Effect of soil properties and hydrology on Archaeal community composition in three temperate grasslands on peat

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Abstract

Grasslands established on drained peat soils are regarded as negligible methane (CH4) sources; however, they can still exhibit considerable soil CH4 dynamics. We investigated archaeal community composition in two different fen peat soils and one bog peat soil under permanent grassland in Denmark. We used terminal restriction fragment length polymorphism (T-RFLP) fingerprinting and clone libraries to characterize the soils’ archaeal community composition to gain a better understanding of relationships between peat properties and land use, respectively, and CH4 dynamics. Samples were taken at three different depths and at four different seasons. Archaeal community composition varied considerably between the three peatlands and, to a certain degree, also with peat depth, but seemed to be quite stable at individual sampling depths throughout the year. Archaeal community composition was mainly linked to soil pH. No methanogens were detected at one fen site with soil pH ranging from 3.2 to 4.4. The methanogenic community of the bog (soil pH 3.9–4.6) was dominated by hydrogenotrophs, whereas the second fen site (soil pH 5.0–5.3) comprised both aceticlastic and hydrogenotrophic methanogens. Overall, there seemed to be a significant coupling between peat type and archaeal community composition, with local hydrology modifying the strength of this coupling.

Introduction

Peatlands that have been drained for agricultural use, a common practice, for example, in Europe, are regarded as negligible CH4 sources, because this involves in most cases active drainage that results in aeration of formerly anaerobic peat layers (Drösler et al., 2008; Couwenberg, 2011). The drastic reduction in CH4 emissions upon peatland drainage is due to a downward movement of the anaerobic CH4 production zone into more highly decomposed peat layers, but also to an enlargement of the aerated part of the peat profile in which upward diffusing CH4 can be oxidized to CO2 by methanotrophic bacteria before reaching the atmosphere (Byrne et al., 2004).

Although several studies have shown negligible CH4 emissions, or even a small uptake of atmospheric CH4, in drained peatlands that were either used for forestry or agriculture (Langeveld et al., 1997; Flessa et al., 1998; Maljanen et al., 2010), drained peatlands should not be dismissed a priori as potential CH4 sources. Ditches and ditch edges, which only constitute a small proportion of drained peatland areas, have been shown to be hotspots of CH4 emission (Schrier-Uijl et al., 2010; Teh et al., 2011). Schäfer et al. (2012) observed in two permanent grasslands on peat substantial dynamics in CH4 turnover, also at sites with negligible CH4 emission. Significant CH4 quantities were even measured above the water table, and for one of the sites, the presence of viable methanogens in peat layers that were drained throughout most of the year was confirmed in anaerobic incubation assays. In areas infested with the deep-rooting aerenchymous plant species Juncus effusus L., soil CH4 was liberated from the peat at rates comparable to those from natural peatlands.

Spatial and temporal variation in CH4 dynamics in peatlands has often been explained by changes in environmental conditions such as temperature, pH, and vegetation. However, little is known about the variability in composition of methanogenic communities between sites, and it is not yet clear whether variations in soil CH4 dynamics are mainly due to environmental factors, or
whether the composition of methanogenic communities must also be taken into account (Basiliko et al., 2003). For a series of undisturbed peatlands in North America, Yavitt et al. (2011) concluded that methanogenic communities are more shaped by present-day environmental conditions than by a region’s glacial history (thus Holocene peatland development). Environmental conditions in managed peatlands are dominated by the land-use type, which determines vegetation cover, hydrological regime, and nutrient status. For croplands and grasslands on mineral soils, organic fertilization has been shown to increase archaeal biomass, especially the methanogenic one. Additionally, cattle excreta might introduce rumen methanogens to the soil, further inducing shifts in archaeal community composition (Gattinger et al., 2007; Radl et al., 2007; Elhottová et al., 2012). Also, in rewetted drained peat soils, the growth and activity of methanogens seems to be mainly stimulated by fresh organic matter input (Hahn-Schöler et al., 2011), but it is not clear to what extent this impacts methanogenic community composition in comparison with the peat type.

Characterization of methanogenic archaea and associated environmental variables in different types of drained peat might help in evaluating their CH$_4$ production potentials. This is especially of interest for drained peatlands under grassland management because this land-use type covers a wide range of different vegetation types as well as hydrological and fertilization regimes. Here, we present the archaeal community composition in three different peatlands under grassland management which covered a wide range of physical and chemical peat properties, as well as hydrological regimes (Schäfer et al., 2012), but were all used as pastures and dominated by the same grass species. It was anticipated that the analysis of archaea at these sites could help to elucidate the relative importance of land use, peat properties, and soil environmental conditions for methanogenic potentials. T-RELFP fingerprinting and clone libraries were used to characterize the archaeal community composition at each of the grasslands at three different depths, and at four different times during a year, parallel to the study on soil CH$_4$ dynamics conducted by Schäfer et al. (2012). We hypothesized that the archaeal community composition at the three sites would be most similar in the rhizosphere because of similar fresh organic matter input, but that dissimilarities between sites would increase with depth as the peat properties become more important.

Materials and methods

Study sites

Soil archaeal community composition was studied at three permanent grasslands on peat in parallel with a 1-year greenhouse gas emission monitoring program. All three sites were located on the Danish mainland Jutland and are hereafter referred to by their location within Jutland (W = Western Jutland, E = Eastern Jutland, N = Northern Jutland). Detailed descriptions of the sites and the monitoring program are presented by Petersen et al. (2012) and Schäfer et al. (2012). This section gives a brief overview of the sites’ main characteristics.

Site W (55°56′ N, 8°26′E) was a fen with about 1-m-deep, highly decomposed peat corresponding to H8–H10 on the Von Post scale (Verry et al., 2011). The only recognizable plant remains were wood pieces, and the peat had a reddish color indicating the presence of large quantities of iron. The groundwater contained more than 150 mg SO$_4^{2-}$ L$^{-1}$ (Petersen et al., 2012). The site was partly flooded during winter, but the groundwater table dropped to a depth of about 80 cm during the summer. The grassland had been used for cattle grazing, but during the period of this study, it was only cut once in autumn. No fertilizer had been applied to the field for at least 3 years. The vegetation consisted of a mixture of conventional grassland species, grasses from the ditches, and weeds from the adjacent grain cropland. The dominant species were Lolium perenne L., Deschampsia cespitosa Desv., Alopecurus geniculatus L., and Elymus repens (L.) Gould.

Site E (56°22′ N, 10°24′ E) was also a fen with a peat decomposition degree of H8–H10. The terrain was strongly undulating featuring distinct microsites. Included in the monitoring program were a dry ridge and a wet depression which were adjacent to each other. The peat profile in the depression seemed to be disturbed and contained varying quantities of sand. The vegetation was dominated by L. perenne, but a few scattered J. effusus tussocks were also present. Peat depth was about 60–80 cm in the depression and about 120 cm at the dry ridge. The ridge peat was undisturbed and did not contain any sand. Plant remains were not identifiable in the field, but Sphagnum remains were visible under the stereo microscope. The vegetation was dominated by Agrostis capillaris L., Poa pratensis L., and Rumex acetosella L. The groundwater table at this grassland fluctuated by < 50 cm throughout the monitoring year. Summer drainage depth was about 40 cm in the depression and 90 cm at the ridge. During the wintertime, standing water could be observed in the lowest lying areas of the depression. The field was extensively used as cattle pasture and had not received any fertilizer for at least 3 years.

Site N (57°13′ N, 9°49′ E) was a drained bog with a 20- to 30-cm-thick Sphagnum peat layer at the surface (H4–H5), a well-preserved Sphagnum peat layer with Eriophorum spp. (H2) at about 30–70 cm depth, and a 30- to 40-cm-thick layer of highly decomposed radicell...
peat (H7–H8) with remains of *Phragmites australis* (Cav.) Trin. ex Steud. at the bottom of the profile. Summer drainage depth was more than 1 m, but water ponding on the surface occurred after rain events due to peat compaction. During the wintertime, the site was partially flooded. The grassland was a cattle pasture without additional fertilizer application, but during the monitoring program, it was only mowed. The vegetation was dominated by *L. perenne*, and part of the area was covered with *J. effusus* tussocks.

**Soil sampling**

Methane emissions at the three sites were monitored at six sampling points (55 cm × 55 cm), organized in three blocks separated by about 10–15 m, from September 2008 to October 2009. Environmental variables monitored in parallel included soil temperature at 10 and 50 cm depth and groundwater table depth at the center of each site (for more details, see Petersen et al., 2012). At < 1-m distance from each block, two 1-m-deep soil cores were taken in spring, summer, autumn, and winter to determine soil CH₄ concentrations (Schäfer et al., 2012) and archaeal community composition. The soil cores were extracted in three steps with a 30-cm-long stainless steel cylinder (5-cm-diameter, 04.15 SA/SB liner sampler, Eijkelkamp, Giesbeek, Netherlands) holding a transparent plastic tube. Depending on the water table depth and the peat composition, it was not always possible to retrieve core sections from the deeper parts of the peat profile. Immediately, in the field, 2-cm-thick slices were cut off the peat cores at 10-cm intervals for soil CH₄ extraction. For this procedure, the peat cores were not removed from the plastic tubes, and the remaining unwrapped core sections were sealed with plastic caps and transported to the laboratory in coolers within two to six hours (Schäfer et al., 2012). Immediately upon arrival in the laboratory, one core was selected from each block and 2-cm-thick peat slices were cut from the cores at 12–14, 42–44, and 72–74 cm depth for DNA extraction. Approximately 50 mL of peat was taken from the middle of each slice and homogenized in a glass bowl with a spatula, and a subsample was transferred into a 10-mL sterile plastic tube. The entire equipment was sterilized with ethanol between samples. The peat samples were stored field-moist at −20 °C until the DNA extraction. The main characteristics of the soil at each sampling depth are summarized in Table 1.

**DNA extraction**

DNA was extracted from the peat samples using the FastDNA Spin Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA). The original extraction protocol was not sufficient to remove all co-extracted humic acids, and in some cases also iron, from the DNA samples. Therefore, based on He et al. (2005), all samples were washed prior to the extraction. Two 200-mg subsamples were taken from each peat sample, mixed with 1 mL 0.1 M Na₂PO₄ solution and placed for one hour on a rotary shaker (Thermomixer comfort; Eppendorf, Hamburg, Germany) at 18 °C and 300 r.p.m. Subsequently, the samples were centrifuged for 10 min at 18 °C and 14 000 r.p.m. (Centrifuge 5417R; Eppendorf) and the supernatant was discarded. Centrifuge temperature and speed were the same in all of the following centrifugations. Between 140 and 310 mg of each prefwashed sample was filled into a Lysing Matrix E tube, and the DNA was extracted according to the FastDNA Spin Kit for Soil protocol with the following specifications and modifications. The samples were homogenized in the FastPrep® Instrument for 45 s at a speed setting of 5.5 (2500 r.p.m.) and subsequently centrifuged for 10 min. DNA was extracted from each prefwashed sample three consecutive times. At the second and third time, only fresh sodium phosphate buffer was added to the Lysing Matrix E tube. After precipitating the proteins, the three DNA extracts were pooled and 1 mL binding matrix was added. Additionally, 2 mL 5.5 M guanidine thiocyanate was added to remove any co-extracted humic acids from the binding matrix. 1.5 mL of the suspension was transferred into a 2-mL cup and centrifuged for about 6 s, and the supernatant was discarded. This was repeated until the entire binding matrix was in the 2-mL cup. The pellet was then gently washed on a Vortex mixer in 1 mL 5.5 M guanidine thiocyanate, subsequently centrifuged for about 5 s and the supernatant discarded. The washing procedure was repeated one to three times until the binding matrix had regained its original color. After the last washing, the binding matrix was resuspended in 600 μL guanidine thiocyanate and transferred onto a SPIN Filter inserted into a catch tube. The SPIN Filter was centrifuged for 1 min and the content of the catch tube discarded. After reinserterion of the SPIN Filter into the catch tube, the original extraction protocol was followed to the end. At last, the SPIN Filter was air-dried at room temperature for 10 min, and then the binding matrix was resuspended in 100 μL DES and incubated for 5 min. DNA samples were stored at −20 °C until further analysis. Immediately after extraction, the total DNA yield and quality was assessed with a NanoDrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany; see Supporting Information for further information).

**PCR amplification of archaeal 16S rRNA genes**

Archaeal 16S rRNA genes were amplified from the DNA extracts using the following Archaea primers: Ar109f
(5'-ACKGCTCAGTAAACAGT-3') and Ar915r (5'-GTGC TCCCCGGCCAATTCCT-3') (Großkopf et al., 1998). The reverse primer had a 6-carboxy-flourescein (FAM)-labeled 5’ end. The 50-µL PCR mixture contained 10 µL 5X Green GoTaq® Flexi Buffer, 1.5 mM MgCl₂, 0.2 µL (1 U) GoTaq® Flexi DNA Polymerase (Promega, Mannheim, Germany), 200 µM deoxynucleotide triphosphates (dNTP; Fermentas, St. Leon-Rot, Germany), 0.5 µL bovine serum albumin (Roche, Mannheim, Germany), 29.3 µM nuclease-free water (Sigma-Aldrich, Taufkirchen, Germany), 0.33 µM of each primer, and 1 µL template DNA. The PCR was performed in a MWG-Biotech Primus 96 Plus Thermal Cycler (MWG-Biotech, Munich, Germany) with 24–34 cycles as described in Metje & Frenzel (2005). The amplification products were purified with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO).

**T-RFLP analysis**

Between 50 and 100 ng of each purified PCR product was digested with 5 U TaqI and 1X buffer (Fermentas) for 3 h at 65 °C. The digests were purified with Sigma-Spin™ Sequencing Reaction Clean-Up, Post-Reaction Purification Columns (Sigma-Aldrich). A 2-µL aliquot of each purified digest was mixed with 11 µL HiDi formamide (Applied Biosystems, Applera, Darmstadt, Germany) and 0.2 µL MapMarker 1000 (BioVentures, Murfreesboro, TN) and subsequently denatured for 2 min at 94 °C. The DNA fragments in the diluted digests were resolved by capillary electrophoresis on a DNA Sequencer 3130 with 36 cm capillary length (PE Applera, Darmstadt, Germany). Terminal restriction fragment (T-RF) sequence length, peak height, and peak area were determined using the GENEMAPPER V4.0 software. Threshold for peak height integration was 30 relative fluorescent units (RFU) for the samples and 10 RFU for the standards. The integration was performed with the Local Southern Method. Peaks from the electropherograms were manually aligned. For the entire dataset, the total peak areas of the T-RFLP profiles were normalized to the value of the T-RFLP profile having the overall smallest total peak area by proportionally reducing the single peak areas within each profile.
(Dunbar et al., 2001). T-RFs with a relative peak area of < 1% were discarded.

**Cloning and phylogenetic analysis**

To aid the interpretation of the T-RFLP profiles, clone libraries were constructed for each sampling depth at site N and site W, whereas at site E, one clone library was constructed for the ridge, one for the 12-cm sampling depth in the depression and one combined library for 42- and 72-cm depth in the depression. For each clone library, DNA aliquots of all samples were pooled, and subsequently archaeal 16S rRNA genes were amplified with PCR as described above, but without fluorescent-labeled primers. The PCR products were cloned in *E. coli* competent cells (JM109) using the pGEM-T Easy Vector Kit (Promega) and agar plates with *E. coli* FastMedia™ Agar Amp IPTG/X-Gal (Fermentas). The clones were sequenced by GATC Biotech (Konstanz, Germany) using primers M13-RP (5′-CTATGACC-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). The sequences were assembled with SEQMAN 4.05 (DNA-STAR, Madison, WI) and compared with sequences in the BLAST database 'Nucleotide collection (nr/nt)' using Megablast (Zhang et al., 2000). The search results were sorted by total score. Sequences were also aligned in ARB (Ludwig et al., 2004) and added with the ARB parsimony tool to the SILVA small subunit rRNA reference database (version 102, www.arb-silva.de) to determine the approximate phylogenetic affiliations. The sequences were deposited into the GenBank (www.ncbi.nlm.nih.gov/genbank/) under the accession numbers JX984803 to JX984935.

**Statistical analysis**

Different ordination techniques were applied to assess the effect of peat properties on archaeal community composition, using the software R (version 2.13.0; R Development Core Team, 2011) with the packages ‘vegan’ (Oksanen, 2011) and ‘packfor’ (Dray et al., 2007) according to the guidelines of Borcard et al. (2011) and McCune & Grace (2002). First, dissimilarities between the soil samples used for DNA extraction were visualized by computing a principal component analysis (PCA) on the correlation matrix of 11 environmental variables extracted from Petersen et al. (2012) and Schäfer et al. (2012) to identify the major gradients in soil properties between sites and sampling depths. The variables used were soil organic matter content (SOM), volumetric water content (VWC), soil pH (soilpH), dry bulk density (DB), C : N ratio (CN), groundwater pH (GW_pH), groundwater NO3_− (GW_NO3) and SO4_2− (GW_SO4) concentration, electrical conductivity of the groundwater (GW_EC), soil CH4 concentration (CH4conc), and CH4 flux (CH4flux). The first four variables, as well as soil CH4 concentrations, were included as averages of the four soil samplings for each sample depth and block, whereas the other variables were represented by annual averages for each block. The PCA was then compared to an ordination of the T-RFLP profiles to assess whether dissimilarities in archaeal community composition between sites and sampling depths overlapped with gradients in soil properties.

Dissimilarities in the T-RFLP profiles were visualized with nonmetrical multidimensional scaling (NMDS) using the function ‘metaMDS’ and the Bray–Curtis dissimilarity matrix. Prior to the ordination, the total peak areas were logarithmically transformed (log(x + 1)) to reduce the importance of T-RF sizes with very large peak areas. The starting configuration for the NMDS was randomly generated and the ordination was then run with a maximum of 50 random starts. The whole procedure was conducted three times – each time with a different random starting configuration. The ordination was tested with one to six dimensions, and the appropriate dimension for this dataset was chosen with the help of scree and Shepard plots. To assess whether observed differences between the T-RFLP-based and the soil property-based ordination could be attributed to single soil properties, thereby neglecting all other soil property gradients, an environmental vector containing the 11 variables used in the PCA was fitted onto the NMDS using the functions ‘envfit’ and ‘ordisurf’. The P-values for the correlations between the NMDS ordination and the single environmental variables were based on permutation tests. Additionally, the same 11 environmental variables were used as constraints in the ordination of the T-RFLP profiles with the eigenvector-based redundancy analysis (RDA). Because Euclidean distances are not appropriate for ordination of taxonomic data, the log-transformed peak areas were additionally subjected to a Hellinger transformation prior to the RDA (Legendre & Gallagher, 2001). The number of explanatory variables in the RDA was reduced by forward selection using the function ‘forward.sel’. Significance of the ordination and the single axes was tested with permutation tests. Eigenvector-based ordinations are methodologically very different from NMDS. Thus, the degree of similarity between the NMDS and RDA ordination could be used to evaluate the strength of the conclusions drawn from the T-RFLP-based ordinations. Furthermore, the RDA gave also insights into correlations between individual T-RFs and soil properties. The clone library database was not subjected to the same ordination techniques because of its lower spatial resolution in comparison with the T-RFLP data.
Results

Environmental gradients

Mean annual soil temperatures at 10 and 50 cm soil depth were similar between the three sites (8.9–9.2 °C and 9.0–9.1 °C, respectively) as were the seasonal soil temperature fluctuations. Minimum temperature ranges were 0.2–0.5 °C and 2.2–3.8 °C at 10 and 50 cm depth, respectively; and maximum ranges were 20.1–21.5 °C and 14.0–14.7 °C at 10 and 50 cm depth, respectively. In contrast, the other measured peat properties showed large gradients between the three sites (Table 1). The available soil data comprised two groups, descriptors of physical and chemical characteristics of the peat substrate itself and descriptors of groundwater chemistry. The SOM represented the largest gradient, covering a range from 1% to 97%. SOM was strongly negatively correlated with dry bulk density. In the ordination of the different sampling depths in environmental space, the dataset could be divided into two main subsets along the SOM gradient (Fig. 1). The largest variation within each subset was observed along the groundwater SO$_2^-$ concentration gradient, which ranged between 1 and 217 mg L$^{-1}$. The soil CH$_4$ concentration was strongly negatively correlated with this variable. The PCA showed that peat heterogeneity was largest at site E, as the soil samples from the three blocks did not group together. The Sphagnum peat samples from the ridge (E1) grouped together with the Sphagnum peat samples from site N. The soil samples from site N all grouped together, although three distinctly different soil horizons were visible in the field. The opposite was true for site W, where no distinct soil horizons were visible in the field, but the PCA showed two different groups separating soil at 10-cm depth from 40- and 70-cm depth. Soil analyses revealed that there was much less SOM at 10-cm depth than at deeper peat layers.

T-RFLP profiles

In total, 24 different T-RFs were identified across all sites and depths with on average six T-RFs at each of the 27 sampling depths as defined in Table 1. The majority of T-RFs in the dataset were found at all three grassland sites; however, one, two, and five T-RF sizes were only found at site W, the depression at site E, and site N, respectively. In contrast to our hypothesis, the T-RFLP profiles did not only differ considerably between the sites at the lower sampling depths, but also between the sites’ rhizospheres. The least diverse T-RFLP profiles were found in peat of the ridge at block E1 (Fig. 2). Here, the profiles comprised altogether six different T-RFs, but T-RF 186 was dominant and the combined relative abundance of the other five T-RF sizes never exceeded 9% regardless of sampling depth and season. The five rare T-RF sizes were 80, 393, 447, 740, and 810 bp. At site W, T-RF of 186 bp was again the most abundant T-RF, albeit not as abundant as at E1. The number of T-RFs in the vertical profiles at site W increased with sampling depth, but within each depth the abundance of the individual T-RFs seemed to be quite stable throughout the year despite fluctuations in soil temperature and groundwater table depth. It was not possible to assess the statistical significance of the sampling time due to the overall low number of samples.

At sites N (N1–N3) and E (E2, E3), the T-RF of 186 bp was also quite abundant, and changes with soil depth and over the year were similar to site W. However, apart from that, each site had its own distinct T-RFLP profiles. At site N, the most abundant T-RF sizes besides 186 bp were 92 and 393 bp. The amount of rare T-RFs differed between the three sampling depths. The most diverse T-RFLP profiles of all three grassland sites were found at site N at 72- to 74-cm sampling depth with 13 different T-RF sizes, of which the majority had relative abundances below 10%. Very rare T-RF sizes not shown in Fig. 2 for site N were 100, 201, 228, 247, 380, 492, and 858 bp. The least variation in the profiles with depth (apart from E1) was observed in the depression at site E (E2, E3), where T-RFs of 80, 92, 186, 284, 393, and 740 bp were found at all depths.
Clone libraries

The majority of the T-RFs could be identified from clone sequences (Table 2), but most of the clone libraries were not sampled near saturation (see Supporting Information for further information). As a result, no corresponding clone sequence was found for the rare T-RFs of 62, 76, 100, 201, 228, 247, 258, 314, 380, 492, and 858 bp. The number of sequences corresponding to each T-RF size was generally consistent with the relative abundance in the T-RFLP profiles for site W and the two lower depths at site N. However, for the other depths, the relative abundance of the four most dominant T-RFs differed partly between T-RFLP-based and clone sequence-based profiles. This might have been due to the fact that the clone libraries had been constructed from combined DNA samples.

The lowest clone diversity was found for depth 42–44 cm at site W, whereas the clone library for depth 72–74 cm at site N and the combined clone library for the two lower depths of the depression at site E were the most diverse ones. In contrast to the T-RFLP profiles, all clone libraries were characterized by a high evenness (see Supporting Information for further information). Several of the abundant T-RFs were associated with more than one phylotype, both within the Euryarchaeota and within Crenarchaeota. Therefore, individual T-RFs could not be unambiguously assigned to only one particular phylotype. However, the detection of either euryarchaeotal or crenarchaeotal clone sequences was distinct between the different sites. While the site W and the ridge of site E (E1) contained almost exclusively crenarchaeotal clone sequences, the other two sites also contained euryarchaeotal clones sequences, many of them affiliated with known taxa of methanogens. Sites W and E1 vs. sites N, E2, and E3 were distinguished by their potential to produce CH₄, as summarized in Table 1.

The majority of methanogens at site N belonged to the hydrogenotrophic groups Methanocella, Methanobacterium or Methanoregula, as well as Rice Cluster II whose members are assumed to be methanogens, but not yet confirmed by culturing. The clone sequences retrieved from site N at 12- and 42-cm depth were all related (99–100% identity) to sequences in the database that had been isolated from natural oligotrophic and acidic Sphagnum bogs (e.g. AF524853.1, JN649321.1, FJ822562.1, AJ459882.1), except for one sequence that had been retrieved from tank bromeliads (GU223485.1). Only one clone of the strictly acetoclastic group Methanosaeta was found at 72-cm depth (HM244253.1). The other sequences obtained at this depth were most closely related to sequences retrieved from lake sediment, rice roots, and boreal fen peat (98–99% identity), but not to sequences from bog peat. Overall, only three clones at site N had a name match within the first 50 blast hits, namely Methanobacterium sp. AL-21 (99% identity); for T-RF 186 Methanosarcina vacuolata (99% identity); for T-RF 83 Methanobrevibacter barkeri (99% identity); for T-RF 116 Methanobrevibacter smithii (99% identity); for T-RF 233 Methanobrevibacter smithii (99% identity); for T-RF 234 Methanobrevibacter smithii (99% identity).
Methanoculleus chikugoensis (97%), Methanosarcina barkeri (99%), and Methanosarcina horonobensis; for T-RF 284 Methanosaeta concilii (97%); and for T-RF 393 M. formicica (98% identity) and Methanolinea tarda (95%).

About 71% and 42% of all clones from site N and the depression at site E, respectively, were classified as Crenarchaeota. Many of the crenarchaeotal clones from site N were similar (98–100% identity) to clones isolated from natural oligotrophic and acidic peatlands (e.g. AB262707.1, HQ614091.1), but also to clones from, for example, acidic red soils (FJ584327.1), rice field soils (AB182714.1), gold mine waters (AB050208.1), tree rhizospheres (GU815318.1), and freshwater sediments (HM244115.1). The related clones from natural peatlands comprised both members of the crenarchaeotic groups 1.1c (FJ822579.1) and 1.3 (AM905988.1). Only one of the crenarchaeotal clone sequences identified at site N (AB050208.1) was also found in the depression at site E. At this site, 70% of the clones were highly similar (identity 98–100%) to clone sequences retrieved from either rice field soil or rice roots. Two clones were highly similar to sequences from a peatland (EU155993.1, EU155994.1).

The sampling blocks at the ridge and the depression at site E were only about 10–20 m apart, but the libraries did not share any of the crenarchaeotal clones. Instead, crenarchaeotal clones were shared with those found at sites N and W. The clones from the ridge were predominantly related to clones found in acidic soil samples, of which one was from a temperate fen (FR745101.1). At site W, the clone libraries consisted mainly of sequences from the soil crenarchaeotal group, and in total, only 12 different clones were isolated from the peat. Highly similar matches (98–100% identity) to database sequences were primarily from marine sediments (DQ831590.1), rice field soils (AB182772.1), and the rhizosphere of trembling aspen (EF020898.1). At 42- and 72-cm depth, however, four clones were also highly similar to clones from acidic peat (AB262709.1) including Crenarchaeota of group 1.1c (FJ822579.1).

### Table 2. Phylogenetic affiliations of T-RFs at the three study sites based on megablast and ARB.

The samples were separated along the depth profiles, but the different blocks were pooled. For block E1, all samples along the depth profile were pooled. The letter of each clone library label indicates the site and the numbers – apart from block E1 – the sampling depth.

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<tr>
<th>Phylogenetic lineage</th>
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<th>Clone libraries</th>
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</tr>
<tr>
<td>Thermoplasmatales</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>SAGMCG-1</td>
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<tr>
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</tr>
<tr>
<td>Total number of Crenarchaeota clones</td>
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<td>9</td>
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None of the crenarchaeotal and nonmethanogenic euryarchaeotal sequences retrieved in this study were closely related to a cultured archaeon.

**Environmental gradients in T-RFLP profiles**

The ordination of samples based on the T-RFLP profiles resulted in a somewhat different grouping as compared to the ordination based on environmental variables presented in Fig. 1. The first dimension separated the T-RFLP profiles containing both *Crenarchaeota* and methanogenic *Euryarchaeota* (NMDS1 with positive values) from the profiles that were completely dominated by *Crenarchaeota* (NMDS1 with negative values; Fig. 3). As several T-RFs had been assigned to both *Crenarchaeota* and *Euryarchaeota*, only a few T-RFs were located on the crenarchaeotal side of the plot. The sampling depths of 42 and 72 cm at site W were spaced further apart than in Fig. 1 based on the abundance of T-RF 447 and T-RF 314, but they were still clearly separated from the 12-cm sampling depth. The *Sphagnum* peat samples (N1–N3, E1), which grouped together in Fig. 1, were completely split up into six smaller groups. On the other hand, the T-RFLP profiles of the depression at site E (E2 and E3) were less variable than the peat and groundwater properties, and their composition was in between the profiles obtained at 72-cm depth at site N and those obtained at 12- and 42-cm depth at site N.

Fitting the environmental variables used for ordination in Fig. 1 onto the NMDS ordination revealed that the variation in the T-RFLP profiles along the first dimension was best described by changes in soil pH (P-value = 0.001; Fig. 4). The *Crenarchaeota*-dominated profiles were found in the most acidic peat samples, whereas profiles containing methanogenic *Euryarchaeota* were found in peat samples with a soil pH of at least 4.0. Other important environmental variables were groundwater SO$_2^-$ concentration (P-value = 0.002), soil CH$_4$ concentration (0.002), and electric conductivity of the groundwater (0.003). VWC was the least significant environmental variable with a P-value of 0.09; however, it was the variable that best described the increase in the number of T-RFs per profile along the second dimension.

The RDA of the T-RFLP profiles also identified soil pH as the most significant and groundwater SO$_2^-$ as the second most significant environmental variable (P-value = 0.001 and 0.002, respectively). Furthermore, the forward selection procedure included groundwater nitrate concentration (P-value = 0.007), soil CH$_4$ concentration (0.005), C : N ratio (0.011), and VWC (0.011) in the reduced environmental dataset. Together, these six variables explained 42% of the variance in the T-RFLP profiles which was only 1% less as when the RDA was run with the full set of 11 environmental variables. The RDA triplot showed a positive correlation between the T-RF of 393 bp and soil pH, the T-RF of 186 bp and groundwater SO$_4^{2-}$ concentration, the T-RFs of 80 and 284 bp and soil pH.
CH₄ turnover (N, E₂, E₃) also contained methanogenic methanogenesis. Theoretically, aceticlastic methanogenesis has always been observed in rice field soils (Conrad, 2007), but in peatlands, aceticlastic methanogenesis has not always been observed (Avery et al., 1999; Horn et al., 2003; Sizova et al., 2003; Rooney-Varga et al., 2007; Tian et al., 2011; Yavitt et al., 2011). Aceticlastic methanogenesis seems to prevail in minerotrophic fens, where fresh labile organic matter is readily available in the form of root exudates from vascular plants. In contrast, nutrient-poor fens and bogs with a low cover of vascular plants, and more recalcitrant peat, are mainly inhabited by hydrogenotrophic methanogens (Artz, 2009). In line with this is our observation that the methanogenic community in the depression at the minerotrophic fen (E₂ and E₃) comprised both aceticlastic and hydrogenotrophic methanogens, whereas the methanogenic community in the Sphagnum peat at the bog (site N) was dominated by hydrogenotrophs. The community composition at site N was similar to the compositions previously observed in pristine oligotrophic, acidic Sphagnum peatlands (Horn et al., 2003; Rooney-Varga et al., 2007), but site N was neither pristine nor oligotrophic anymore. The remaining Sphagnum peat layers were highly recalcitrant, as indicated by the high C : N ratios, but the vegetation was no longer characteristic for a pristine Sphagnum peatland, but resembled the vegetation in the depression at site E.

The dominant presence of hydrogenotrophs at site N might have been the combined result of peat aeration and peat pH. The summer-drainage depth at site N was more than 1 m, but the VWC in the zone above the water table fluctuated little throughout the year, and viable methanogens were found throughout the peat profile (Schäfer et al., 2012). The presence of the Methanocellales at 12-cm depth at this site can be seen as an indicator of oxidative stress. The order Methanocellales, also known as Rice Cluster I, has been identified as key producer of CH₄ from plant-derived carbon in the rice rhizosphere (Lu & Conrad, 2005). They possess a set of antioxidant enzymes to deal with oxidative stress, and it seems to give them an advantage over other methanogenic groups (Erkel et al., 2006). Apart from rice fields, Methanocellales has also been found to be abundant in the rhizoplane of two bog-inhabiting plants (Cadillo-Quiroz et al., 2010). In contrast, clones of Methanoregula, found at 72-cm depth at site N, were closely related to M. formicica that is strictly anaerobic (Yashiro et al., 2011). Overall, methanogenic diversity at site N increased with depth and thus with decreasing oxidative stress. For reference, the most diverse methanogenic communities in this study were observed in the depression at site E, where the summer drainage depth was only about 40 cm. However, oxidative stress alone is not sufficient to explain the patterns in

CH₄ concentrations, as well as the T-RF of 810 bp and VWC (Fig. 5). The sampling depths were distributed slightly different in Fig. 5 compared to Fig. 3, but overall both ordinations showed similar correlations between the T-RFs and the environmental variables.

**Discussion**

Archaeal communities, as reflected in DNA-derived T-RFLP profiles and clone libraries, differed clearly between the three permanent grassland sites, and within the grasslands depth-related differences in archaeal community composition were observed, which is only partly in agreement with our hypothesis. Sites with no detectable CH₄ turnover (W, E₁) apparently did not contain methanogenic archaeal sequences, but only crenarchaeotal sequences and Thermoplasmatales. In contrast, sites with CH₄ turnover (N, E₂, E₃) also contained methanogenic archaeal sequences. The depression at site E with the highest methanogenic potential also exhibited the most complex community structure of methanogenic archaea, containing both putatively hydrogenotrophic and aceticlastic methanogens.

One important aspect of archaeal community composition with regard to CH₄ production dynamics is the relative contribution of aceticlastic and hydrogenotrophic methanogenesis. Theoretically, aceticlastic methanogenesis should contribute twice as much CH₄ as hydrogenotrophic methanogenesis (Conrad, 2007). This ratio has indeed been observed in rice field soils (Conrad, 2007), but in peatlands, aceticlastic methanogenesis has not always been observed (Avery et al., 1999; Horn et al., 2003; Rooney-Varga et al., 2007; Tian et al., 2011; Yavitt et al., 2011). Aceticlastic methanogenesis seems to prevail in minerotrophic fens, where fresh labile organic matter is readily available in the form of root exudates from vascular plants. In contrast, nutrient-poor fens and bogs with a low cover of vascular plants, and more recalcitrant peat, are mainly inhabited by hydrogenotrophic methanogens (Artz, 2009). In line with this is our observation that the methanogenic community in the depression at the minerotrophic fen (E₂ and E₃) comprised both aceticlastic and hydrogenotrophic methanogens, whereas the methanogenic community in the Sphagnum peat at the bog (site N) was dominated by hydrogenotrophs. The community composition at site N was similar to the compositions previously observed in pristine oligotrophic, acidic Sphagnum peatlands (Horn et al., 2003; Rooney-Varga et al., 2007), but site N was neither pristine nor oligotrophic anymore. The remaining Sphagnum peat layers were highly recalcitrant, as indicated by the high C : N ratios, but the vegetation was no longer characteristic for a pristine Sphagnum peatland, but resembled the vegetation in the depression at site E.

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![Fig. 5. RDA triplot of the T-RFLP profiles constrained by the six environmental variables chosen by forward selection. T-RFs are shown in black and environmental variables are shown in gray. Symbols have the same meaning as in Fig. 3. The plot is scaled in a way that the angles between T-RFs and environmental variables reflect the correlations. The bottom and left-hand scales are for the sampling depths and the T-RFs, the top and right-hand scales are for the environmental variables. Sampling depths are represented as fitted site scores.](image-url)
Archaeal community composition in grasslands on peat

mehanogenic community composition between the grassland sites. For example, it has been shown that besides *Methanocella* also, *Methanosarcina* can survive in different types of aerated soils (Angel et al., 2011), but this group was not detected at site N.

In this study, the T-RFLP profiles comprising methanogenic archaea were primarily separated in the unconstrained ordination along the soil pH gradient, with soil pH ranging between 4.0 and 4.8 at site N and between 4.6 and 5.2 at the depression of site E. The pH values at site W, and at the ridge of site E, ranged from 3.2 to 4.0, and the T-RFLP profiles were completely dominated by *Crenarchaeota*, that is, no methanogenic *Euryarchaeota* were included in the clone libraries. Soil pH has been shown to be a main predictor of bacterial community composition in soils across long environmental gradients (Fierer & Jackson, 2006; Lauber et al., 2009). Yavitt et al. (2011) found a strong correlation between soil water pH and euryarchaeotal community composition across a set of six different North American peatlands. Most cultured methanogens grow at pH 6–8 (Garcia et al., 2000), and a decrease in pH seems to filter out especially aceticlastic methanogens. For instance, Horn et al. (2003) showed that acetate and other volatile fatty acids inhibited methanogenesis at pH 4.5 in a methanogenic enrichment culture from bog peat and that in situ CH₄ production was conducted by acido-tolerant hydrogenotrophic methanogens which seemed to receive H₂ from acetate-oxidizing bacteria. Likewise, acido-tolerant (pH 4.0–6.5) methanogens isolated from *Sphagnum* peat by Sizova et al. (2003) were all hydrogenotrophs, and one of the cultured clones was highly similar to the three *Methanocella* clones isolated from site N. Soil pH seems to be also the main driver of archaeal community composition in pastures on mineral soils. According to Radl et al. (2007) and Elhottová et al. (2012), massive and long-term application of cattle manure can increase soil pH, which in turn provides more suitable conditions for methanogens to utilize the fresh organic matter input, subsequently leading to a shift in archaeal community composition from *Crenarchaeota* to methanogenic *Euryarchaeota*. Thus, in contrast to our hypothesis, the input of similar fresh organic matter does not seem to be the main driver of rhizospheric archaeal community composition at the three investigated peatlands. Also, the land use has not been strong enough to alleviate differences in soil pH, and perhaps subsequently in archaeal community composition, in the rhizosphere between the study sites.

Evidence was presented above to suggest that soil pH – in conjunction with the degree of oxidative stress – can sufficiently explain the main differences in methanogenic community composition between site N and the depression at site E. However, the occurrence of T-RF 284, which is only associated with aceticlastic methanogens of the order *Methanosetaeaceae*, did not correlate strongly with increasing soil pH in the constrained ordination. Instead, T-RF 393, which in this study represented hydrogenotrophic methanogens and *Crenarchaeota*, showed a strong positive correlation with soil pH. One has to keep in mind that soil pH is a very complex variable, and the publications mentioned so far all investigated unmanaged peatlands that differ quite strongly in soil chemistry from our study sites. Soil pH does not only impose a physiological stress on methanogens (Yavitt et al., 2011), but it also influences the availability of nutrients and toxic substances and may serve as a proxy for acid-generating processes. For example, pH values below 3.5 at site W might be explained by the presence of H₂SO₄ as a result of pyrite oxidation (Blume et al., 2002), and thus, peat aeration may partly be reflected in the soil pH gradient.

The absence of methanogens at site W, and at the ridge of site E, could have been a result of the very low soil pH, but at site W also the high SO₄²⁻ and iron concentrations alone might have been responsible. For instance, sulfate- and iron-reducing microorganisms can outcompete methanogens for electron donors, because they have an energetically more favorable metabolism (Watson & Nedwell, 1998; Roden & Wetzel, 2003). On the other hand, the ridge at site E was the overall driest site in this study, and here oxidative stress might have simply been too high to allow the development of methanogenic communities large enough to be detectable with the applied methods (Nicol et al., 2003a).

The *Crenarchaeota* clones isolated in this study were similar to clones from a wide variety of environments. It is not clear if differences in crenarchaeotal community composition between the three different grassland sites can be solely attributed to differences in the chemical and physical peat properties, as management has also been shown to influence the structure of crenarchaeotal communities (Nicol et al., 2003b). Overall, nonthermophilic *Crenarchaeota* seem to be the dominant archaeal group in aerated pasture soils (Nicol et al., 2003b), but the ecophysiology of *Crenarchaeota* in soils is still largely unknown (Pester et al., 2011).

Apart from soil pH and groundwater SO₄²⁻ concentration, the variables identified as significant in both types of ordination of the archaeal T-RFLP profiles were VWC and soil CH₄ concentration. The VWC of the peat described well the separation of the T-RFLP profiles on the second axis of the unconstrained ordination; however, the interpretation of its effect on archaeal community composition is not straightforward. The overall VWC of peat is dependent on dry bulk density and organic matter content, which influence the available total pore space and the water-holding capacity, respectively (Verry et al., 2011).
indeed increase with depth; however, for site E, only soil
CH$_4$ production at site E can be assumed to have been
production, transportation, and oxidation. Nevertheless,
positive correlation between T-RF 284 and soil CH$_4$
concentration. Methanosetae were only identified in the most
diverse methanogenic communities at 72-cm depth at site
N and at the depression of site E. Yavitt et al. (2011) found a positive correlation between the diversity of
methanogenic communities and potential CH$_4$ production
rates. For site N, CH$_4$ production potential did
indeed increase with depth; however, for site E, only soil
CH$_4$ concentration profiles were available (Schäfer et al.,
2012). Soil CH$_4$ concentration is not a direct measure of
soil CH$_4$ production, but the combined result of CH$_4$
production, transportation, and oxidation. Nevertheless,
CH$_4$ production at site E can be assumed to have been
substantial; otherwise, the CH$_4$ fluxes and soil CH$_4$
concentrations measured at block 2 in the depression at site
E (Schäfer et al., 2012) would have been very difficult to
maintain.

Significant CH$_4$ fluxes at both sites N and E were
restricted to blocks containing the aerenchymous plant
$J$. effusus, and soil CH$_4$ concentration profiles also differed between $Juncus$-containing blocks and blocks with
conventional grassland vegetation (Schäfer et al., 2012). It
has been shown that methanogenic communities in the
upper peat layers (0- to 30-cm depth) can be significantly
different between different vegetation types in the same
peatland (Galand et al., 2003; Cadillo-Quiroz et al., 2010;
Tian et al., 2011), which may partly account for differences in soil CH$_4$ dynamics (Cadillo-Quiroz et al., 2010).
Significant horizontal changes in methanogenic
community composition between vegetation types were not
observed at site N or at the depression of site E, but this
could have been a methodological problem, because the
peat samples from the $Juncus$-containing blocks were not
taken directly below the tussocks, but about 20–50 cm
next to them in the conventional grassland vegetation.
However, horizontal homogeneity of the methanogenic
community composition in peat layers below the main
rooting zone is in line with observations made by Galand
et al. (2003). So it appears that spatial differences in soil
CH$_4$ dynamics and CH$_4$ fluxes within a grassland site
were foremost due to differences in environmental condi-
tions and the gas transport capacity of $J$. effusus, as
discussed by Schäfer et al. (2012), although horizontal
differences in the composition of important soil bacterial
communities, for example, methanotrophs, cannot be
excluded. Nevertheless, horizontal homogeneity of archa-
eal community composition within a peatland indicates
stable physicochemical conditions at the sampled depth
(Galand et al., 2003). An important factor influencing
spatial variability in physicochemical conditions is peat-
land hydrology (Eurola & Kaakinen, 1984). Depending on
the hydrological conductivity and infiltration capacity of
the peat, water movement might be able to alleviate verti-
cal differences, for example, in the availability of metha-
nogenic substrates within the peat profile (Putkinen et al.,
2009). This could explain the similarity between T-RFLP
profiles obtained from the depression at site E, where the
peat substrate was quite heterogeneous, but hydraulic
conductivity was very high. The opposite observation was
made for site N, where the measured soil properties
seemed to be quite uniform throughout the peat profile,
but the T-RFLP profiles of the different depths were
divided in the unconstrained ordination. The hydraulic
conductivity of the compressed Sphagnum peat was very
low, and it also had a low infiltration capacity, resulting
in water ponding after precipitation events.

Overall, soil properties and groundwater chemistry
seemed to describe the main differences in archaenal
community composition between and within the three
grassland sites fairly well. Still, 58% of the variance in the
T-RFLP profiles remained unexplained in the constrained
ordination. The data presented here only provide a first
insight into the system. The selection of environmental
variables covered a wide range of physicochemical peat
properties, but important variables could have been easily
missed. For instance, groundwater pH was not identified
as a significant environmental variable in the forward
selection procedure of the constrained ordination. If soil
pH values had not been available, the most significant
environmental variable would have been groundwater
SO$_4^{2-}$ concentration. With regard to the DNA-based
T-RFLP profiles, one has to keep in mind that they only
provide an overview of the structure of the archaenal
community, but not of the actual activity of different groups
of archaea, as would have the use of RNA. The apparent
stability of archaenal community structure throughout the
year observed in this study has, for example, also been
shown by Juottonen et al. (2008) and Tian et al. (2011).
However, Juottonen et al. (2008) observed higher vari-
bility in RNA-based T-RFLP profiles throughout the year
and showed that archaenal groups with a small population
size, but a high activity, might only be detectable in
RNA-based community fingerprints.

In conclusion, differences in archaenal community com-
position between the three studied permanent grasslands
on peat were best described by soil pH, which can serve
as a proxy for different environmental variables. Overall,
there seemed to be a significant coupling between peat
type and archaenal community composition, with local
hydrology modifying the strength of this coupling.
However, more than 50% of the variation in the T-RFLP
profiles remained unexplained. The data presented here
provide only a first overview. To better elucidate the importance of specific methanogenic groups and certain peat properties for soil CH$_4$ dynamics in peat under permanent grassland management, information on the seasonal activity of the methanogenic archaea would also be needed.

**Acknowledgements**

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**References**


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Data S1.** DNA yields and ecological indices for the clone libraries.