rochefort 2014; Putkinen et al. 2018, Kreyling et al. 2021). Our study shows that the CH_4-cycling microorganisms follow this trend. Therefore, without additional fen reference sites as an alternative natural end-point, or older restored sites, ORBA analysis would not allow us to predict a time to recovery. We are limited to the age of restored sites due to the relatively short time that peatland restoration has been applied in Canada. The Seba Beach site restored in 1991 is one of the oldest restored peatlands in Canada (Strack et al. 2016) and therefore represents the current maximum age limit of restored sites available for analysis. Future research could build upon this dataset by including a wider diversity of reference site conditions and more restored peatlands to further investigate how the CH_4-cycling microbial community can act as an indicator of ecosystem recovery in restored peatlands.

Our study was a part of a larger multidisciplinary project on CH_4 cycling in natural and managed peatlands from CH_4 production, to its storage in peat deposits, oxidation and emission to the atmosphere (see Bieniada and Strack 2021; Bieniada et al.

2021 dataset). We targeted CH_4-community characteristics in deep peat profiles with the inclusion of the stability of hydrological conditions. This limited the number of cores that we could collect at each site. A larger number of sample locations could be more appropriate for tracking the recovery of the microbial community over time (e.g., using ORBA, Rydgren et al. 2019; Auestad et al. 2020). However, previous research showed high spatial variation of microbial community in soils (Weedon et al. 2017). Thus, capturing the variation for the whole site could not be guaranteed even with a large number of cores/samples and unlimited budget. This renders microbial community analyses less practical in routine monitoring of the restoration outcome compared to plant communities, but facilitates better understanding of the underlying carbon cycling processes occurring in restored peatlands.

Aside from a relevant reference site to evaluate the effectiveness of restoration actions (Rydgren et al. 2019), the restoration goal must be established. If the goal of peatland restoration is to rebuild the carbon pool removed during peat extraction, the full recovery would take about 2000 years (Cleary et al. 2005). If the goal is the restoration of carbon accumulation and the development of a functional acrotelm, this could be achieved within < 20 years (Lucchese et al. 2010; Nugent et al. 2018). Reumer et al. (2018) concluded that at least additional 15 years would be needed to reverse the changes made by peat harvesting in CH_4-cycling communities based on studies of 15–16 year old restored sites. Although RES-1991 was 25 years at the time of sampling, the CH_4-cycling community remained distinct from the reference bog due to the fen-like conditions present on site and these differences are likely to persist until peat accumulation supports a succession to more acidic, bog-like conditions.

Environmental Conditions as Potential Drivers of the Methanogenic and Methanotrophic Development

The development of CH_4-cycling communities that departed from the Natural site with time post restoration was linked to changing hydrological, ecological, and chemical conditions with time. This was reflected in similarities in site distribution in the CCA ordination (Fig. 7B) and the weighted UniFrac for methanogens (Fig. 6A). Low core sample numbers precluded identifying significant relationships with local vegetation cover. Cadillo-Quiroz et al. (2010) found that the archaeal community structure varied in peat depending on the species of plant and the presence of roots. Andersen et al. (2013b) reported more similarities in microbial functions in natural and vegetated unrestored sites compared to a restored site with greater importance of vegetation than restoration-related physicochemical features. For Seba Beach peatlands, we cannot ignore the importance of peat chemistry, as it

clearly had an impact on the similarities and dissimilarities between sites regardless of a potential role of vegetation.

The concentrations of Fe^{3+} were the major factor influencing the CCA ordination with separation of RES-2009 from other sites. Higher $[Fe^{3+}]$ at restored sites than at the Natural site at Seba Beach followed a pattern observed by Aggenbach et al. (2013) at a natural and a restored fen, only the authors looked at total iron and did not consider its form or oxidation state. We presume that high concentrations of Fe^{3+} in bottom peat of RES-2009 was supplied from underlying clay and WT fluctuations down to 90 cm depth could mobilize iron to shallower depths and change its oxidation state (Knorr et al. 2009). High concentrations of Fe^{3+} and SO_4^{2-} at RES-2009 and the Unrestored sites had a potential to limit methanogenesis when active bacterial reducers are present resulting in lower CH_4 emission (Conrad 1999). In fact, potential rates of CH_4 production, oxidation, and CH_4 emissions from RES-2009 and the Unrestored sites were very low, with random CH_4 production hotspots at RES-2009 (Bieniada and Strack 2021; Bieniada et al. 2021). Jeffrey et al. (2019) reported a significant negative relationship between CH_4 emission from wetlands and the concentration of Fe^{3+} and SO_4^{2-} in underlying sediment, which is likely also the case at Seba Beach sites.

Relatively low C:N was expected at RES-2009 and RES-1991 as low rates are characteristic for fens rather than bogs and indicate more labile organic matter (Hodgkins et al. 2014). It was also expected that the majority of methanotrophs and many methanogens would find favorable conditions around the WT (Sundh et al. 1994, 1995; Segers 1998; Clymo and Bryant 2008; Martí et al. 2015) with moderate to high concentrations of PO_4^{3-}. Lin et al. (2014) also found the highest abundance of microorganisms in the zone of WT fluctuation, where they mobilize PO_4^{3-}, reducing its limitation. Although microorganisms require PO_4^{3-} for physiological processes, the ion can inhibit acetoclastic methanogenesis. In our study, $[PO_4^{3-}]$ was at least an order of magnitude below the inhibition threshold of 20,000 μM (Conrad et al. 2000) or 70,000 μM (Paulo et al. 2005), ranging from < 0.4 (LOD) to 70.3 μM, with mean of 3.9 μM. These conditions were associated with high concentrations of propionate. Some methanotrophs can use short chain fatty acid ions such as acetate, propionate, succinate or pyruvate instead of CH_4 or simultaneously (Dedysh et al. 2005; Belova et al. 2011; Zuñiga et al. 2013). EC was of relatively lower importance for the abundance of CH_4-cycling microorganisms but its average values were in range of EC found at poor and moderately-rich fens in Alberta, Canada (Bieniada et al. 2020).

Conclusions

This work is one of small number studies focused on the post-restoration development of CH_4-cycling microbial communities at former peat extraction sites. We show that

methane-cycling microbial communities found in restored sites at Seba Beach diverge from those found in a natural bog nearby. Although this observation could be site-specific or dependent on the restoration method, it is consistent with a general trend observed in other young restored peat extraction sites in Canada that post-restoration wetlands resemble fens more than bogs, and will likely undergo a succession through fen-like stages before becoming bogs once again.

As our study lacked a fen reference site that might have been appropriate at this restoration stage (< 30 years post-restoration) and had limited number of sampling locations, we could not predict the time to full recovery of microbial communities. Therefore, in future studies focused on this aspect, we would recommend 1) a larger number of sampling locations with a focus on shallow/surface peat, 2) analysis of more plots with different dominant vegetation and 3) inclusion of fens as reference sites.

Similarities in the abundance and composition of methanogenic and methanotrophic taxa between sites were linked to their location in relation to the water table (redox conditions) and peat chemistry. Our results demonstrate that high concentrations of non-oxygen TEAs, originating from underlying mineral soil did not seem to affect methanogen abundance even though CH_4 production (unpublished results) was hindered.

Our results indicate that restoration is necessary for the development and activity of CH_4-cycling communities. Although some evidence of the recovery is related to the presence of spontaneously encroaching sedges, this is not reflected in the activity of these microbes (Bieniada et al. 2021). Since abundance of methanogens and methanotrophs does not translate directly to CH_4-cycling activity, we recommend the use of molecular methods that can distinguish between microbe presence vs activity and identify gene expression in future studies. Microbial analyses are unlikely to be incorporated into routine monitoring of restored sites due to technical constraints and cost. However, our results show that the assessment of microbial community development combined with peat chemistry, hydrology and vegetation can provide insight into the factors that slow down greenhouse gas balance recovery in restored peatlands. Therefore, we recommend the inclusion of microbial analyses in future studies of restoration progress in post-extraction peatlands.

Declarations

Conflict of Interest   The authors declare that they have no conflict of interest that could have appeared to influence the work reported in this paper.

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