Archaea, Bacteria, and methane production along environmental gradients in fens and bogs

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Front cover picture: *Eriophorum* on a mire in Persböle, Pohja, Finland
(photo by Hannu Juottonen)
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This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.


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Summary

Methanogens are anaerobic Archaea with unique energy metabolism resulting in production of methane (CH$_4$). In the atmosphere CH$_4$ is an effective greenhouse gas. The largest natural sources of atmospheric CH$_4$ are wetlands, including peat-forming mires. Methane emissions vary greatly between and within mires, depending on season and hydrological and botanical characteristics. The aim of this work was to elucidate the microbiology underlying the variation.

Methanogens and potential CH$_4$ production were assessed along spatial and temporal gradients of ecohydrology, season, ash fertilization, and peat depth in three Finnish boreal mires. Non-methanogenic Archaea and Bacteria were additionally addressed as potential substrate producers and competitors to methanogens. Characterization of microbial communities targeted the mcrA gene, essential in CH$_4$ production, and archaeal or bacterial 16S ribosomal RNA gene. The communities were differentiated by analysis of clone libraries, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP) fingerprinting.

Methanogen communities and CH$_4$ production changed markedly along an ecohydrological gradient from fen to bog, with changing vegetation and pH. The most acidic Sphagnum bog showed mainly Methanomicrobiales-associated, hydrogenotrophic Fen cluster methanogens, whereas the oligotrophic and mesotrophic fens with higher pH and sedge coverage had more diverse communities including acetoclastic methanogens. Season had a minor effect on the archaeal community in an acidic oligotrophic fen, but the temporal variation of CH$_4$ production potential was substantial. Winter potential was unexpectedly high, and active methanogens were detected in winter peat. Ash fertilization, a forestry practice for promoting tree growth, had no substantial effects on CH$_4$ production or methanogen communities in a drained bog, but the communities changed with peat depth. Comparison of three mcrA primer sets revealed that their coverage of methanogens from the drained bog was similar, but the quantitative representations of communities were primer-dependent. Bacterial and non-methanogenic archaeal groups detected in mires included Deltaproteobacteria, Acidobacteria, Verrucomicrobia, and Crenarchaeota of groups 1.1c and 1.3. Their detection forms a starting point for further studies to distinguish possible interactions with methanogens.

Overall, the results indicate that methanogen community composition reflects chemical or botanical gradients that affect CH$_4$ production, such as mire hydrology. Predictions of CH$_4$ production in the spatially heterogeneous mires could thus benefit from characterization of methanogens and their ecophysiology.
Abbreviations

aa amino acid(s)
ANME anaerobic methane-oxidizing Archaea
ANOSIM analysis of similarity
ANOVA analysis of variance
bp base pair(s)
DGGE denaturing gradient gel electrophoresis
FC Fen cluster; a group of methanogens named after detection in a fen
FISH fluorescence in situ hybridization
gdw grams dry weight
MCR methyl-coenzyme M reductase
mcrA gene encoding methyl-coenzyme M reductase I α-subunit
mRNA messenger ribonucleic acid
mrtA gene encoding methyl-coenzyme M reductase II α-subunit
OTU operational taxonomic unit
PCA principal component analysis
PCR polymerase chain reaction
ppb parts per billion
qPCR quantitative polymerase chain reaction
RC Rice cluster; group of Archaea named after detection in rice field soil
RDA redundancy analysis
RFLP restriction fragment length polymorphism
rRNA ribosomal ribonucleic acid
spp. species
SSCP single-strand conformation polymorphism
TGGE temperature gradient gel electrophoresis
T-RF terminal restriction fragment
T-RFLP terminal restriction fragment length polymorphism
1 Introduction

1.1 Methanogenic Archaea

Methanogens are anaerobic prokaryotes belonging to the domain *Archaea*, the third domain of life in addition to *Eucarya* and *Bacteria* (Woese *et al.* 1990). They are metabolically unique among *Archaea* and all other organisms due to their ability to obtain energy from selected low molecular weight carbon compounds and hydrogen with stoichiometric production of methane (CH$_4$).

In the classification of *Archaea* into two main phyla of *Euryarchaeota* and *Crenarchaeota*, methanogens occupy the euryarchaean branch together with non-methanogenic halophilic, thermoacidophilic, and hyperthermophilic *Archaea* (Boone and Castenholz 2001). A number of genes of the extensively studied, complex methanogenic pathway are found in non-methanogenic organisms, particularly in euryarchaeal *Archaeoglobales*, but methanogens are the only organisms employing the whole pathway with CH$_4$ production (Vornolt *et al.* 1995, Klenk *et al.* 1997, Chistoserdova *et al.* 1998, Thauer 1998). Methanogenesis has thus been suggested as an ancestral feature of euryarchaea which has subsequently been lost in non-methanogens (Bapteste *et al.* 2005, Gribaldo and Brochier-Armanet 2006).

Methanogens can only grow with a limited set of one- or two-carbon compounds and hydrogen. Unable to gain energy from complex compounds, methanogens are dependent on substrate supply from associated anaerobic microbial communities or geological sources. Three types of methanogenic pathways are recognized, differing in their substrates (Deppenmeier 2002):

- **Hydrogenotrophic** methanogens grow with hydrogen (H$_2$) as the electron donor and carbon dioxide (CO$_2$) as the electron acceptor. Some hydrogenotrophs also use formate, which is the source of both CO$_2$ and H$_2$.
- **Acetoclastic** methanogens cleave acetate into a methyl and a carbonyl group. Oxidation of the carbonyl group into CO$_2$ provides reducing potential for reduction of the methyl group into CH$_4$.
- **Methylotrophic** methanogens grow on methylated compounds such as methanol, methylamines, and methylsulphides, which act as both electron donor and acceptor or are reduced with H$_2$.

Some methanogens are also able to use alcohols such as ethanol and propanol as a source of H$_2$ for reduction of CO$_2$ or grow on CO (O’Brien *et al.* 1984, Zellner and Winter 1987). Taxonomically methanogens form five orders: *Methanosarcinales* (9 genera), *Methanomicrobiales* (8), *Methanobacteriales* (5), *Methanococcales* (4), and *Methanopyrales* (1 genus) (Boone and Castenholz 2001). The majority of described methanogens are able to produce CH$_4$ from H$_2$ and CO$_2$, and orders *Methanomicrobiales*, *Methanococcales* and *Methanopyrales* contain only hydrogenotrophic methanogens. Members of *Methanobacteriales* are also hydrogenotrophic, except the methylotrophic genus.
Methanosphaera. Other methylotrophic methanogens and all acetotrophs belong to Methanosarcinales, including the only known obligate acetotrophs forming the family Methanosarcinaceae. The order Methanosarcinales includes the most metabolically versatile methanogens; several members of the family Methanosarcinaceae possess all three methanogenic pathways (Garcia et al. 2000, Galagan et al. 2002).

Beyond their shared energy metabolism, methanogens are physiologically and morphologically divergent. For example, most cultured methanogens grow optimally at mesophilic temperatures (Garcia et al. 2000), but the temperature range of methanogenic activity reaches from psychrophilic growth of Methanogenium frigidum (Franzmann et al. 1997) and Methanosarcina lacustris (Simankova et al. 2001) at 1 °C to hyperthermophilic growth of Methanopyrus kandleri at 110 °C (Kurr et al. 1991). Several thermophilic genera are found in orders Methanobacteriales and Methanococcales. Cell forms of methanogens are highly variable even within one order and range from cocci, rods, and spirilla to sarcina and irregular plate forms (Garcia et al. 2000). Like cell walls of all Archaea, those of methanogens lack peptidoglycan and consist of pseudomurein, protein units, or a unique polymer called methanochondroitin (Kandler and König 1998).

The variety of methanogenic habitats reflects their physiological diversity and requirement of anoxic conditions. Methanogenic ecosystems include (Garcia et al. 2000, Chaban et al. 2006):

- Anaerobic environments with decomposing organic matter. These include temporarily or permanently flooded wetlands such as mires, rice fields, and salt marshes; freshwater and marine sediments, landfills, and waste digesters. In freshwater environments methanogenesis is generally acetoclastic or hydrogenotrophic but in marine sediments often methylotrophic. These environments harbour a wide range of methanogens of the orders Methanosarcinales, Methanomicrobiales, Methanobacteriales, and Methanococcales (see Chaban et al. 2006).

- Digestive tracts of diverse organisms including ruminants, humans, and arthropods such as termites. Anaerobic protozoa also have endosymbiotic methanogens. Because the host organism absorbs intermediates of decomposition such as acetate, methanogenesis in digestive tracts is mostly hydrogenotrophic and frequently carried out by methanogens of the order Methanobacteriales (Lange et al. 2005).

- Geothermal environments, such as hot springs, petroleum reservoirs, and seafloor hydrothermal vents, where the substrates (H₂, CO₂) originate from geological activity. Thermophilic and hyperthermophilic strains belonging to the orders Methanobacteriales, Methanococcales, and Methanopyrales have been isolated from these environments (e.g. Jones et al. 1983, Lauerer et al. 1986, Kurr et al. 1991).
1.2 Methane as a greenhouse gas

When released from methanogenic ecosystems into the atmosphere, CH$_4$ is a reactive and radiatively active trace gas. After water vapour and carbon dioxide (CO$_2$), CH$_4$ is the next most abundant greenhouse gas (Wuebbles and Hayhoe 2002). The global warming potential of CH$_4$, i.e., effectiveness as a greenhouse gas, is 25 times that of CO$_2$ with a 100-year time horizon (IPCC 2007). The contribution of CH$_4$ to the total climate warming effect of greenhouse gases is 18%. The increase in atmospheric CH$_4$ concentrations from 700-715 parts per billion (ppb) in 1750 to 1775 ppb in 2005 has been attributed to anthropogenic CH$_4$ sources (IPCC 2007).

Annual CH$_4$ emission is estimated to be 503-610 Tg CH$_4$ year$^{-1}$, and more than 70% of this is biogenic CH$_4$ originating from activity of methanogens (IPCC 2007). Natural biogenic sources include northern and tropical wetlands, termites, and oceans. Anthropogenic biogenic sources include rice fields, ruminants, landfills and other waste treatment facilities. Abiogenic emissions originate from fossil fuels, incomplete biomass burning, CH$_4$ hydrates, and geological sources (Wuebbles and Hayhoe 2002, IPCC 2007). Plants have been argued to emit 0-236 Tg CH$_4$ year$^{-1}$, but the magnitude and mechanism of these emissions remain unresolved (Keppler et al. 2006, Kirschbaum et al. 2006, Dueck et al. 2007). The primary CH$_4$ sinks are oxidation to CO$_2$ in the atmosphere and oxidation by methanotrophic bacteria in aerobic soils (Wuebbles and Hayhoe 2002, IPCC 2007).

The largest single source of CH$_4$ is natural wetlands, including peat-forming mires. Estimations of emissions range from 100 to 231 Tg CH$_4$ year$^{-1}$ (IPCC 2007). Northern wetlands, which are predominantly located >50°N, are estimated to account for 15-30% of the total CH$_4$ emissions from wetlands (Matthews and Fung 1987, Cao et al. 1996, Hein et al. 1997, Walter et al. 2001, Chen and Prinn 2006). Wetland emissions have been suggested to contribute significantly to the interannual variability of global CH$_4$ emissions (Mikaloff Fletcher et al. 2004, Bousquet et al. 2006). Permafrost melting due to rising temperature in Siberia and Alaska is expected to increase wetland CH$_4$ emissions from the northern hemisphere (Christensen et al. 2004, Turestsky et al. 2007).

1.3 Methanogenesis in mires

1.3.1 Mires

Mires are wetlands with permanently high water level, peat-forming vegetation, and accumulation of partially degraded organic matter as peat (Laine and Vasander 1996). Low rates of decomposition in anoxic peat lead to extensive carbon storage (Clymo 1984, Gorham 1991). Peatlands are important long-term carbon sinks, storing ~300 Pg of carbon, which is approximately 13% of total soil carbon (Turunen et al. 2002, Vasander and Kettunen 2006). Northern mires comprise only 3% of the global land area, but they cover >20% of land area of some countries in the boreal region such as Finland and Estonia (Rydin and Jeglum 2006). A considerable share of the original mire area in Finland has been drained for forestry (Paavilainen and Päivänen 1995).
Mires are acidic, nutrient poor environments with vegetation adapted to the harsh conditions. Two main types defined based on hydrology are minerotrophic fens, which receive water and nutrients from groundwater, and ombrotrophic bogs, which rely solely on atmospheric deposition. Consequently, bogs have lower nutrient and cation levels, particularly Ca, and lower pH than fens (Laine et al. 2000). Vegetation of northern bogs is characterized by Sphagnum mosses, which further acidify their surroundings (Rydin and Jeglum 2006). Fens typically have higher coverage of graminoids such as sedges (Carex spp.) with root systems reaching anoxic peat. As an adaptation to anoxia, Carex and other typical mire vascular plants have aerenchyma, intercellular spaces which form a gas conduit and allow transport of oxygen into roots (Koncalova 1990, Armstrong et al. 1991).

![Figure 1](image_url). Schematic overview of carbon and CH\(_4\) cycling in mires.

1.3.2 Anaerobic decomposition

The high water level in mires leads to vertical stratification with a shallow oxic layer and up to several meters of anoxic peat (Fig. 1). Above the water level, aerobic fungi and bacteria degrade organic matter to CO\(_2\). Below the water level, oxygen level declines rapidly with depth (Lloyd et al. 1998). Under anoxic conditions, decomposition requires several guilds of anaerobic microbes acting in interconnected successive stages. Organic matter is converted into fermentation products, including organic acids and acetate, and finally into CH\(_4\) and CO\(_2\) (Fig. 2).
In the first stage, hydrolytic enzymes of anaerobic bacteria and fungi break the organic polymers (e.g. cellulose, hemicellulose, starch, proteins) into monomers (sugars, amino acids). The monomers are fermented into acetate, fatty acids, alcohols, CO$_2$, and H$_2$. In peat, acetate, phenyl acetate, phenyl propionate, caproate, butyrate, and ethanol have been detected as intermediates in CH$_4$ production (Kotsyurbenko et al. 2004, Metje and Frenzel 2005, 2007). Syntrophic bacteria ferment the fatty acids and alcohols to acetate, CO$_2$, and H$_2$. Syntrophs produce H$_2$ and often occur in tight interaction with hydrogenotrophic methanogens; consumption of H$_2$ by methanogens makes the fermentation process of the syntrophs energetically feasible (Schink 1997). Acetogens produce acetate from organic monomers or from H$_2$ and CO$_2$. Acetate, CO$_2$, and H$_2$ generated at the fermentative and acetogenic steps are substrates for terminal decomposers. Because availability of oxygen and alternative electron acceptors such as sulphate (SO$_4^{2-}$), nitrate (NO$_3^-$), and ferric iron (Fe$^{3+}$) is generally limited in anoxic peat, the prevalent terminal process is methanogenesis.

Methane produced in the water-submerged peat layers is emitted into the atmosphere by diffusion in water, bubbling, or through the aerenchyma of vascular plants (Whalen 2005). When CH$_4$ passes through the oxic surface layer, depending on the thickness of the layer, more than 90% of the methane produced in anoxic peat may be oxidized into CO$_2$ by aerobic methanotrophic bacteria (Segers 1998, Frenzel and Karofeld 2000, Pearce and Clymo 2001). Methane transported through aerenchymatous plants largely escapes oxidation (Schimel 1995).
1.3.3 Pathways of CH$_4$ production

In freshwater environments, the principal precursors of CH$_4$ are acetate and H$_2$/CO$_2$. Acetate is considered to account for two thirds of the produced CH$_4$ (Whiticar et al. 1986, Conrad 1999). In mires, however, either hydrogenotrophic methanogenesis (Lansdown et al. 1992, Metje and Frenzel 2005) or acetoclastic methanogenesis may dominate (Kotsyurbenko et al. 2004, Metje and Frenzel 2007). Stimulation of CH$_4$ production by methanol in permafrost peat additionally implies potential for the methylotrophic pathway in some peat soils (Ganzert et al. 2007).

The pathway of methanogenesis has been observed to shift from acetoclastic in surface peat, rhizosphere, and Carex fens to hydrogenotrophic in more oligotrophic deeper peat and Sphagnum-dominated bogs (Kelly et al. 1992, Hornibrook et al. 1997, Bellisario et al. 1999, Popp et al. 1999, Chasar et al. 2000a, Galand et al. 2005). Hence, availability of fresh organic matter favours acetoclastic methanogenesis, whereas hydrogenotrophic pathway dominates in more oligotrophic and recalcitrant peat (Miyajima et al. 1997, Hornibrook et al. 2000, Ström et al. 2003). When acetoclastic production is substantial, the pathways appear to have seasonal shifts, with acetoclastic production being particularly important in summer when acetate levels and CH$_4$ production rates are high (Kelly et al. 1992, Avery et al. 1999, Chasar et al. 2000b).

1.3.4 Environmental factors controlling methanogenic activity

The occurrence and activity of methanogens can be assessed by measuring potential CH$_4$ production, i.e., microbial formation of CH$_4$ from endogenous or added substrates in anoxic laboratory incubations. Although sampling and preparation of peat slurries disturb the samples, potential CH$_4$ production estimates methanogenic activity better than CH$_4$ emissions or gas concentrations measured in the field. Unlike the latter methods, measurements of production potential are not affected by aerobic CH$_4$ oxidation or transport of old CH$_4$ from deeper peat. An unknown factor is anaerobic CH$_4$ oxidation, which occurs coupled to sulphate reduction in marine sediments (Hoehler et al. 1994, Orphan et al. 2001) and to denitrification in agriculture-influenced freshwater sediments (Raghoebarsing et al. 2006). Anaerobic CH$_4$ oxidation has only recently been reported in peat (Smemo and Yavitt 2007), and its extent and mechanism in mires is unknown. If it is prevalent, measurements of CH$_4$ production without considering the simultaneous anaerobic oxidation underestimate the actual methanogenic activity.

The growth and activity of methanogens occurs mainly in the anoxic portion of the peat profile and is thus regulated by water table depth. The largest CH$_4$ production potentials are generally measured 10-20 cm below the water level, and production declines in deeper, more decomposed peat (Williams and Crawford 1984, Sundh et al. 1994, Krumholz et al. 1995, Saarnio et al. 1997, Edwards et al. 1998). Methane production potentials and methanogens have, however, been detected in unsaturated peat and soils (Peters and Conrad 1995, Wagner and Pfeiffer 1997, Kettunen et al. 1999, Kobabe et al. 2004, Høj et al. 2006), suggesting methanogens survive in anoxic microenvironments or tolerate temporary

In anoxic peat, the main regulator of methanogenic activity and CH$_4$ production is substrate supply (Svensson and Sundh 1992, Valentine et al. 1994, Segers 1998). Plant primary production ultimately regulates the input of organic matter, and recently fixed carbon from fresh litter or root exudates of vascular plants has been shown to support CH$_4$ production (Chanton et al. 1995, Bellisario et al. 1999, van den Pol-van Dasselaar and Oenema 1999, Chasar et al. 2000a, King and Reeburgh 2002, Ström et al. 2003). Fresh organic matter has higher quality, i.e., higher amount of labile carbohydrates readily available to decomposers, opposed to older material rich in recalcitrant compounds such as lignin and humic substances (Valentine et al. 1994, Yavitt et al. 2000). Water level affects substrate quality. If the level is close to surface, organic matter reaches water-saturated peat virtually undecomposed; when the level is lower, the labile compounds are degraded extensively in the thicker layer of aerobic peat, and the fraction of organic matter available for CH$_4$ production is more recalcitrant. However, vascular plants such as Carex and Eriophorum allocate labile carbon directly into water-saturated layer as root exudates (Joabsson et al. 1999). Vegetation also influences substrate quality through differences in litter chemistry and decomposability. Sphagnum mosses are particularly resistant to decomposition (Aerts et al. 1999, Kuder and Krüge 2001).

Methane production is strongly dependent on temperature and usually temperature-limited in northern mires, with maximal production at 20-35 °C (Svensson 1984, Segers 1998, Kotsyurbenko et al. 2004, Metje and Frenzel 2005). Incubation of acidic peat (pH 4-5) at elevated pH has lead to higher production with maximal production at pH 6-7 (Williams and Crawford 1983, Goodwin and Zeikus 1987, Dunfield et al. 1993, Valentine et al. 1994, Kotsyurbenko et al. 2007), suggesting that also pH limits methanogenic activity in peat. Exceptions are known where higher pH had no effect or even inhibited CH$_4$ production (Yavitt et al. 1987, Bergman et al. 1998, Bräuer et al. 2004).

If alternative electron acceptors are present, methanogens compete for substrates, particularly H$_2$, with other terminal decomposers. For example, sulphate reduction due to air-borne sulphate deposition may decrease CH$_4$ production (Nedwell and Watson 1995, Dise and Verry 2001, Gauci et al. 2004). When the available electron acceptor is CO$_2$, hydrogenotrophic methanogens compete with acetogens. In some northern mires, an acetate-accumulating terminal process has been observed in connection to low CH$_4$ production levels (Hines et al. 2001, Duddleston et al. 2002). Although acetogenesis from H$_2$ and CO$_2$ occurs in cold soils and sediments (Schulz and Conrad 1996, Kotsyurbenko 2005), no direct evidence exists of its occurrence in peat. Acetogenesis has even been calculated to be thermodynamically unfavourable in peat (Metje and Frenzel 2007).
1.4 Detection of methanogen diversity

Biogeochemical processes, including carbon cycling, are vital for sustaining life on Earth. The ecology, physiology, and taxonomy of the microbes carrying out the processes remain, however, largely uncharacterized. As microbial activity forms the basis of these processes, unravelling how microbial diversity affects them and how biotic and abiotic factors in the ecosystem influence the microbes is essential.

Microbial communities in the environment are exceedingly complex (Torsvik et al. 2002, Gans et al. 2005). Traditional culture-dependent methods have proved inadequate to describe the vast microbial diversity; they may miss >99% of the organisms and enrich those thriving in cultures but not numerically or functionally important in the environment (Torsvik et al. 1990, Amann et al. 1995). Introduction of culture-independent, molecular methods has vastly improved the potential to describe microbial diversity (DeLong and Pace 2001). Since their introduction over 20 years ago, the methods have increased the number of recognized bacterial phyla from 12 to over 50 (Hugenholtz et al. 1998, Rappe and Giovannoni 2003), and recovered, for instance, a wide diversity of mesophilic Archaea with unknown function (Schleper et al. 2005).

A standard approach in molecular analysis of microbial communities starts with extraction of DNA from environmental samples, followed by PCR amplification of marker genes, differentiation of amplicons by molecular fingerprinting or cloning, and identification of the populations by DNA sequencing and phylogenetic analysis (Head et al. 1998). A challenge in microbial ecology has been to relate the molecular fingerprints and sequence data to ecosystem functions (Gray and Head 2001, Torsvik and Øvreås 2002). Another challenge is accurate and comprehensive description of the vast microbial diversity and its components. The high diversity and high numbers of prokaryotes pose challenges for detection of species richness, and only the most abundant species may be retrieved. Detection of community composition by PCR-based methods is highly dependent on the coverage of the primers (Baker et al. 2003, Forney et al. 2004). The major weakness of PCR-based methods is the recovery of relative abundances of taxa, because amplicon ratios may become biased during amplification (Suzuki and Giovannoni 1996, von Wintzingerode et al. 1997, Ishii and Fukui 2001, Lueders and Friedrich 2003).

Methanogen communities have been characterized by employing the 16S ribosomal RNA (rRNA) gene or the methyl-coenzyme M reductase gene mcrA as molecular markers in a wide variety of environments. These include rice field soil, wetlands, freshwater and marine sediments, hydrothermal environments, deep subsurface habitats, rumen and other digestive tracts, termites, anaerobic digesters, and landfills (reviewed by Chaban et al. 2006). The studies have differentiated communities by analysis of clone libraries or by community fingerprinting by denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single-strand conformation polymorphism (SSCP), which separate DNA fragments according to sequence-based melting behaviour, or by terminal restriction fragment length polymorphism (T-RFLP), which relies on differences in restriction fragment lengths between taxa (Moyer et al. 1994, Liu et al. 1997, Muyzer and Smalla 1998, Schwieger and Tebbe 1998). Additionally, fluorescence in situ hybridization (FISH) and membrane hybridization have been used (Raskin et al. 1994, Purdy et al. 2003).
Active methanogens have been targeted by analysis of environmental RNA (Lueders and Friedrich 2002, Koizumi et al. 2004, Shigematsu et al. 2004).

Application of molecular methods has revealed novel methanogenic or putatively methanogenic lineages. A notable example is Rice cluster I (RCI), which was first detected in rice field soil as 16S rRNA gene sequences only distantly related to Methanomicrobiales and Methanosarcinales (Grosskopf et al. 1998a). Subsequently, the group has been shown to be an important CH4 producer in rice fields (Lu and Conrad 2005, Conrad et al. 2008), and the complete genome of a RCI methanogen has been sequenced (Erkel et al. 2006). The first RCI strain was recently isolated from rice field soil (Sakai et al. 2007).

1.4.1 Ribosomal 16S RNA gene as a molecular marker

The 16S rRNA gene encodes the small subunit of prokaryotic ribosomal RNA. As a part of the protein synthesis machinery, it has an essential function conserved across all prokaryotes, ubiquitous distribution, and lack of extensive horizontal gene transfer. It was integral for defining the three domains of life, and it has become a major tool in identification of prokaryotes. The widespread application of 16S rDNA as a molecular marker in microbial ecology has been central to discovery of numerous novel prokaryotic lineages (Hugenholtz et al. 1998, Rappe and Giovannoni 2003). Conserved sequence regions allow design of primers for different taxonomic levels, and interspersed variable regions and the length of the gene (~1500 bp) provide phylogenetic resolution for distinguishing taxa. Sequence similarity of <97% has been adopted to indicate that the 16S rDNA sequences represent members of different species (Stackebrandt and Goebel 1994). This threshold was later raised to 98.7-99% (Stackebrandt and Ebers 2006). The presence of multiple copies of the rRNA operon with slightly differing sequence in one organism may lead to overestimation of diversity, although mostly the sequences differ only by <1% (Acinas et al. 2004).

The 16S rRNA gene sequence provides the phylogenetic affiliation of the organism but tells nothing explicit of its function. Many functional microbial groups, including methanogens, are not monophyletic in 16S rRNA gene phylogeny, which hampers their detection and identification. Methanogen-specific 16S rRNA gene primers have been designed (Marchesi et al. 2001, Wright and Pimm 2003), but in silico analysis by Banning et al. (2005) indicated that these primer pairs amplify also non-methanogenic Euryarchaeota and Crenarchaeota. As a solution the authors developed three primer pairs, which together cover most known methanogen 16S rDNA sequence diversity. A primer set covering at least Methanobacteriales, Methanosarcinales and Methanomicrobiales has been developed for DGGE analysis (Watanabe et al. 2004). Group-specific probes and primers have also been developed for hybridization studies (Raskin et al. 1994) and quantitative PCR (qPCR) (Hori et al. 2006). A more straightforward approach for detection of methanogen 16S rDNA is application of general archaeal primers (e.g. DeLong 1992, Embley et al. 1992, Øvreås et al. 1997, Grosskopf et al. 1998b) and identification of methanogens by phylogenetic analysis.
1.4.2 mcrA as a specific marker gene for methanogens

Marker genes encoding functions specific to a functional microbial guild overcome the problem of phylogenetic dispersal. Methyl-coenzyme M reductase (MCR, EC 2.8.4.1) is an essential enzyme in CH$_4$ production. It catalyzes the final step of methanogenesis in which the methyl group linked to coenzyme M is reduced with formation of CH$_4$ (Ellermann et al. 1988, Deppenmeier 2002). This enzyme is present in all known methanogens, and unlike many other enzymes in the methanogenic pathway, it is absent from non-methanogenic Archaea and Bacteria (Chistoserdova et al. 1998, Thauer 1998, Baptiste et al. 2005). MCR is composed of three subunits, α, β, and γ, encoded by the operon mcrBDCGA (Reeve et al. 1997). The gene encoding the α-subunit, mcrA, contains conserved sequence regions, which have been related to catalytic sites of MCR (Weil et al. 1988, Hallam et al. 2003). The phylogeny of mcrA follows the 16S rRNA phylogeny (Springer et al. 1995, Lueders et al. 2001, Luton et al. 2002), allowing identification of methanogens based on mcrA sequences. Most methanogens possess only one copy of mcrA, except members of the orders Methanobacteriales and Methanococcales, which additionally have an isoenzyme MCR-II and the corresponding mrtA gene (Thauer 1998). Anaerobic CH$_4$-oxidizing Archaea (ANME-1 and ANME-2) harbour phylogenetically distinct mcrA genes (Hallam et al. 2003). In ANME, MCR is hypothesized to catalyze the reverse reaction of methanogenesis (Kruger et al. 2003, Hallam et al. 2004).

Several degenerate primer pairs have been designed for detection of the mcrA gene (Ohkuma et al. 1995, Springer et al. 1995, Hales et al. 1996, Lueders et al. 2002). The primers differ in amplicon length, target site, and the level of degeneracy (Fig. 1 and Table 1 in II). Studies using two primer pairs have reported differences or limitations in their coverage of methanogen taxa (Lueders et al. 2001, Banning et al. 2005, Nercessian et al. 2005). There are also group-specific primers for quantitative PCR (Denman et al. 2007) or for detection of the ANME mcrA genes (Hallam et al. 2003, Nunoura et al. 2006). In addition to cloning and sequencing, the ME primer pair of Hales et al. (1996) has been applied in DGGE (Galand et al. 2002), the ML primers of Luton et al. (2002) in T-RFLP, DGGE and TGGE (Castro et al. 2005, Sheppard et al. 2005, Wilms et al. 2007), and the MCR pair (Springer et al. 1995) in T-RFLP (Lueders et al. 2001). The ML pair or its modification has also been used in qPCR (Radl et al. 2007, Goffredi et al. 2008).

The MCR primer pair has mainly been employed in studies of rice field soil or rice root methanogens (Chin et al. 1999, Ramakrishnan et al. 2001, Conrad et al. 2008), but also in floodplain wetland (Kemmitch et al. 2004) and hydrothermal sediment (Dhillon et al. 2005). The ME primers and the most recent ML primer pair have been used in a wider range of environments, ranging from wetlands and freshwater sediments (Earl et al. 2003, Castro et al. 2004, Banning et al. 2005, Smith et al. 2007) to hydrothermal, hypersaline, deep subseafloor, and CH$_4$ hydrate habitats (Inagaki et al. 2004, Newberry et al. 2004, Nercessian et al. 2005, Parkes et al. 2005, Smith et al. 2008) and rumen and animal fecal material (Tatsuoka et al. 2004, Ufnar et al. 2007). The ME pair has been used to detect mcrA of anaerobic methane oxidizers (Hallam et al. 2003, Lloyd et al. 2006, Lösekann et al. 2007).
1.5 Methanogen communities in mires

Methanogens in peat have been investigated with the 16S rRNA and mcrA genes as molecular markers, mainly by RFLP or sequence analysis of clone libraries or community fingerprinting, but also by probe hybridization. The studies are summarized in Table 1, together with studies of methanogenic enrichments. Members of orders Methanosarcinales (families Methanosarcinaceae and Methanosaetaceae), Methanomicrobiales, Methanobacterales, and of Rice cluster I have frequently been detected in peat (Table 1). The first molecular study assessing mire methanogens detected two novel groups, R10 associated with Methanomicrobiales and R17 distantly related to Methanosarcinales (Hales et al. 1996). These groups have occurred in other mires as well. The R10 group has also become known as Fen cluster (FC), named based on the type of mire where the mcrA genes of the group were first detected as a novel lineage (Galand et al. 2002), or as the E2 group (Cadillo-Quiroz et al. 2006). The R17 group is commonly referred to as Rice cluster II (RCII) after its discovery in rice field soil (Grosskopf et al. 1998a).

Methanogen communities generally change with peat depth (Galand et al. 2002, Galand et al. 2003, Høj et al. 2005, Cadillo-Quiroz et al. 2006, Ganzert et al. 2007). Shifts related to vegetation have also been reported: communities differed between Sphagnum-dominated hummocks and Eriophorum lawns (Galand et al. 2003), along a successional gradient on land-uplift coast (Merilä et al. 2006), and between Sphagnum- and Carex-dominated Alaskan mires (Rooney-Varga et al. 2007). The study of Alaskan mires also suggested correlation of communities with pH and temperature. Temporal patterns during the growing season have been addressed in arctic peat (Høj et al. 2005, 2006). In addition to peat samples, methanogen communities have been characterized in enrichment cultures (Horn et al. 2003, Sizova et al. 2003), and in incubations where the effect of temperature (Metje and Frenzel 2005, 2007, Høj et al. 2008) or pH (Kotsyurbenko et al. 2007) on community composition has been assessed.

Several isolates affiliated with Methanobacterales have been obtained from peat, many of these active at low pH (Williams and Crawford 1985, Zellner et al. 1988, Kotsyurbenko et al. 2007). Recently, novel strains affiliated with Methanomicrobiales have been isolated from North American mires (Bräuer et al. 2006a, Cadillo-Quiroz et al. 2008). In these studies, the detection of uncultured methanogen lineages in peat has been followed by their successful isolation. “Candidatus Methanoregula boonei” is the first cultured member of the Fen cluster/R10/E2 group, and “Candidatus Methanosphaerula palustris” is the first isolate of an E1 group within Methanomicrobiales.
Table 1. Summary of molecular studies of methanogen communities in northern peatlands.

<table>
<thead>
<tr>
<th>Marker gene (primers)</th>
<th>Method</th>
<th>Site and latitude</th>
<th>Detected methanogens</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcrA(^a) archaeal 16S</td>
<td>sequencing (16S), PCR (mcrA)</td>
<td>Moorhouse blanket bog, England, 54(^\circ)N</td>
<td>Methanomicrobiales (R10 group/FC), R17 group/RCII</td>
<td>two novel methanogen lineages detected in peat</td>
<td>Hales et al. 1996</td>
</tr>
<tr>
<td>mcrA(^a) archaeal 16S</td>
<td>PCR</td>
<td>Ellergower Moss, Scotland, 55(^\circ)N</td>
<td>no identification</td>
<td>methanogens detected 9 cm below water level in a peat profile</td>
<td>Lloyd et al. 1998</td>
</tr>
<tr>
<td>archaeal 16S mcrA(^a)</td>
<td>PCR, membrane hybridization</td>
<td>Moorhouse blanket bog, England, 54(^\circ)N</td>
<td>Methanosarcinaceae, Methanococcaceae, Methanobacteriaceae</td>
<td>minor differences in depth distribution of groups</td>
<td>McDonald et al. 1999</td>
</tr>
<tr>
<td>mcrA(^a)</td>
<td>sequencing</td>
<td>Moorhouse, Ellergower Moss, UK, 54-55(^\circ)N</td>
<td>Methanosarcinales(^d)</td>
<td>sequences differed between sites but not with depth</td>
<td>Nercessian et al. 1999</td>
</tr>
<tr>
<td>archaeal 16S mcrA(^a)</td>
<td>FISH, PCR, membrane hybridization</td>
<td>Ellergower Moss, Scotland, 55(^\circ)N</td>
<td>Methanomicrobiales, Methanosarcinaceae, Methanococcaceae, Methanobacteriaceae</td>
<td>methanogens detected below 14 cm in a 25-cm peat profile</td>
<td>Upton et al. 2000</td>
</tr>
<tr>
<td>mcrA(^a)</td>
<td>clone RFLP, sequencing, DGGE</td>
<td>oligotrophic Salmisuo fen, Finland, 62(^\circ)N</td>
<td>Methanomicrobiales (FC), RCI</td>
<td>communities changed with depth</td>
<td>Galand et al. 2002</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>clone RFLP, sequencing</td>
<td>Labrador Hollow conifer swamp, ombrotrophic McLean bog, NY, USA, 42(^\circ)N</td>
<td>Methanosarcinaceae, Methanosaetaceae, RCII, Methanomicrobiales (FC), Methanobacteriaceae</td>
<td>same groups but different RFLP patterns in swamp and bog</td>
<td>Basiliko et al. 2003</td>
</tr>
<tr>
<td>methanogen 16S</td>
<td>clone RFLP, sequencing, DGGE</td>
<td>oligotrophic Salmisuo fen, Finland, 62(^\circ)N</td>
<td>Methanomicrobiales (FC), Methanosarcinaceae</td>
<td>hummock and lawn communities differed at surface but not in deeper peat</td>
<td>Galand et al. 2003</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>enrichment, sequencing</td>
<td>Sphagnum-Picea bog, Germany, 50(^\circ)N</td>
<td>Methanomicrobiales (FC), Methanobacteriaceae, Methanosarcinaceae</td>
<td>methanogens detected from serial peat dilutions and enrichments</td>
<td>Horn et al. 2003</td>
</tr>
<tr>
<td>Method</td>
<td>Location</td>
<td>Community Structure</td>
<td>Findings</td>
<td>Reference</td>
<td></td>
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<tr>
<td>archaeal 16S enrichment, sequencing</td>
<td>ombrotrophic Bakchar Bog, Siberia, 57°N</td>
<td><em>Methanomicrobiales</em> (FC), <em>RCI, Methanobacteriaeae</em></td>
<td>methanogens detected in peat enrichments</td>
<td>Sizova et al. 2003</td>
<td></td>
</tr>
<tr>
<td>archaeal 16S sequencing</td>
<td>Bakchar Bog, Siberia, 56°N, Akaiyachi Mire, Japan, 37°N, Okefenokee Swamp, USA, 30°N</td>
<td><em>Methanosarcinaceae</em>, <em>Methanomicrobiales</em> (including FC), <em>Methanosaetaeae</em></td>
<td>communities of subarctic, temperate, and subtropical sites differed</td>
<td>Utsumi et al. 2003</td>
<td></td>
</tr>
<tr>
<td>archaeal 16S T-RFLP, sequencing</td>
<td>ombrotrophic Bakchar Bog, Siberia, 56°N</td>
<td><em>RCI, Methanobacteriaeae</em>, <em>Methanosarcinaceae</em>, <em>Methanomicrobiales</em></td>
<td>predominantly acetoclastic methanogenesis in an acidic bog (pH 4.2-4.8)</td>
<td>Kotsyurbenko et al. 2004</td>
<td></td>
</tr>
<tr>
<td>mcrA&lt;sup&gt;b&lt;/sup&gt; clone RFLP, sequencing</td>
<td>Lakkasuo mire complex, Finland, 61°N</td>
<td>FC, <em>Methanosaetaeae</em>, <em>RCI</em></td>
<td>pathway and community differences between bog and two fens</td>
<td>Galand et al. 2005</td>
<td></td>
</tr>
<tr>
<td>archaeal 16S DGGE, sequencing of bands</td>
<td>Solvatnet and Stuphallet, Spitsbergen, 78°N</td>
<td><em>Methanomicrobiales</em>, <em>Methanosaetaeae</em>, <em>Methanobacteriaeae</em>, <em>Methanosarcinaceae</em>, <em>RCI</em></td>
<td>differences with depth, site, and sampling time; community variation related to CO&lt;sub&gt;2&lt;/sub&gt; emission</td>
<td>Høj et al. 2005</td>
<td></td>
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<tr>
<td>mcrA&lt;sup&gt;b&lt;/sup&gt; T-RFLP</td>
<td>a chronosequence of five peatlands, Siikajoki, Finland, 64°N</td>
<td>FC, <em>RCI, Methanosarcinaceae</em></td>
<td>community change in succession gradient</td>
<td>Merilä et al. 2006</td>
<td></td>
</tr>
<tr>
<td>archaeal 16S mcrA&lt;sup&gt;a&lt;/sup&gt; sequencing, incubations, T-RFLP</td>
<td>mire in eastern Finnish Lapland, 68°N</td>
<td><em>Methanobacteriaeae</em></td>
<td>low diversity, no substantial change in incubations at 4-45 °C, hydrogenotrophic methanogenesis</td>
<td>Metje and Frenzel 2005</td>
<td></td>
</tr>
<tr>
<td>archaeal 16S mcrA&lt;sup&gt;a&lt;/sup&gt; enrichment, sequencing</td>
<td>ombrotrophic McLean Bog, NY, USA, 42°N</td>
<td><em>Methanomicrobiales</em> (FC)</td>
<td>enrichment of two similar strains, isolation of <em>Methanoregula boonei</em> (FC)</td>
<td>Bräuer et al. 2006a, 2006b</td>
<td></td>
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<tr>
<td>archaeal 16S T-RFLP, sequencing</td>
<td>oligotrophic Chicago Bog, ombrotrophic McLean Bog, NY, USA, 42°N</td>
<td><em>Methanomicrobiales</em> (FC, E1), <em>RCI, RCII, RCI, Methanosarcinaceae</em>, <em>Methanosaetaeae</em></td>
<td>communities of bogs similar at surface but different in deeper peat</td>
<td>Cadillo-Quiroz et al. 2006</td>
<td></td>
</tr>
<tr>
<td>Marker gene (primers)</td>
<td>Method</td>
<td>Site and latitude</td>
<td>Detected methanogens</td>
<td>Main findings</td>
<td>Reference</td>
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<tr>
<td>archaeal 16S</td>
<td>DGGE, sequencing of bands</td>
<td>Solvatnet, Stuphallet, and Sassen Valley, Spitsbergen, 78°N</td>
<td>Methanosetaeaceae, Methanobacteriaceae</td>
<td>shifts in methanogen communities along a moisture gradient in upper peat layer but not deeper</td>
<td>Høj et al. 2006</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>clone RFLP, sequencing</td>
<td>continental bog, permafrost mound, and internal lawn, western Canada, 55°N</td>
<td>RCII, Methanomicrobiales (FC), Methanosetaeaceae, Methanosarcinaceae, Methanobacteriaceae</td>
<td>differences between continental bog and internal lawn</td>
<td>Yavitt et al. 2006</td>
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<tr>
<td>archaeal 16S</td>
<td>T-RFLP, sequencing</td>
<td>Chicago Bog and Michigan Hollow, NY, USA, 42°N</td>
<td>Methanomicrobiales (FC, E1), RCI, RCI, Methanosetaeaceae</td>
<td>different communities in acidic Sphagnun bog and neutral Carex fen</td>
<td>Dettling et al. 2007</td>
</tr>
<tr>
<td>methanogen 16S</td>
<td>DGGE, sequencing of bands</td>
<td>Laptev Sea coast, Siberia, 72-73°N</td>
<td>Methanosarcinaceae, Methanomicrobiales, RCI</td>
<td>vertical shift of communities, differences between permafrost formations</td>
<td>Ganzert et al. 2007</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>incubations, T-RFLP, sequencing</td>
<td>ombrotrophic Bakchar Bog, Siberia, 57°N</td>
<td>Methanobacteriaceae, Methanomicrobiales, Methanosarcinaceae</td>
<td>Methanobacteriaceae at low pH, three strains isolated, similar community at 15 and 25 °C</td>
<td>Kotsyurbenko et al. 2007</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>sequencing, incubations, T-RFLP</td>
<td>mire in north-western Siberia, 67°N</td>
<td>Methanobacteriaceae, Methanosarcinaceae</td>
<td>acetoclastic methanogenesis, Methanobacteriaceae more important in incubations with higher temperature</td>
<td>Metje and Frenzel 2007</td>
</tr>
<tr>
<td>archaeal 16S</td>
<td>DGGE, sequencing of bands and clones</td>
<td>12 Alaskan and 2 midlatitudes peatlands, USA, 42-69°N</td>
<td>Methanobacteriaceae, Methanomicrobiales (FC), Methanosetaeaceae</td>
<td>archaeal community variation related to vegetation, also to pH and temperature</td>
<td>Rooney-Varga et al. 2007</td>
</tr>
<tr>
<td>Archaeal 16S</td>
<td>Method</td>
<td>Location</td>
<td>Discussion</td>
<td>Reference</td>
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<tr>
<td>T-RFLP, sequencing, enrichment and isolation</td>
<td>Michigan Hollow minerotrophic fen, NY, USA, 42°N</td>
<td>Methanomicrobiales (E1, FC, Methanospirillaceae), Methanosphaeraceae, Methanosarcinaceae, Methanobacteriaceae, RCI, RCII</td>
<td>Cadillo-Quiroz <em>et al.</em> 2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>archaeal 16S incubation, DGGE, sequencing</td>
<td>Solvatnet, Spitsbergen, 78°N</td>
<td>Methanomicrobiales, Methanosarcinaceae, Methanosaetaceae, Methanobacteriaceae</td>
<td>greater contribution of methanogens with increasing incubation temperature</td>
<td>Høj <em>et al.</em> 2008</td>
<td></td>
</tr>
</tbody>
</table>

* ME primers (Hales *et al.* 1996)
* ML primers (Luton *et al.* 2002)
* FC, Fen cluster; RCI, Rice cluster I; RCII, Rice cluster II
* Phylogenetic affiliation uncertain because sequences are not available for analysis
* Identification based on sequences from Lakkasuo mire complex
1.6 Non-methanogenic Archaea in peat

Non-methanogenic archaea in peat have been reported in methanogen studies with general archaeal 16S rRNA gene primers. Group 1.3 crenarchaea (or Rice cluster IV) have been found in several peat ecosystems (Galand et al. 2003, Kotsyurbenko et al. 2004, Høj et al. 2005, 2008). In a recent study, group 1.3 was the most common archaeal group in Alaskan mires (Rooney-Varga et al. 2007). It also occurred in arctic wet soils and peat (Høj et al. 2006). Other archaeal groups detected in mires include crenarchaeal Rice cluster VI and euryarchaeal groups Rice cluster V, Lake Dagow Sediment group, Marine Benthic group D, and a subaqueous cluster (Kotsyurbenko et al. 2004, Cadillo-Quiroz et al. 2008, Høj et al. 2008).

1.7 Bacterial communities in anoxic peat

The most studied bacteria in mires are methanotrophs (e.g. Dedysh et al. 1998, Jaatinen et al. 2005, Raghoebarsing et al. 2005, Chen et al. 2008). Factors controlling aerobic bacterial activity (Fisher et al. 1998, Fisk et al. 2003, Jaatinen et al. 2007) and bacteria associated with Sphagnum mosses have also been characterized (Opelt et al. 2007a, Opelt et al. 2007b). The bacterial communities in anoxic peat have received much less attention, despite their role in carbon cycling as substrate producers and competitors to methanogens. The few molecular studies that have characterized bacterial communities in anoxic or undefined but most likely anoxic peat have recovered mainly members of Alphaproteobacteria, Acidobacteria, Planctomycetes, Verrucomicrobia, Deltaproteobacteria, Actinobacteria, Firmicutes, and Chloroflexi (Rheims et al. 1996, Dedysh et al. 2006, Morales et al. 2006). Several of these groups have also been recovered in methanogenic peat enrichments (Horn et al. 2003, Sizova et al. 2003, Bräuer et al. 2006b), and strains of Acidobacteria and Planctomycetes have been isolated (Dedysh et al. 2006, Kulichevskaya et al. 2006). A study of 24 Sphagnum bogs in New England attempted to relate bacterial 16S rDNA T-RFLP fingerprints from oxic or deep anoxic (1 m) peat to a wide range of environmental variables, finding highly similar communities and a weak connection to Ca$^{2+}$ level (Morales et al. 2006).
2 Aims of the study

Mires exhibit horizontal and vertical patterns of peat chemistry, vegetation, surface topography, and water level, reflected in microbial activities. Spatial and temporal variability of methane emissions has partially been related to environmental factors (Blodau 2002), but understanding of the underlying microbiology is more limited. Characterization of methanogen communities in a range of mires has revealed varied community compositions (Table 1), but when this work was initiated, studies attempting to link methanogen community composition to environmental variables were few.

The general aim of this work was to investigate methanogen communities and their activity in northern mires in relation to specific environmental gradients, and concomitantly compare methods for detecting community dynamics. Bacteria and non-methanogenic Archaea, which have received even less attention in mires, were assessed because they are potential substrate producers and competitors to methanogens. The specific objectives were to address:

- variation of methanogen communities and CH$_4$ production in respect to
  - ecohydrological gradient from fen to bog (III)
  - season in a fen where CH$_4$ emissions are closely monitored (IV)
  - ash fertilization in a drained bog (I)
- Bacteria and non-methanogenic Archaea in mires (III, IV)
- performance of PCR primers for the mcrA gene in analysis of mire methanogens (II)
3 Materials and methods

3.1 Sample collection

Study sites comprised three Finnish boreal mires: Lakkasuo mire complex in Orivesi and Siikaneva in Ruovesi, which are two closely situated pristine mires in southern Finland, and a drained site Pelso-Resula in Muhos, northern Finland (Table 2).

<table>
<thead>
<tr>
<th>Mire</th>
<th>Location</th>
<th>Type</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakkasuo mire complex</td>
<td>61°48'N, 24°19'E</td>
<td>ombrotrophic bog</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>oligotrophic fen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mesotrophic fen</td>
<td></td>
</tr>
<tr>
<td>Siikaneva</td>
<td>61°50'N, 24°12'E</td>
<td>oligotrophic fen</td>
<td>IV</td>
</tr>
<tr>
<td>Pelso-Resula</td>
<td>64°30'N, 26°18'E</td>
<td>drained bog</td>
<td>I, II</td>
</tr>
</tbody>
</table>

Table 2. Sampled mires.

The sampled areas of the Lakkasuo mire complex form an ecohydrological gradient from minerotrophic fens to ombrotrophic bog. The *Sphagnum*-shrub bog has lower pH and lower levels of N, P, Ca, and Fe than the *Carex-Sphagnum* fens (Laine et al. 2002). Three replicate peat cores from lawn microsites at each site were collected in October 2002.

Siikaneva is an open fen, where seasonal fluctuations of CH$_4$ and CO$_2$ emissions, including winter fluxes, have been investigated (Rinne et al. 2007, Riutta et al. 2007). The samples were collected in 2005-2006 in October (end of growing season before snowfall), February (midwinter with snow cover of 35 cm), May (spring after snowmelt and temperature rise), and August (late summer after a warm, dry period). On each occasion, a peat profile was collected from three marked lawn or hollow locations.

The Pelso-Resula bog is a drained cottongrass pine bog with small Scots pines (*Pinus sylvestris*) and birches (*Betula pendula*). Ash fertilization was conducted in 1997 with 15 000 kg ha$^{-1}$ of wood ash applied on 30×30-m plots. After five years, fertilized plots had higher pH and levels of B, Ca, and K in surface peat, enhanced tree growth, and higher abundance of *Eriophorum vaginatum* and *Rubus chamaemorus* but reduced abundance of *Sphagnum* mosses (Moilanen and Silfverberg 2004). Three replicate peat profiles were collected from fertilized plots and unfertilized control plots in May 2002.

Peat profiles were collected with a box corer (8×8×90 cm). Samples were taken as 4-cm peat slices from selected depths. The depths were measured from water table level (I, II, III) or peat surface (IV). Vegetation of the study sites is described in more detail in the articles.
3.2 Methods

The methods used in characterization of peat samples, methanogenic potential and microbial communities are described in detail in the original articles and listed in Table 3.

Table 3. Overview of chemical, molecular, and data analysis methods. Roman numerals refer to the articles I-IV.

<table>
<thead>
<tr>
<th>Method</th>
<th>Described and used in:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical analyses</strong></td>
<td></td>
</tr>
<tr>
<td>Potential CH₄ production</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Temperature response of CH₄ production</td>
<td>IV</td>
</tr>
<tr>
<td>Peat pH</td>
<td>I, III, IV</td>
</tr>
<tr>
<td><strong>Nucleic acid methods</strong></td>
<td></td>
</tr>
<tr>
<td>DNA extraction</td>
<td>I-IV</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>IV</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>IV</td>
</tr>
<tr>
<td>mcrA PCR</td>
<td></td>
</tr>
<tr>
<td>ME primers (Hales et al. 1996)</td>
<td>I, II</td>
</tr>
<tr>
<td>MCR primers (Springer et al. 1995)</td>
<td>II</td>
</tr>
<tr>
<td>ML primers (Luton et al. 2002)</td>
<td>II-IV</td>
</tr>
<tr>
<td>Archaeal 16S rRNA gene PCR</td>
<td>IV</td>
</tr>
<tr>
<td>Bacterial 16S rRNA gene PCR</td>
<td>III</td>
</tr>
<tr>
<td>DGGE</td>
<td>I</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>II, IV</td>
</tr>
<tr>
<td>Cloning</td>
<td>I-IV</td>
</tr>
<tr>
<td>RFLP screening of clone libraries</td>
<td>I-IV</td>
</tr>
<tr>
<td>Plasmid extraction</td>
<td>I, II, III</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>I-IV</td>
</tr>
<tr>
<td><strong>Data analysis and statistics</strong></td>
<td></td>
</tr>
<tr>
<td>Phylogenetic analysis</td>
<td>I-IV</td>
</tr>
<tr>
<td>Bootstrapping</td>
<td>I-IV</td>
</tr>
<tr>
<td>Rarefaction analysis</td>
<td>I, II</td>
</tr>
<tr>
<td>Coverage values</td>
<td>I, III</td>
</tr>
<tr>
<td>Cluster analysis</td>
<td>I, III</td>
</tr>
<tr>
<td>Diversity indices</td>
<td>I-III</td>
</tr>
<tr>
<td>Multivariate analysis (PCA, RDA) and ANOSIM</td>
<td>IV</td>
</tr>
<tr>
<td>Two-way ANOVA</td>
<td>I, III</td>
</tr>
</tbody>
</table>
4 Results

4.1 Potential CH$_4$ production in relation to environmental gradients (I-IV)

Methane production rates from endogenous substrates at temperatures close to *in situ* conditions (10 or 14 °C) varied from 0 to ~30 nmol g$^{-1}$ h$^{-1}$ (Table 4). The lowest rates were measured in the drained and pristine bogs and in autumn and summer samples from Siikaneva fen. The rates were highest in the minerotrophic fens of the Lakkasuo mire complex, both less acidic than Siikaneva fen (Table 4).

<table>
<thead>
<tr>
<th>Mire</th>
<th>Gradient</th>
<th>pH</th>
<th>Depth from (cm)</th>
<th>CH$_4$ production (nmol gdw$^{-1}$ h$^{-1}$)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>water table</td>
<td>peat surface</td>
<td></td>
</tr>
<tr>
<td>Lakkasuo mire complex</td>
<td>ombrotr. bog</td>
<td>4.0-4.3</td>
<td>-20</td>
<td>-45</td>
<td>4.8 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>oligotrophic fen</td>
<td>4.9-5.0</td>
<td>0</td>
<td>-16</td>
<td>19.3 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>mesotrophic fen</td>
<td>5.0-5.5</td>
<td>-10</td>
<td>-10</td>
<td>16.2 ± 14.9</td>
</tr>
<tr>
<td>Siikaneva</td>
<td>autumn</td>
<td>3.9-4.3</td>
<td>-24</td>
<td>-20</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>winter</td>
<td></td>
<td>-b</td>
<td>-20</td>
<td>11.9 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>spring</td>
<td></td>
<td>-16</td>
<td>-20</td>
<td>7.4 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td></td>
<td>3</td>
<td>-20</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>Pelso-Resula</td>
<td>control (no ash)</td>
<td>3.7-4.2</td>
<td>-20</td>
<td>-46</td>
<td>2.4 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>ash-fertilized</td>
<td>3.7-4.7</td>
<td>-10</td>
<td>-45</td>
<td>5.8 ± 7.4</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation, n=3. Incubation temperature for Pelso-Resula and Lakkasuo 10 °C and for Siikaneva 14 °C. Gdw, grams dry weight.

The production potential of Lakkasuo fens was approximately four times that of the bog (Table 4, Table 1 in III). In Siikaneva fen, there were seasonal differences in the production at the depths of 10 and 20 cm, with unexpectedly large potential in winter (Table 4, Fig. 3). There was no difference in the CH$_4$ production potential between the control and ash-fertilized sites of the drained Pelso-Resula bog (Table 4, Table 1 in I).

In the seasonal study on Siikaneva, the sampling depths of 10, 20, and 50 cm were kept constant from peat surface to allow sampling the same layer despite water level fluctuations. Production was generally largest at 20 cm (Fig. 3). This depth was above water table in August sampling, but even samples from the depth of 10 cm produced substantial amounts of CH$_4$ (Fig. 3). In Pelso-Resula and Lakkasuo, depth distribution of CH$_4$ production potential was examined from the water table to 40 cm below it. Production was greater 0-20 cm below the water table than in deeper peat, 30 and 40 cm below the water table (Table 1 in I; Table 1 in III).
Temperature response of CH₄ production potential in Siikaneva was determined at temperatures from 5 to 43 °C. No obvious seasonal shift in the temperature of maximal production was observed for peat from the depth of 20 cm (Fig. 4 in IV), and the depth distribution of CH₄ production remained similar with temperature (Fig. 3). Production was low at 5 and 14 °C, resembling the field temperature range, and substantially higher from 25 to ~35 °C with apparent optimum at ~30 °C and clear reduction above 35 °C (Fig. 4 in IV).

4.2 Methanogen groups (I-IV)

The methanogen groups detected as clones or T-RFLP peaks and identified by sequencing and phylogenetic analysis are summarized in Table 5, Fig. 4, and Fig. 5. Methanomicrobiales-associated Fen cluster (FC), Rice cluster I (RCI) or Rice cluster II (RCII), and Methanosarcinaceae occurred at all three mires. Methanosaetaceae and Methanobacteriaceae were only detected in the fens. Methanosaetaceae in Siikaneva fen were detected from RNA but not from DNA (Fig. 1 in IV). The year-round occurrence of FC in Siikaneva was additionally verified by PCR with specific 16S rRNA gene primers (IV). Lakkasuo bog and Siikaneva fen revealed similar FC and RCII 16S rRNA gene sequences with identities of 98-99%, but other groups detected in Siikaneva were absent from the bog (Table 5, Fig. 5). In Lakkasuo fens, one mcrA sequence type could not be assigned to any known group of methanogens (Ug in Table 5, sequence Lak19 in Fig. 4). This unidentified sequence cluster showed a very distant affiliation with mcrA sequences of anaerobic methane-oxidizing archaea (ANME-1) (Fig. 4).
Table 5. Methanogen groups detected in three boreal mires.

<table>
<thead>
<tr>
<th>Mire</th>
<th>Marker gene</th>
<th>Gradient</th>
<th>Methanogen group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakkasuo mire complex</td>
<td>mcrA</td>
<td>ombrotrophic bog</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>oligotrophic fen</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mesotrophic fen</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
<tr>
<td>Siikaneva</td>
<td>16S rRNA</td>
<td>autumn</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>winter</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>spring</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>summer</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
<tr>
<td>Pelso-Resula</td>
<td>mcrA</td>
<td>control</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ash-fertilized</td>
<td>FC: +, RCI: +, RCII: +, Ms: +, Mt: +, Mb: +, Ug: +</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> FC Fen cluster, RCI Rice cluster I, RCII Rice cluster II, Ms Methanosarcinaceae, Mt Methanosaetaceae, Mb Methanobacteriaceae, Ug unidentified mcrA group, • prominent group, + minor group based on relative proportion of clones or terminal restriction fragments

<sup>b</sup> Detection in Siikaneva based on a shared terminal restriction fragment and RFLP pattern of Methanobacteriaceae and Lake Dagow Sediment euryarchaea

Figure 4. Maximum likelihood tree of mcrA sequences from the drained Pelso-Resula bog (PR) and Lakkasuo mire complex (LS). The tree was constructed from inferred amino acid sequences (130 aa) as in Study II. Scale indicates 0.1 changes per position. Filled circles mark nodes with bootstrap values >75% from 100 replicates. The sequences were selected from Studies I-III.
Figure 5. Maximum likelihood tree of methanogen 16S rRNA gene sequences from Siikaneva fen (SN) and Lakkasuo bog (LS). The tree was constructed from partial (~776 bp) nucleotide sequences as in Study II. Scale indicates 0.1 changes per position. Filled circles mark nodes with bootstrap values >75% from 100 replicates. The Siikaneva sequences were selected from Study IV. The Lakkasuo bog sequences originate from an unpublished clone library.

4.3 Methanogen communities in relation to environmental gradients

4.3.1 Ecohydrology (III)

Methanogens of sites forming an ecohydrological gradient from ombrotrophic bog to mesotrophic fen in the Lakkasuo mire complex were compared by RFLP and sequence analysis of mcrA clone libraries. The bog showed distinct communities from the fens. In the upper layer of the bog, nearly all clones belonged to Fen cluster (Fig. 6). The FC sequence types characteristic to the bog (Lak15, Lak16 in Fig. 4) were also dominant in the deeper bog layer, but rare in the oligotrophic fen and absent from the mesotrophic fen (Fig. 1 in III). The oligotrophic fen had 22% and the mesotrophic fen 11% of FC clones, but the sequences grouped separately from the bog sequences (Fig. 6; Fig. 1 and 3 in III). An archaeal 16S rDNA library was constructed and analyzed as in Study IV for the bog upper layer (unpublished). In the RFLP analysis, 74% of 39 clones were assigned to FC (Fig. 5), 15% to RCII, and 10% to non-methanogenic Archaea, hence supporting the predominance of Fen cluster in the bog.
The oligotrophic and mesotrophic fens revealed a wider range of methanogen groups, and their communities showed no substantial divergence. The largest group, constituting 38-40% of all fen clones, was *Methanosetaeaceae*. This group was not detected in the upper layer of the bog and it occurred only as a rare group in the deeper bog layer (Fig. 6; Fig. 1 in **III**). RCI was detected in the fens at both depths and in the deeper bog layer, constituting 15-25% of the clones (Fig. 6). Interestingly, the unpublished bog 16S rRNA gene library revealed a small number of sequences affiliated with RCII instead (Fig. 5).

4.3.2 Season (IV)

Seasonal variation of methanogen communities in Siikaneva fen was assessed by archaeal 16S rRNA- and rDNA-based T-RFLP fingerprinting and cloning to determine whether fluctuations of temperature and CH₄ production were reflected in community composition. The analysis focused on the peat depth of 20 cm, which showed the highest methanogenic potential (Fig. 3). The major terminal restriction fragments (T-RFs) represented FC and RCII (T-RF length 393 bp), and *Methanosarcinaceae* and group 1.1c Crenarchaeota (186 bp). These T-RFs were detected around the year, but their relative proportions exhibited moderate temporal variation (Fig. 1 and 2 in **IV**). Redundancy analysis tentatively connected variation of DNA-derived communities to season (P=0.088). RNA-derived communities, which showed higher overall variability (Fig. 1 in **IV**), reflected differences in the CH₄ production potential (P=0.020).
One objective in the seasonal study was to determine whether methanogens are active during winter, when small CH$_4$ emissions have been observed at the site (Rinne et al. 2007, Riutta et al. 2007). Because detection of 16S rRNA may not conclusively indicate active archaea, winter activity of methanogens was addressed by PCR detection of mcrA mRNA. Successful amplification confirmed activity in winter (Fig. 5 in IV).

4.3.3 Peat depth (I-III)

Most of the sites showed a shift in methanogen communities between the layers with the highest CH$_4$ production and the deeper layers having lower capacity to produce CH$_4$. In the drained bog, the ME primers detected different RCI sequence types in the upper and deeper peat layer, and the ML and MCR primers supported the depth distribution (Fig. 2 in I, Fig. 2 in II). In Lakkasuo, the deeper bog layer had higher mcrA diversity than the FC-dominated upper layer. In the oligotrophic fen, the stratification of communities was less pronounced, and the mesotrophic fen showed no apparent stratification (Fig. 1 and 2 in III).

4.3.4 Ash fertilization (I, II)

Methanogen communities in the drained Pelso-Resula bog were studied by DGGE and RFLP and sequence analysis of clone libraries with mcrA as marker gene. Comparison of fertilized and unfertilized peat from two depths in Study I with the ME primers showed no major changes in the communities with ash (Fig. 2 and 3 in I). The most prominent sequence types were the same in unfertilized and fertilized peat, and they were affiliated with RCI (Fig. 4 in I). Less frequent FC sequence types forming a separate phylogenetic cluster were nearly exclusively detected in the fertilized plots (sequences T, II, and III in Fig. 2 in I; Fig. 4). In the four samples selected for Study II, where the focus was on mcrA primer comparison, the ML and MCR primer sets supported the detection of the main sequence types and emphasized the occurrence of the specific FC sequences (E and G in Fig. 3 in II) in fertilized peat.
4.4 Comparison of \textit{mcrA} primers (II)

The ability of three \textit{mcrA} primer sets, MCR (Springer et al. 1995), ME (Hales et al. 1996) and ML (Luton et al. 2002), to differentiate methanogen communities was tested with ash-fertilized and unfertilized peat sampled from two depths of the drained bog. The amplicons were compared by RFLP and sequence analysis of clone libraries. Instead of comparing the RFLP groups of individual primer sets, a sequence similarity cut off was applied to combine groups into operational taxonomic units (OTUs) to enable comparisons between the primer sets. All primer sets detected the same major OTUs affiliated with RCI and FC, but the proportions of the OTUs varied (Fig. 3 in II). The MCR primer set indicated presence of Fen cluster in the upper peat layer, whereas the ME and ML sets detected mainly RCI sequences (depth 1, Fig. 3 in II). A fifth of the sequences the MCR set recovered from upper layer peat turned out not to be \textit{mcrA}. In the deeper layer (depth 2), the community structure depended less on the primer pair, but the ME set emphasized RCI and failed to detect \textit{Methanosarcinaceae} when the other primers recovered the group. Each primer set also failed to detect one or more rare FC OTUs.

Several genomic sequences of \textit{Methanosarcinales} and \textit{Methanomicrobiales} have become available, including Fen cluster ("Candidatus \textit{Methanoregula boonei}") and RCI genomes. The genomic full length \textit{mcrA} sequences, previously unavailable for these groups, allow \textit{in silico} determination of mismatches at primer binding sites. The most degenerate MCR primers showed none or single mismatches (Table 6). Primer ME1 had six mismatches to the only available \textit{mcrA} sequence for the \textit{Methanosarcinales} family \textit{Methanosaetaceae}. The longest primer, MLf, had several mismatches to sequences of Rice cluster I, \textit{Methanomicrobiales}, and \textit{Methanosarcinales}. Hence, even the ML set, which otherwise performed best of the three primer pairs, may have shortcomings of coverage.

Table 6. Comparison of primer sequences to \textit{mcrA} sequences from genomes of Rice cluster I, \textit{Methanomicrobiales} and \textit{Methanosarcinales}, and selected members of \textit{Methanobacteriales} and \textit{Methanococcales}.

<table>
<thead>
<tr>
<th>Methanogen</th>
<th>Accession number</th>
<th>Mismatches against primer sequence$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCRf MCRr ME1 ME2 MLf MLr</td>
<td></td>
</tr>
<tr>
<td>Rice cluster I</td>
<td>AM114193</td>
<td>- - 1 1 4 1</td>
</tr>
<tr>
<td>\textit{Methanocorpusculum labreanum}</td>
<td>CP000559</td>
<td>1 - - 1 4 1</td>
</tr>
<tr>
<td>\textit{Methanolobus marisnigri}</td>
<td>CP000562</td>
<td>1 - 1 2 3 2</td>
</tr>
<tr>
<td>&quot;\textit{Methanoregula boonei}&quot;</td>
<td>CP000780</td>
<td>1 - - 1 3 1</td>
</tr>
<tr>
<td>\textit{Methanospirillum hungatei}</td>
<td>CP000254</td>
<td>1 - - 1 3 1</td>
</tr>
<tr>
<td>\textit{Methanococcoides burtonii}</td>
<td>CP000300</td>
<td>- - 1 - 4 1</td>
</tr>
<tr>
<td>\textit{Methanosaeta thermohalina}</td>
<td>CP000477</td>
<td>- - 6 2 3 2</td>
</tr>
<tr>
<td>\textit{Methanosarcina acetivorans}</td>
<td>AE010299</td>
<td>- - - 1 3 1</td>
</tr>
<tr>
<td>\textit{Methanosarcina barkeri}</td>
<td>CP000099</td>
<td>- - - - - 1</td>
</tr>
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<td>\textit{Methanosarcina mazei}</td>
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<td>- - - - - 1</td>
</tr>
<tr>
<td>\textit{Methanobrevibacter smithii}</td>
<td>CP000678</td>
<td>- - - 2 -</td>
</tr>
<tr>
<td>\textit{Methanobrevibacter smithii}</td>
<td>CP000678</td>
<td>- - - 2 -</td>
</tr>
</tbody>
</table>

$^a$ Full length \textit{mcrA} sequences from the genomes were aligned with ClustalW and inspected against the primer sequences in GeneDoc software.
4.5 Bacteria in Lakkasuo (III)

In sequencing of bacterial 16S rDNA clones from the upper layer of the Lakkasuo bog and both fens, the main groups were *Deltaproteobacteria*, *Acidobacteria*, and *Verrucomicrobia* (Fig. 4 in III). Also sequences affiliated with *Planctomycetes*, other proteobacteria, *Spirochaetes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, and three uncultured candidate divisions were retrieved. The number of bacterial phyla detected in the oligotrophic fen (9 phyla) and mesotrophic fen (10 phyla) exceeded the number recovered from the bog (4 phyla). Only 10 of the clones showed high sequence similarity to cultured species (*Deltaproteobacteria* or *Alphaproteobacteria*), but several resembled environmental sequences from peat or other acidic soils.

4.6 Non-methanogenic Archaea (III, IV)

*Crenarchaeota* of groups 1.3 and 1.1c were detected in Siikaneva at different seasons (Fig. 3 in IV). Non-methanogenic *Euryarchaeota* were less abundant in clone libraries, and they were related to *Thermoplasmatales*, Lake Dagow Sediment cluster (Glissman et al. 2004), or exceptionally small archaea from acid mine drainage (Baker et al. 2006) (Fig. 3 in IV). Among the bacterial sequences from Lakkasuo were also some crenarchaeal sequences, indicating that the applied 16S rRNA gene primers were not strictly specific to *Bacteria*. Nine sequences were recovered which showed 98-99% sequence similarity to group 1.3 crenarchaeal sequences from Siikaneva, a Siberian bog (Kotsyurbenko et al. 2004), and Finnish Salmisuo fen (Galand et al. 2003).
5 Discussion

5.1 Spatial and temporal patterns of methanogen communities and CH$_4$ production

Four aspects in boreal mires were considered in relation to methanogen communities and CH$_4$ production: 1) ecohydrological gradient from ombrotrophic bog to minerotrophic fens, 2) seasonal variation, 3) vertical distribution in peat profiles, and 4) effect of wood ash fertilization on mires drained for forestry.

The strongest variation of CH$_4$ production and methanogen community composition was associated to the shift from fen to bog in the Lakkasuo mire complex (III). The bog showed lower rates of CH$_4$ production than the fens and had distinct, low methanogen diversity dominated by the *Methanomicrobiales*-associated Fen cluster. The same pattern was detected in Lakkasuo in the following year (Galand *et al*., 2005), showing it was not transient. Similar dominance of FC has been observed in North American *Sphagnum* bogs with pH <4.3 (Cadillo-Quiroz *et al*., 2006). Low microbial activity in bogs has been related to low pH, low nutrient levels, and recalcitrant or even inhibitory nature of *Sphagnum* residue, making bog peat poor substrate for microbes (Van Breemen 1995, Verhoeven and Toth 1995, Bergman *et al*. 1999). Yet, comparisons of CH$_4$, CO$_2$, and acetate production rates in *Sphagnum*-dominated mires have suggested that the restriction of activity may concern methanogenesis in particular rather than total anaerobic microbial activity (Bridgham *et al*. 1998, Yavitt *et al*. 2005, Hines *et al*. 2008). Low pH as such could shape the communities and restrict acetoclastic production. Decrease of pH from 4.8 to 3.8 in incubations of Siberian bog peat reduced CH$_4$ production and shifted the pathway from acetoclastic to hydrogenotrophic (Kotsyurbenko *et al*. 2007). Methanogenic growth in bog peat could also be limited by lack of required trace elements such as Ni, Fe, and Co (Basiliko and Yavitt 2001). A third possibility is a competitive process, for example acetogenesis, which in some soils inhibits hydrogenotrophic methanogenesis especially at low temperatures (Schulz and Conrad 1996, Kotsyurbenko *et al*. 2001). The limitation of CH$_4$ production has been particularly severe at temperatures <15 °C (Bräuer *et al*. 2004, Kotsyurbenko *et al*. 2007, Hines *et al*. 2008). Similar pattern of conspicuously low CH$_4$ production potential at ≤15 °C and strong increase with temperature was also observed in Siikaneva fen, which has a low pH that is comparable to pH of bogs.

The low pH optimum of 5 of the isolated FC strain, “Candidatus *Methanoregula boonei*” (Bräuer *et al*. 2006a), indicates that the group is adapted to unusually low pH for methanogens, which could explain the prominence of FC in bogs. Another competitive advantage may be tolerance of low nutrient and cation levels. Strains were enriched from *Sphagnum* peat using acidic media mimicking the low ionic strength in bog pore water, and NaCl and KCl inhibited CH$_4$ production (Sizova *et al*. 2003, Bräuer *et al*. 2004, Bräuer *et al*. 2006b). Fen cluster sequences have been recovered in nearly all published studies of mires with pH <5.5 (Table 1) with few exceptions (Kotsyurbenko *et al*. 2004, Metje and Frenzel 2005). In peat with higher pH, other members of *Methanomicrobiales* such as the E1 group have been more prominent (Høj *et al*. 2005, 2006, Ganzert *et al*. 2007, Cadillo-Quiroz *et al*. 2006).
2008). The wide occurrence suggests that FC may be ubiquitous in the methanogenic layers of acidic, *Sphagnum*-dominated boreal and temperate mires.

In fens, the higher abundance of vascular plants such as sedges entails allocation of labile carbon as root exudates to the methanogenic layer. Compared to relatively homogenous bog peat, the root system creates a more heterogeneous environment. Root exudates have been shown to support CH$_4$ production from acetate (Ström et al. 2003). Accordingly, the oligotrophic and mesotrophic Lakkasuo fens had higher CH$_4$ production potential and more diverse methanogen communities including also acetoclastic *Methanosetaeaceae*. The mesotrophic fen has also exhibited a higher fraction of acetoclastic methanogenesis than the bog (Galand et al. 2005). The nearby Siikaneva fen had lower pH and CH$_4$ production rates than the Lakkasuo fens, but the methanogen community was diverse and included acetoclastic groups (Table 5). A similar difference between *Sphagnum* bogs with FC-dominated community and a *Carex* fen with higher diversity and *Methanosetaeaceae* has been observed in North America (Cadillo-Quiroz et al. 2006, 2008, Dettling et al. 2007). Archaeal communities in Alaskan mires also varied according to *Sphagnum* or *Carex* cover (Rooney-Varga et al. 2007). In a chronosequence of mires of Finnish land-uplift coast, methanogen communities of younger fens differed from a site in fen-bog transition stage (Merilä et al. 2006). The fen-bog transition is reflected in both pH and vegetation. However, different communities in *Eriophorum* lawn and poorer hummock with similar pH in an oligotrophic fen (Galand et al. 2003) further imply that not only pH but also the botanical composition of peat and substrate quality shape methanogen community composition.

The smaller pH shift and differences in vegetation and surface peat chemistry in the drained bog due to ash-fertilization (Moilanen and Silfverberg 2004) did not have substantial effects on methanogen communities or CH$_4$ production. Ash has affected archaeal and bacterial communities in forest humus (Fritze et al. 2000, Perkiömäki and Fritze 2002, Yrjälä et al. 2004), but at the Pelso-Resula bog the effect may have been restricted to surface peat, with the exception of a specific FC cluster occurring in fertilized peat. Although different mcrA primers gave to some extent contradicting results, the most prominent methanogen group in the drained bog was Rice cluster I. This hydrogenotrophic group also occurred in deeper peat of Lakkasuo bog (III) and Salmisuo fen (Galand et al. 2002). When draining lowers the water level, the methanogenic layer is lowered into more decomposed peat, and a higher fraction of organic matter is degraded aerobically. The low substrate availability may benefit RCI, because it has been enriched and isolated under low H$_2$ levels (Lu et al. 2005, Sakai et al. 2007). Another factor benefiting the group in the drained bog could be tolerance to oxygen. The genome sequence of a RCI archaeon revealed a large number of genes for oxygen detoxification (Erkel et al. 2006).

Season had a strong effect on CH$_4$ production potential, but the archaeal community composition was largely stable; temporal variation in rDNA- and rRNA-derived communities was observed as variation of relative proportions of T-RFs but not as their presence or absence. These results suggest that the population size or activity of methanogens varied substantially without a marked change in community structure. High production potential in winter has not been observed in previous studies that have included a winter sampling (Yavitt et al. 1987, Avery et al. 1999). The result was the opposite of what was expected: temperature, CH$_4$ emission and plant productivity would all favour high
potential activity in summer. The summer was, however, exceptionally dry, and the sampled layer was above water level, most likely diminishing methanogenic activity. A possible explanation for the high winter potential is substrate accumulation, which has been suggested as a reason for increasing potential towards autumn (Saarnio et al. 1997, Kettunen et al. 1999). When temperature declines in autumn, substrate-producing activity could exceed methanogenesis, leaving unused substrates in peat. Although high potential in the laboratory would not necessarily mean active production in the field at <2 °C, the detection of mcrA mRNA confirmed the presence of active methanogens in winter peat. 

Seasonal pattern of CH₄ production differed in Swedish mire sites with distinct plant communities, and the difference was attributed to substrate supply (Bergman et al. 2000). A temporal community pattern in arctic peat was suggested to result from substrate availability (Høj et al. 2005). As there was some spatial variation in communities and CH₄ production even between the relatively similar sampling sites (Fig. 1 and 4 in IV), it would be worthwhile to compare substrate levels and seasonality of methanogens under a range of specific plant communities with a higher resolution fingerprinting method or a quantitative approach. 

The archaeal 16S rRNA gene analysis of Siikaneva revealed RCII instead of RCI found in the mcrA studies of other mires (Table 5). This could simply be a difference in the occurrence of the groups, but because to date no mcrA sequences have been assigned to RCII, the possibility that mcrA primers do not detect RCII or that the sequences have erroneously been assigned to RCI should also be considered. As no members of RCII have been isolated, the methanogenic phenotype is currently assumed based on its phylogenetic position and occurrence in methanogenic soil enrichments (Grosskopf et al. 1998a, Lehmann-Richter et al. 1999).

5.2 Detection of methanogen communities – methodological considerations

Methanogen community analyses targeting the mcrA gene have the great advantage that the detected organisms are known to be CH₄ producers (or anaerobic CH₄ oxidizers). Several studies using the published primers have, however, questioned or revealed failings in the primers’ species coverage or quantitative robustness (Lueders et al. 2001, Lueders and Friedrich 2003, Galand 2004, Banning et al. 2005, Nercessian et al. 2005). The comparison of three primer sets (II) showed that, in case of the drained bog, the choice of primer set had a minor effect on the recovered methanogen community composition but a major influence on the relative proportions of OTUs. Because each primer pair detected similar proportions in two different samples, the differences did not result from random PCR drift during amplification but more likely represented primer-dependent PCR selection (Wagner et al. 1994). The differing extent of primer-dependent variation between peat depths indicated that the properties of template such as species composition affected the outcome of amplification. 

The ME primers have detected Methanosarcinaceae in the drained bog in Study I and in other environments (e.g. Lueders et al. 2001, Newberry et al. 2004), but the lack of detection in the samples of Study II and in Salmisuo fen (Galand 2004) suggests failings in amplification of this family. The ME set has also failed to detect Methanosetaeaceae (Lueders et al. 2001, Banning et al. 2005), most likely due to mismatches in the forward primer
(Table 6). As these families comprise all acetoclastic methanogens, at worst the ME set could miss the entire acetoclastic population. Despite the poor performance of the MCR primers with our peat samples, they had the lowest number of mismatches to genome sequences and could therefore have the widest species coverage. The coverage is, however, achieved with high degeneracy, which may enhance quantitative bias in PCR when sequence variants with GC-rich primer binding sites are amplified preferentially over AT-rich ones (Polz and Cavanaugh 1998). Although the least degenerate ML primers showed several mismatches to mcrA sequences (Table 6), the primers have been shown to amplify mcrA from 23 methanogen strains representing all five orders (Luton et al. 2002). Our studies confirmed that they also detect Fen cluster and Rice cluster I. The ML set is thus currently the best choice for detection of methanogens in peat, although the effect of the abundant mismatches should be evaluated. The recent increase in availability of sequence data suitable for primer design, particularly for the orders Methanosarcinales and Methanomicrobiales which were previously poorly represented, makes modifying the existing primers or even designing entirely new mcrA primers a noteworthy option.

In addition to primers with good coverage, assembling a meaningful representation of methanogen communities requires a fingerprinting method with an appropriate level of resolution. Among the approaches used in this work, the two extremes in terms of resolution and the effort required per sample were the analysis of mcrA clones with two restriction enzymes (III), and the archaeal 16S rRNA gene T-RFLP (IV). The use of two restriction enzymes yielded a fine level of resolution, dividing most methanogen groups into several OTUs, but required time-consuming analysis of high numbers of clones. The 16S rRNA gene T-RFLP required considerably less time per sample, allowing analysis of a larger number of samples and replicates, but both main T-RFs were shared by two archaeal groups. The same combination of primers and restriction enzyme has extensively been used in T-RFLP analysis of rice field soil and even mire methanogens (Ramakrishan et al. 2001, Kotsyurbenko et al. 2004), but the T-RF of 393 bp shared between FC, RCII, and RCI (IV, Conrad et al. 2008), all groups commonly found in peat, makes the approach less ideal for differentiation of mire methanogens. Separating methanogen groups into OTUs revealed differences along the studied gradients, for example the change of RCI sequence types with depth in the drained bog (I), and the distinct FC OTUs occurring in Lakkausuo bog and fens (III). The mcrA gene has higher sequence divergence than the 16S rRNA gene (Springer et al. 1995), and therefore mcrA analysis should provide better prospects for differentiating smaller groups within methanogenic clusters and, for example, defining ecotypes (Palys et al. 1997, Cohan 2001). Although detection of only the terminal fragment lowers the resolution in T-RFLP, the higher resolution of mcrA combined with the swiftness of T-RFLP analysis may be the ideal compromise (Castro et al. 2005, Merilä et al. 2006).

In Study IV, comparison of DNA- and RNA-derived communities showed differences in T-RF proportions, and Methanosetaeae were only detected from RNA. As the sole obligate acetoclastic methanogens, their detection is an indication of acetoclastic methanogenesis, and analysis of only DNA would have overlooked this group. RNA has been used in analysis of archaea and methanogens from other environments, but prior to this work not from peat. The analysis of mcrA mRNA was here restricted to PCR detection. Currently only one published study, addressing methanogens in a chemostat, has used mcrA mRNA in community analysis (Shigematsu et al. 2004). This approach of fingerprinting
mcrA expression would be interesting also for natural environments, regarding the resolution mcrA analysis offers. Combining the mRNA approach with qPCR could be particularly valuable, considering the proposal that cellular activity of methanogens may be a better predictor of CH₄ fluxes than changes of population size (Röling 2007).

5.3 Bacteria and non-methanogenic Archaea – interactions with methanogens

In methanogenic peat layers, non-methanogenic microbes, as substrate producers and competitors to methanogens, are essential for the regulation of methanogenic activity. The members of Deltaproteobacteria in Lakkasuo were related to syntrophic fermenters and could hypothetically function with hydrogenotrophic methanogens, although mere sequence similarity is insufficient to establish this conclusion. In a Florida wetland, hydrogenotrophic methanogens were visualized in vicinity of putative syntrophs (Chauhan et al. 2004). The other prominent groups, Verrucomicrobia and Acidobacteria, are abundant in soils but characterized isolates are few (Hugenholtz et al. 1998, Janssen 2006). Until recently, all known Verrucomicrobia isolates were carbohydrate degraders, but recent isolates from acidic hot springs are CH₄ oxidizers (Dunfield et al. 2007, Islam et al. 2008). The described members of Acidobacteria include heterotrophs and a phototroph (Liesack et al. 1994, Bryant et al. 2007). In accordance to their detection in highly acidic peat in Lakkasuo, Acidobacteria from soil have been shown to be more abundant and grow preferably at pH <6 (Sait et al. 2006). A study on bacteria in an acidic Sphagnum-Carex bog in Siberia later reported nearly exactly the same phyla as those found in Lakkasuo, including even some of the rarer groups such as Planctomycetes, Chloroflexi, Bacteroidetes, and candidate division OP3 (Sizova et al. 2006). The FISH analysis of the study suggested that, in contradiction to clone library data, Planctomycetes and Alphaproteobacteria were more abundant than Acidobacteria and Verrucomicrobia. Interestingly, the detected phyla did not markedly differ from those commonly observed in mineral soils (Janssen 2006).

Crenarchaea of group 1.3 occurred at Siikaneva and all three Lakkasuo mires, supporting the wide occurrence of the group not only in mineral soils (Ochsenreiter et al. 2003) but also in peat (Høj et al. 2006, Rooney-Varga et al. 2007). Group 1.1c crenarchaea occur in mesophilic, acidic soils (Jurgens et al. 1997, Yrjälä et al. 2004, Bomberg and Timonen 2007, Kemnitz et al. 2007, Hansel et al. 2008), but have not previously been detected in anoxic peat. In related crenarchaeal groups 1.1a and 1.1b, ammonium oxidizing organisms have been identified (Leininger et al. 2006, Nicol and Schleper 2006), but function of groups 1.1c and 1.3 is yet unknown. Members of group 1.3 were observed in close association with acetoclastic methanogens in anaerobic sludge (Collins et al. 2005), and it is tempting to speculate this group could be acetogenic.
6 Conclusions and future directions

Methanogen communities and CH$_4$ production potential differed strongly between sites with distinct ecohydrological status, namely fens and bogs. Fens revealed more diverse methanogen communities than bogs. The pristine and drained bog harboured hydrogenotrophs of Rice cluster I and Fen cluster. The results suggest that Fen cluster is ubiquitous in various types of acidic mires and particularly prominent in highly acidic Sphagnum bogs. In fens, root exudates of sedges supposedly promoted the obligate acetoclastic methanogens of the family Methanosaetaceae and potentially acetoclastic Methanosarcinaceae. The wide range of methanogens detected at Siikaneva fen with a bog-like pH (~4) demonstrated that pH alone did not define the community composition of fens and bogs. To identify the specific variables behind the influence of hydrology, further studies are needed addressing the effects of pH, other chemical properties, and vegetation on methanogen diversity. Such studies could also unravel the restrictions of methanogenesis in bogs and illuminate ecophysiology of mire methanogens.

The seasonal study demonstrated substantial temporal variation in potential CH$_4$ production and minor changes in archaeal DNA- and RNA-derived communities with season. However, due to low resolution, the T-RFLP analysis most likely missed some community shifts. Presumably, the seasonal temperature shifts primarily affected the size or activity of the methanogen community rather than its composition. Fingerprinting and quantifying mcrA mRNA would be a promising but methodologically challenging approach to clarify this issue. Temporal comparison of methanogen communities between distinct mire types or microsites could further resolve the extent of seasonal variation of methanogens. Another future objective could be determining whether the communities of bacterial substrate producers or substrate levels vary with season. The finding of high methanogenic potential and active methanogens in winter stresses the need to acknowledge microbial activity outside growing season.

The drained bog revealed a clear change of methanogen communities with peat depth, but ash fertilization had no substantial effects in the methanogenic peat layer. Comparison of three mcrA primer sets demonstrated that their coverage for methanogens from the drained bog was similar, but the quantitative representations of communities were primer-dependent. Particular care should therefore be taken in interpretation of mcrA-based abundance data, as opposed to merely assessing the presence or absence of taxa. One solution could be developing more quantitatively robust methods, for instance qPCR assays to monitor specific populations.

Detection of bacteria and non-methanogenic archaea showed that several wide groups commonly occurring in mineral soils, most with unknown function, also exist in acidic, anoxic peat. The next step would be assessing the occurrence and function of specific groups and identifying those interacting with methanogens either by supporting or inhibiting methanogenesis.

Overall, the results indicate that methanogen community composition reflects chemical or botanical gradients that affect CH$_4$ production, such as mire hydrology. Ecophysiological characterization of methanogens could thus benefit predictions of CH$_4$ production. The spatial heterogeneity of mires makes knowledge of peat chemistry indispensable for relating communities to CH$_4$ production capacity.
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8 References


