Methanogen communities along a primary succession transect of mire ecosystems

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Abstract

Peat accumulating mires are important sources of the greenhouse gas methane. Methane emissions and methanogenic *Archaea* communities have been shown to differ between fens and bogs, implying that mire succession includes an ecological succession in methanogen communities. We investigated methane production and the methanogen communities along a chronosequence of mires (ca. 100–2500 years), which consisted of five sites (1–5) located on the land-uplift coast of the Gulf of Bothnia. Methane production was measured in a laboratory incubation experiment. Methanogen communities were determined by amplification of a methyl coenzyme M-reductase (*mcr*) gene marker and analyzed by terminal-restriction fragment length polymorphism. The terminal-restriction fragment length polymorphism fingerprinting resulted in 15 terminal restriction fragments. The ordination configuration of the terminal restriction fragments data, using nonmetric multidimensional scaling, showed a clear gradient in the methanogen community structure along the mire chronosequence. In addition, fingerprint patterns of samples from the water table level and 40 cm below differed from one another in the bog site (site 5). Methane production was negligible in the three youngest fen sites (sites 1–3) and showed the highest rates in the oligotrophic fen site (site 4). Successful PCR amplification using *mcr* gene primers revealed the presence of a methanogen community in all five sites along the study transect.

Introduction

Mire ecosystems are characterized by a high water table which creates anoxia in submerged layers. These conditions lead to accumulation of peat as a consequence of the partial decomposition of organic matter. The pathway of mire development is determined by several factors, which are both allogenic and autogenic in origin. Autogenic factors include biotic processes involved in the accumulation of organic sediments as peat. The most important allogenic processes are connected to hydrological properties of the basin and to climatic variations (Bunting & Warner, 1998). Primary succession of mires begins with the initial colonization of bare mineral soil by pioneer species that are adapted to temporarily or permanently waterlogged conditions. During the successional development of mires, accumulation of peat typically leads to a shift from minerotrophic, groundwater-fed fens to ombrotrophic, rainwater-fed bogs. This shift is reflected in the vegetation community structure and has been observed in the vertical layering of the peat stratigraphy, where the minerotrophic sedge-dominated fen plant communities are gradually replaced by *Sphagnum*-dominated ombrotrophic vegetation (Tolonen, 1967; Korhola, 1992; Hughes & Dumayne-Peaty, 2002). Moreover, the relative proportion of bog communities has been shown to increase over time during the development of mires (Korhola *et al.*, 1996).

Due to anoxic conditions, mires are characterized by anaerobic, carbon dioxide, and methane (CH_4)-forming microbiota (Zehnder, 1978; Zinder, 1993; Stams, 1994; Schink, 1997). The end product of the anaerobic degradation of organic matter in mires is methane, produced by the methanogenic *Archaea* community. From the viewpoint of production of greenhouse gases, methanogenesis is of

special interest, as mires are important sources of natural methane emissions (Crill *et al.*, 1988; Whiting & Chanton, 1993; Nykänen *et al.*, 1998). Methane emissions have been connected to the successional stage of mires (Korhola *et al.*, 1996). Emission rates, as high as nearly 50 g CH_4 m^{-2} $year^{-1}$, have been reported from pristine fens in Finland, whereas older bog stage peatlands emit an order of magnitude less methane (Nykänen *et al.*, 1998).

Some information is available regarding the succession of plant communities in mire ecosystems at different stages of development (Huikari, 1956; Klinger & Short, 1996) but knowledge of the dynamics, structure and stratification of the microbial communities, including those of methane-producing *Archaea*, is still lacking. This knowledge is crucial for a better understanding of how mire succession, which results in a gradual change in environmental conditions and in availability of substrates, is reflected in the activity and community structure of methanogens. Knowledge about these processes is fundamental for predicting and mitigating undesirable impacts of human activities on these ecosystems.

Generally, succession of methanogenic *Archaea* communities can be hypothesized to be dependent on changes in environmental conditions, on the quantity and quality of substrate, which is ultimately determined by plant community structure, and on the succession of the other microbial communities. Recent studies of methanogen communities, based on the application of both functional (*mcrA*) (Galand *et al.*, 2002, 2005a) and 16S rRNA molecular markers (McDonald *et al.*, 1999; Galand *et al.*, 2003), have revealed differences in the microbial communities between fens and bogs. Moreover, methanogen community structure has also been found to change vertically within a site (Galand *et al.*, 2002, 2005a). These findings led us to hypothesize that mire succession includes a successional change in methanogen communities.

In the coastal area of the Gulf of Bothnia between Finland and Sweden, new land is continuously exposed (8–9 mm $year^{-1}$, Mäkinen *et al.*, 1986) due to ongoing post-glacial rebound. Consequently, a primary successional series of mire ecosystems, ranging from young fens in the first step of primary paludification to ombrotrophic bogs in older stages, can be found when moving inland from the coast. These kinds of spatial continua (Klinger, 1996; Klinger & Short, 1996) of mires offer opportunities to study interdependent successional pathways of plant and microbial communities of mire ecosystems.

We postulated that, as with mire vegetation, there is a successional gradient in methanogenic microbial communities from young fens to bogs. We studied the differences in methane production and in the community structures of methanogenic *Archaea* along a mire chronosequence, which formed a primary succession transect from a site in the mire induction phase to a site already in the fen–bog transition.

Materials and methods

The transect and sampling

The ca. 8-km-long chronosequence transect of peatlands is located in Siikajoki, on the eastern coast of the Gulf of Bothnia, Finland (64°45′N, 24°42′). The transect consists of five peatland sites, with ages varying from approximately 100 to 2500 years (Fig. 1).

The two youngest sites (sites 1 and 2, approximately 100 and 150 years old, respectively) are young fens with vegetation resembling wet meadows. The field layer is dominated by sedges and grasses (*Carex nigra, Carex canescens*, *Agrostis canina*), and herbs such as *Potentilla palustris*. In both sites, *Warnstorfia exannulata* dominates the ground layer, but the moss carpet is not fully developed in the younger site. The thin peat layer (0.0–0.1 m) consists of sedge remnants.

The two intermediate sites (sites 3 and 4, approximately 500 and 1065 ± 70 years old, respectively) are fens characterized by sedge (*Carex chordorrhiza*, *C. canescens* and *Carex rostrata*) communities. The moss carpet is well developed and consists of *Sphagnum fimbriatum* and *Sphagnum papillosum*. The peat layer is already 0.5–0.7 m thick.

The oldest site (site 5), 2520 ± 50 years old, is partly covered by bog vegetation. *Eriophorum vaginatum* dominates the field layer together with the dwarf shrubs *Rubus chamaemorus, Betula nana* and *Empetrum nigrum*. The ground layer is a mosaic, formed of ombrotrophic hummock species *Sphagnum fuscum* and *Sphagnum capillifolium* with oligotrophic lawn level species, such as *Sphagnum fallax*.

From each site, two or three peat (or peat/mineral soil) profiles were collected with box samplers ($8 \times 8 \times 100$ cm for peat cores and $4 \times 7 \times 70$ cm for peat/mineral soil cores) in September 2003. In addition, a set of parallel profiles was sampled for bulk density measurements. At the time of sampling, the mean water table was a depth of 52, 43, 32, 8 and 7 cm below the peat surface in sites 1–5, respectively. Peat or mineral soil samples were cut at intervals of 10 cm (± 1 cm) from the anoxic part of the collected profiles, i.e. at 0, 10, 20, 30 and 40 cm below the water table at the time of sampling (Table 1). The water table level at the time of sampling was selected as a starting point of sampling because the highest methane production rates are generally located in the oxic/anoxic interface just below the water table (Nykänen *et al.*, 1998). Altogether, the sampling resulted in 41 peat or mineral soil samples.

Measurement of methane production

For measurement of the methane production *in vitro,* 15 mL of peat or 30 mL of mineral soil were added to 120 mL

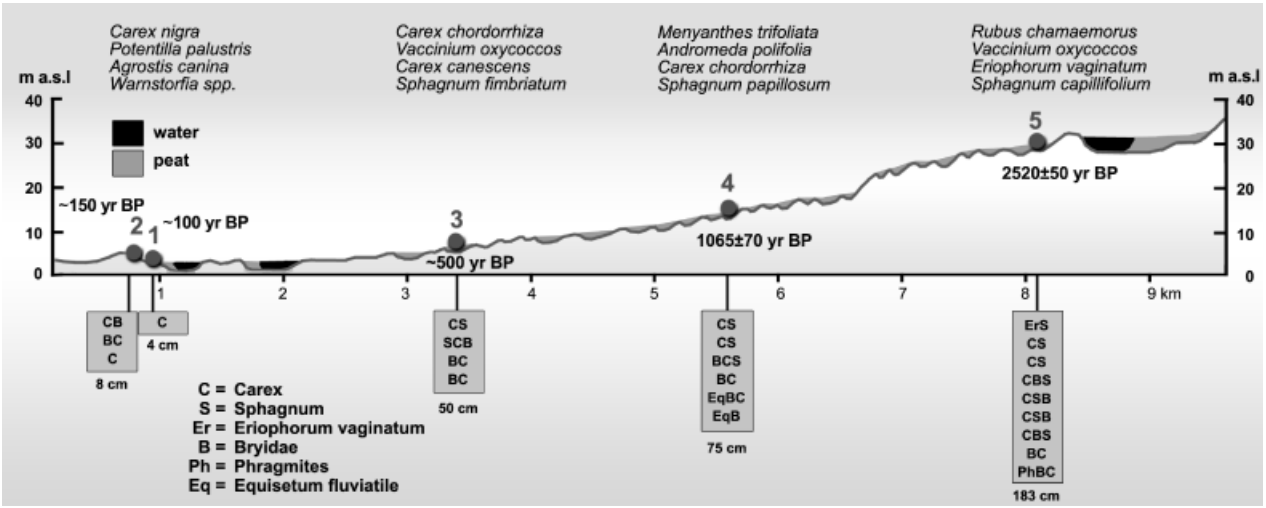

Fig. 1. Profile of the primary successional transect of mires studied consisting of five peatland sites (1–5). The characteristic plant species (above) and thickness and layering of the peat profile (below) in each site are presented. The estimates of site age are based on the land-uplift rate (sites 1–3) or on radiocarbon dating (sites 4 and 5). m a.s.l. = meters above sea level.

infusion bottles containing 30 mL of distilled water. The soil–water suspension material was flushed twice for 1 min with N_2 in order to obtain anoxic conditions, sealed with rubber septa and stored at +4 °C for 3 days. Thereafter, the bottles were flushed with N_2 and incubated at +14 °C in the dark for 22 h, followed again by N_2 flushing to remove residual methane at the beginning of the incubation experiment.

During the incubation of 70 h, methane concentrations in the headspace were monitored four times by injecting a gas subsample into a 0.5 mL loop of a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a 2-m-long column (inner diameter 1.5 mm, packed with HayeSep Q 80/100 mesh, 75 cc; Chromatography Research Supplies Inc. no. 483015, Louisville, KY). Helium was used as carrier gas at a flow of 20 mL min^{-1}. The conditions for the injector were 120 °C, pressure 345 kPa, split ratio 2 : 1, split flow 180 mL/min. The conditions for the detector were 250 °C, with H_2, O_2 and N_2 flows of 60, 450 and 25 mL min^{-1}, respectively. Oven temperature was set at 50 °C.

The rate of methane production was calculated from the slope of the linear regression given by the methane concentration increase over time. After incubation, pH was measured. Samples were filtered and measured for dry weight (105 °C, 24 h) and ignition loss (550 °C, 4 h) (Table 2). The final results were calculated on a volume basis based on the bulk density of the volumetric sample slices (Table 2) taken

Table 1. Description of the peat and mineral soil samples* (Dn) taken along the chronosequence transect and the respective abundance of the PCR product (PCR; ++, abundant; +, minor; –, none/not observed) obtained from the DNA extracted using methyl coenzyme M-reductase specific primers ($n = 41$)

	Successional age increases →																									
	Site 1				Site 2						Site 3						Site 4						Site 5			
	Core 1		Core 2		Core 1		Core 2		Core 3		Core 1		Core 2		Core 3		Core 1		Core 2		Core 3		Core 1		Core 2		Core 3	
Depth b.w.t. (cm)	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR	Dn	PCR
0	**m**	++	m	+	**m**	++	m	–	m	+	m	+	S, m	+	**m**	++	CS	++	CS	+	**SC**	++	NS	–	ErS	+	**NS**	++
10	-	-	-	-	-	-	-	-	-	-	S, m	++	S, m	–	m	+	BC	+	SC	++	(B)C	+	NCS	++	ErS	+	NErS	++
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	BC	+	BC	+	BC	+	SC	–	CNS	+	NCS	++
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	BC	+	BC	+	BC	–	(S)C	–	SC	++	NSC	+
40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	BC	+	BC	++	**BC**	++	BC	–	BC	++	**BC**	++

The samples in bold were subjected to terminal restriction fragment length polymorphism analysis. Depth b.w.t. = sampling depth, cm below water table.

*m, mineral soil (fine sand) including plant roots and their litter; S, *Sphagnum* peat; C, sedge peat; B, *Bryales* peat; N, nanolignine; Er, *Eriophorum* peat; -, not observed.

Table 2. Loss on ignition (OM%), bulk density and pH on the peat and mineral soil samples analyzed (see Table 1)

	Successional age increases →														
	Site 1			Site 2			Site 3			Site 4			Site 5		
Depth (cm)	OM%	Density (g cm^{-3})	pH	OM%	Density (g cm^{-3})	pH	OM%	Density (g cm^{-3})	pH	OM%	Density (g cm^{-3})	pH	OM%	Density (g cm^{-3})	pH
0	0.25	2.67	6.6	0.46	2.83	6.3	0.78	1.64	5.0	88.6	0.05	5.7	97.8	0.02	4.4
10	–	–	–	–	–	–	0.31	2.22	4.9	81.1	0.15	5.6	94.1	0.03	4.5
20	–	–	–	–	–	–	–	–	–	88.6	0.15	5.8	97.7	0.06	4.4
30	–	–	–	–	–	–	–	–	–	89.7	0.12	5.6	90.1	0.07	4.6
40	–	–	–	–	–	–	–	–	–	94.7	0.09	5.6	92.6	0.07	4.7

The values are means of two to three observations measured at 0, 10, 20, 30 and 40 cm below the water table at the time of sampling.

in parallel with the samples subjected to measurement of methane production.

Analyses of methanogen communities

DNA was extracted from peat (0.25 g) and mineral soil (1.00 g) samples by chemical and mechanical cell lysis using the Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). For the peat samples from deeper layers, further DNA purification, as described by (Kåren *et al.*, 1997), was occasionally required. The success of DNA extraction was verified by electrophoresis on 0.8% agarose gel with ethidium bromide staining.

Portions of the methyl coenzyme M reductase (*mcr*) gene were amplified by PCR with a methanogen-specific ML primer pair (Luton *et al.*, 2002). The forward primer was fluorescently labelled with 5′6-carboxyfluorescein (6-FAM; TAG Copenhagen A/S, Copenhagen, Denmark). The 50 μL PCR mixture contained 20 pmol of the primer pairs, 20 μm dNTPs, 1.2 U DNA polymerase (Biotools, B&M Labs SA, Madrid, Spain), PCR reaction buffer and 1–5 μL of template (DNA concentration determined empirically). Bovine serum albumin (0.6 μg μL^{-1} of reaction mixture) was used to prevent PCR inhibition. The reaction conditions were 40 cycles of 95 °C for 1 min, 50 °C for 1 min 30 s, and 72 °C for 2 min. Products were analyzed on 1% agarose gels with ethidium bromide staining.

One sample from the water table level of each site (named as −0) and from the deepest layer of sites 4 and 5 (40 cm below water table, named as −40) was selected for further analysis ($n = 7$, Table 1). The PCR products were purified from the gel by using WIZARD® PCR purification columns (Promega, Madison, WI) and aliquots of amplicons containing 200–300 ng of DNA were digested with the restriction enzyme *Msp*I (+37 °C, 2 h). *Msp*I was found to produce the highest amount of restriction groups when a set of *mcr* sequences (Galand *et al.*, 2005a,b) was digested with a set of restriction enzymes (*Msp*I, *Taq*I, *Rsa*I, *Hha*, *Hae*III, *Sau96*I) using NEBcutter V2.0 (Biolabs Inc., http://tools.neb.com/NEBcutter2/index.php; data not shown). The T-RFLP (terminal restriction fragment length polymorphism) fingerprints of three replicates of each community were determined by electrophoresis with a model ABI 310 automated sequencer (Applied Biosystems Instruments, Foster City, CA). The base pair lengths of the obtained peaks were defined using an internal size standard (GeneScan®-500 [TAMRA]™, Applied Biosystems). The electropherogram analysis was performed with the GeneScan software and peak heights lower than 100 fluorescent units were omitted from the final results. For each community analyzed, the average relative area proportion (%) of each terminal restriction fragment (T-RF) group from the sum total of all T-RF areas present was calculated and used in further analyses.

The Shannon diversity index (H') was calculated as:

$$H' = -\sum_{i=1}^{S} p_i \ln(p_i)$$

where p_i is the proportion of peak area of the ith T-RF group from the total peak area of all T-RFs present in the sample and S is the total number of T-RF groups.

Nonmetric multidimensional scaling (NMDS) was applied to present the reproducibility of T-RFLP analysis and to test the postulate concerning the successional gradient in the methanogen communities. The T-RFLP data were ordered by using PAST 1.23 software (http://folk.uio.no/ohammer/past/ Ryan *et al.*, 1995; Harper, 1999). The Bray–Curtis distance was applied as a measure of dissimilarity in methanogen community structure between the samples.

Results

Methane production along the chronosequence

Methane production was mainly detected in sites 4 and 5 (Fig. 2) and showed the highest rates at depths of 0–20 cm under the water table in those sites. The younger sites 1, 2 and 3 showed extremely low to negligible methane production during the incubation experiment (Fig. 2).

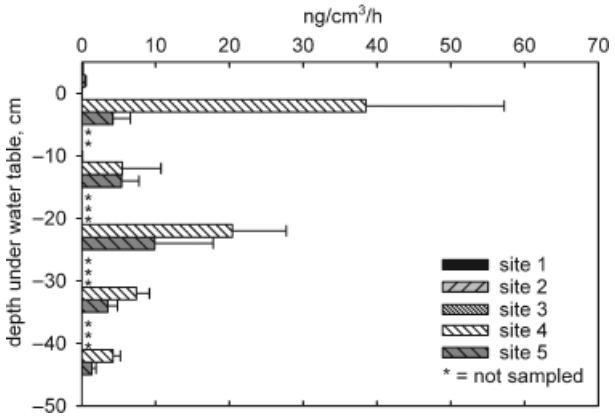

Fig. 2. Methane production at depths of 0–40 cm below water table in peat profiles taken along a successional transect of peatlands. The successional age increases from site 1 to 5. Error bars show SEM (n = 2–3 per site).

PCR amplification of methanogen *mcrA* gene and T-RFLP analysis

PCR amplification resulted in a detectable PCR-product at every depth sampled but not for every replicate sample (Table 1). The samples with an undetectable or very weak PCR product were generally connected with a low methane production, indicating that methanogens were present in low numbers in the samples.

The T-RFLP fingerprinting resulted in 15 T-RFs (*groups a–o*, Fig. 3). The Shannon diversity index (H') was 1.70, 1.63, 1.91, 1.43 and 1.08 at the water table level in sites 1, 2, 3, 4 and 5, respectively; at 40 cm below the water table, H' was 1.70 (site 4) and 1.72 (site 5). Methane production rate and the Shannon diversity index showed no clear relationship with one another.

In ordination configuration of non-metric multidimensional scaling, the three replicates of each sample generally grouped together, showing the reproducibility of T-RFLP analysis (Fig. 4). However, replicate 5–0 c separated slightly from its replicates (Fig. 4). This is due to the dominant T-RF group (group h), which was represented with a greater proportion in this replicate than in the two other replicates analyzed.

The ordination configuration of T-RF data in NMDS showed also a clear successional gradient in the methanogen community structure: the samples taken from the water table level (− 0) were organized according to their position along the chronosequence transect (Fig. 4). The two youngest fens (sites 1 and 2) were overlapping and were located at the one end of the gradient (Fig. 4): their methanogen communities were also almost identical (Fig. 3). The upper layer of ombrotrophic site 5, which showed the lowest methanogen diversity (Fig. 3), was at the other end of the successional gradient. The samples from fen sites 3 and 4 were situated in their successional order between the two extremes, although the community sample in site 3 was taken from mineral soil, as in sites 1 and 2, whereas the sample of site 4 originated from peat, as in site 5. In addition to the successional gradient, the methanogen communities showed a clear depth-related distribution in site 5. In site 4 the stratification was not as pronounced. The stress factor (0.1072) of the NMDS solution was low.

The young fen sites (sites 1–3) were characterized by T-RF *group b*; in sites 4 and 5, this group was not present (4–0) or represented only a very small proportion of the sequences (4–40, 5–0, 5–40). *Groups c, d* and *f* were characteristic for all the fen sites (sites 1–4): in these sites, *group c* showed the greatest relative abundance. *Group d* was retrieved exclusively from them. *Group f* was found in minor proportions in all samples taken from the fen sites. In the bog site 5, *group f* was absent (5–40) or had a very low relative abundance (5–0). With a relative proportion of 40% or more, *group a* was the most abundant in site 4 (−0, −40) and site 5 (−40). *Group h* dominated the surface community of bog site 5 (−0) and was also present in the deeper layer (−40) of fen site 4. In other samples the proportion of this group was extremely small. *Groups e, g* and *k* were the

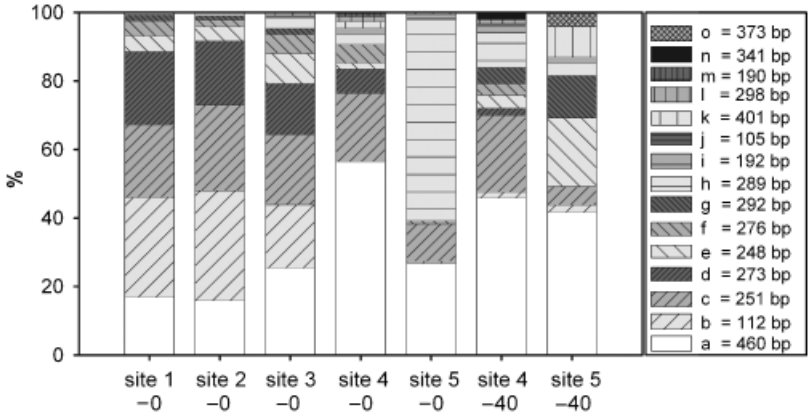

Fig. 3. The mean relative abundances of terminal restriction fragments (T-RFs; a–o) obtained (n = 3 in each sample). The samples were taken along a successional transect of peatlands at the depth of water table level (− 0) and 40 cm below (− 40). The successional age increases from site 1 to 5. bp = the length of terminal fragment in base pairs.

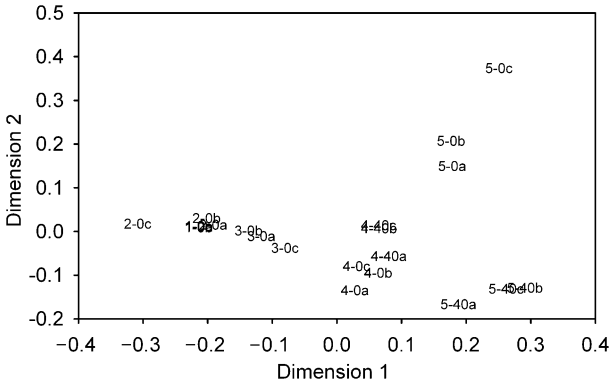

Fig. 4. Ordination configuration of terminal restriction fragment length polymorphism fingerprints of the samples in two-dimensional solution of nonmetric multidimensional scaling (cf. Fig. 3). The samples were taken along a successional transect of peatlands (sites 1–5) at the depth of water level table (− 0) and 40 cm below (− 40). The three replicates analyzed from each sample are shown (a–c).

most abundant at the lower layer of site 5 (−40) and *group o* was found only in this sample. *Group n* was present only in the deeper layer (−40) of site 4. *Groups i, j, l* and *m* were minor groups, with a relative proportion always less than 3.5%.

Discussion

To the best of our knowledge, this is the first study demonstrating the changes occurring in a community structure of an important, functionally defined microbial group during the formation of a characteristic boreal ecosystem, i.e. during the primary succession of a mire ecosystem. As postulated, the methanogen community structure along the chronosequence transect studied showed a clear gradient. This was revealed by NMDS ordination of the T-RF data in which the sites were organized in the order of their position along the transect.

The chronosequence transect that we studied was determined primarily by the age and vegetation type of the sites. When the anaerobic, submerged layer was sampled to study the concurrent changes in the methanogen communities, the transect converted into a sequence from sandy soils, poor in organic matter and with decreasing pH (from site 1 to 3) to a peat layer with decreasing pH (Dunfield *et al.*, 1993) (from site 4 and 5). Thus, there was a gradual change in environmental conditions that was accompanied with a change in the quality and quantity of substrates, which were available to methanogens and the other microbes participating in the anaerobic degradation of organic matter. This change can be interpreted to have originated from ecosystem succession, which includes vegetation changes, podzolization of the uppermost mineral soil layers and accumulation and stratification of the peat layer. The shift of the prevailing matrix from mineral soil (sites 1–3) to organic peat (sites 4–5) inherently belongs to the primary succession of mire ecosystems, which, by definition, begins with the initial colonization of pioneer species on bare mineral soil. We did not sample either the mineral soil of the neighboring sea bottom, still unexposed and thus representing the mineral soil habitat before the start of mire succession, or the mineral soil layer under the peat layer of older successional sites 4 and 5. Therefore, we are not able to assess whether the methanogen community structures found in the anoxic mineral layer of young fen sites (sites 1–3) are specific to these early successional sites or if they also represent communities typical of the anoxic mineral soil layers in the study area. It is, however, important to bear in mind that the mineral soil layers sampled were not completely isolated from the above ground vegetation communities, as small amounts of plant roots and their litter were present even in the mineral soil layers sampled.

In addition to the clear changes in methanogen community structure along the transect, a vertical stratification was also apparent, especially in the oldest site representing the fen–bog transition stage (site 5). This result is consistent with the previous results of Galand *et al.* (2002, 2005a), who found depth-related distribution in methanogen communities in both fen Galand *et al.*, (2002) and bog (Galand *et al.*, 2005a) ecosystems. That the vertical stratification was more apparent in fen–bog site (site 5) than in fen site (site 4) may be due to different types of peat layers affecting the substrate composition for the microbes. The layers at the top of the water table and 40 cm below differed more from each other in site 5 than in site 4: sedge peat was present in both layers sampled in site 4 (Table 1), whereas *Sphagnum* peat dominated the upper (− 0) peat layer in site 5, and sedge peat was present only in the lower (− 40) peat layer.

In this study the sequential change in methanogen community structure along the successional transect was well illustrated. The mechanisms of colonization and spreading during the successional development of methanogen communities in submerged layers remain elusive. Seasonally fluctuating water table can, however, be considered as a primary vector facilitating migration of methanogens to new habitats upwards from deeper layers and vice versa.

The pattern of methane production along the chronosequence transect of five successional mire sites was consistent with earlier studies (Nykänen *et al.*, 1998), showing the highest methane production in the oligotrophic fen site (site 4) and a clearly lower rate in the oldest site representing the bog–fen transition stage (site 5). In the young fen sites (sites 1–3) the rate of methane production was generally near zero. PCR amplification using methanogen-specific *mcr* gene primers however, revealed the occurrence of a methanogen community, even in the young fen sites with negligible methane production. In these sites (sites 1–3) the water-

saturated layer consisted primarily of mineral soil with a small mass of plant roots and their litter (Tables 1 and 2), which apparently provided the substrates (e.g. acetate, carbon dioxide, hydrogen) for the methanogen community. The negligible methane production suggests that methanogenesis in these layers is probably still extremely limited by substrate availability. It could also be possible that sandy soil provides a supply of oxidants, such as Fe(III), Mn(IV) or SO_4^{2-} (originating from brackish water and former sea sediments), for anaerobic oxidation of the methane produced (Valentine, 2002). There is evidence indicating that the anaerobic oxidation of methane is coupled to sulphate reduction (Hoehler *et al.*, 1994), but other electron acceptors theoretically could also act in methane oxidation under anaerobic conditions (Valentine, 2002). Moreover, the particle size of the mineral soil sampled in sites 1–3 was relatively coarse (fine sand), having a low cation exchange capacity and surface area, thus offering poor conditions for sorption of soil organisms (Wagner *et al.*, 1999). The importance of soil texture in methane production was shown in a study of (Wagner *et al.*, 1999), who by using acetate and H_2/CO_2 as substrates for inoculated strains of *Methanobacterium* and *Methanosarcina* found the methane production rate in anoxic conditions to be more than fivefold higher in a clay material than in sandy soil.

The vertical pattern of methane production in bog site 5 agrees with earlier studies (Sundh *et al.*, 1994; Kettunen *et al.*, 1999) showing the highest rate at the depth of 20 cm below the water table at the time of sampling. Site 4, however, showed a somewhat anomalous pattern, with the highest methane production just at the water table level. This is a result of one sampling and may be due to the fact that, after a dry summer with exceptionally low precipitation in the sampling area (data not shown), the water table had fallen below the normally prevailing level. Thus, sampling based on the current water table level may have resulted in an upward transfer, relative to water level, of the layer, which generally showed the highest methane production.

Interpretation of T-RFLP fingerprints and identification of T-RFs

We made an attempt to identify and evaluate the phylogeny of the T-RF groups. This was carried out by comparing T-RFs of the community samples of our study with known sequences recently retrieved from other Finnish peatlands (Galand *et al.*, 2005a, b). The known sequences were selected as references because their theoretical T-RFs corresponded with the T-RF groups found in the community samples analyzed. The known sequences were PCR-amplified and further analyzed by T-RFLP, both alone and mixed with the environmental samples. This analysis resulted in tentative phylogenetic information on five of the 15 T-RF groups found. T-RF *group b* is possibly related with members of Rice cluster-I (Lueders *et al.*, 2001; Galand *et al.*, 2005b); *group c* may correspond either with Rice cluster-I or with *Methanosarcinales* (Galand *et al.*, 2005b). *Group f* resembled a sequence belonging to Fen-cluster (Galand *et al.*, 2005b), recently named by Galand *et al.* (2002). Phylogenetically the members of this cluster were found to stand closest to the order *Methanomicrobiales* (Galand *et al.*, 2002). Fen-cluster is also a probable affiliation for *groups h* and *k* (Galand *et al.*, 2005a, b).

Our tentative approach to identify the phylogeny of T-RF groups resulted in interesting findings. Firstly, the dominant T-RF group in the upper layer of bog site 5 (*group h*) may correspond to the sequence which, as reported by Galand *et al.* (2005b) and Juottonen *et al.* (2005), was also found to dominate the clone libraries retrieved from the ombrotrophic bog site of their studies. Thus, the upper anoxic layer of ombrotrophic bogs, being generally dominated by *Sphagnum* peat, may support only a low diversity of methanogens, reflecting the acid, nutrient-poor environment and low vegetational diversity in these ecosystems. Secondly, the T-RF group abundant in the fen sites (*group b*) tentatively corresponded with earlier extracted sequences related to Rice cluster-I. Members of the Rice cluster-I have been earlier selectively enriched with H_2/CO_2 as an energy source (Lueders *et al.*, 2001; Sizova *et al.*, 2003), indicating that the group includes hydrogenotrophs. This would be in accordance with previous studies which have shown hydrogenotrophy to be an important pathway in peatlands (Lansdown *et al.*, 1992; Hornibrook *et al.*, 1997; Popp & Chanton, 1999; Chasar *et al.*, 2000; Horn *et al.*, 2003). Thirdly, T-RF *groups f, h* and *k* may correspond with sequences related to the Fen-cluster (Galand *et al.*, 2002). The pathway of methanogenesis is not known for the members of this cluster.

We used the ML primer set containing degenerate nucleotides (Luton *et al.*, 2002) for T-RFLP analysis to investigate changes occurring in the structure of methanogen communities along a successional transect of mires. The ML primer set, designed to detect *mcrA* gene present in MCRI operon, can potentially also detect *mrtA,* the equivalent gene of *mcrA* in MCRII operon (Luton *et al.*, 2002), shown to be present only in members of orders *Methanobacteriales* and *Methanococcales*. Thus, the use of ML primers in community analysis has the potential to overestimate the true methanogen diversity. When interpreting the T-RFLP fingerprints obtained, it is important to bear in mind that, especially when using degenerate primers, the amplicon frequencies found in the resulting PCR product are sensitive to the applied annealing temperature (Lueders & Friedrich, 2003). Functional primers in mire studies still have the advantage of strictly amplifying the

genes of functionally unique methane-producing communities. Further, in the evaluation of primers targeting *mcrA* gene, the ML primers proved to be the best for analysis of methanogens in boreal mires both in terms of amplification efficiency and diversity coverage (H. Juottonen, P. E. Galand and K. Yrjälä, unpublished).

Conclusions

In this study, the methanogen community structure was shown to respond clearly to changing resource availability, which resulted from the development of a mire ecosystem. The methane production rate showed no clear relationship with the methanogen diversity described by the abundance and number of T-RF groups that we found. In general, the chronosequence proved to be a promising tool to study the changes occurring in microbial community structure, function and their relationships during the succession of peatlands, as well as interactions between plant and microbial communities. As the sites representing different successional stages were not replicated, the generalizations based on our results should be made with care. Our approach offers, however, an interesting reference to the development of cutaway peatlands under restoration.

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