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Methanogenic *Archaea* in boreal peatlands

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Academic Dissertation in General Microbiology

To be presented, with permission of the Faculty of Biosciences, University of Helsinki, in the auditorium 2, Viikinkaari 11, Viikki Infocenter, on June 11th 2004, at 12 noon.

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ISSN 1239-9469
ISBN 952-10-1895-X printed version
ISBN 952-10-1897-6 pdf version, http://ethesis.helsinki.fi

Yliopistopaino
Helsinki 2004

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals.

I. Galand, P. E., Saarnio, S., Fritze, H. & Yrjälä, K. (2002). Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. FEMS Microbiology Ecology 42**:** 441-449.

II. Galand, P. E., Fritze, H. & Yrjälä, K. (2003). Microsite-dependent changes in methanogenic populations in a boreal oligotrophic fen. Environmental Microbiology 5**:** 1133-1143.

III. Galand, P. E., Juottonen, H., Fritze, H. & Yrjälä, K. (2004). Methanogen communities in a drained bog: effect of ash fertilization. Microbial Ecology (in press).

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ABBREVIATIONS

ARDRA	amplified ribosomal DNA restriction analysis
bp	base pair
CH_4	methane
CLSM	confocal laser scanning microscopy
CO_2	carbon dioxide
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FISH	fluorescence *in situ* hybridization
ha	hectare
HFCs	hydrofluorocarbons
mcr	methyl-coenzyme M reductase
N_2O	nitrous oxide
O_2	oxygen
PCR	polymerase chain reaction
ppm	parts per million
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
16SrRNA	small subunit ribosomal ribonucleic acid

ABSTRACT

Wetlands, including peatlands, are the main source of natural emissions of the green house gas methane (CH_4). The production and release of CH_4, and their regulatory environmental factors have been studied intensively in boreal peatlands, but the communities of methanogenic *Archaea*, responsible for CH_4 formation, remain poorly described.

We investigated the diversity, and activity of methanogens, at the natural Salmisuo fen, at different peat depths of the most representative microsite (*Eriophorum* lawn). Amplification of portions of the *mcr*A gene revealed a change of methane producing populations with depth. Sequences from the upper waterlogged layers of the fen grouped in a novel "Fen cluster" related to *Methanomicrobiales*. Sequences retrieved from the deeper layers of the fen were related to hydrogenotrophs from Rice Cluster-I.

Comparison of methanogen communities from two well-defined fen microsites, *Eriophorum* lawn and hummock, indicated that populations changed with sites in upper fen layers. 16SrRNA phylogenetic analyses revealed that *Methanomicrobiales* related sequences dominated upper layers of hummock, and that members of *Methanosarcinales* occupied upper parts of the *Eriophorum* lawn. In the deeper fen layers, methanogen communities were homogenous across microsites. Novel methanogenic sequences were found at both depths of both sites. Vegetation characterising the microsites probably influences methanogenic communities, and metabolic pathways in the anaerobic layers of the fen.

Samples from all depths of the drained Pelso-Resula bog contained a majority of *mcr*A sequences related to the hydrogenotroph Rice cluster-I. The diversity of methanogens was lower in the drained bog than in the natural fen. An ash fertilisation trial, intended to simulate forestry practices, demonstrated that ash treatment marginally increased peat pH in anaerobic layers without affecting the major methanogen groups, or the potential methane production. Some Fen cluster-related sequences were, nevertheless, retrieved from fertilized plots only, suggesting a group specific response to increase in soil pH.

INTRODUCTION

Methane (CH_4), and the other important green house gases (carbon dioxide (CO_2), nitrous oxide (N_2O), and hydrofluorocarbons (HFCs)) are changing the composition of the atmosphere. They absorb infrared radiations originating from the Earth's surface, the atmosphere, and clouds. Thus, heat is trapped within the surface-troposphere system, and the temperature at Earth's surface, and in the troposphere increases. Responsibility of green house gases in global climate change is now accepted. Since the Industrial Revolution in 1750, the concentration of green house gases in the atmosphere has increased dramatically. Although CH_4 concentration in the atmosphere is low, when compared to the main green house gas CO_2 (1,75 ppm versus 367 ppm), its capacity to absorb infrared radiations, and radiate energy back to earth gives it a 23 times higher global warming potential (GWP) than CO_2 on a 100 years time horizon (IPCC, 2001). After the importance of CH_4 as a green house gas was acknowledged (IPCC, 1990), much attention has been directed toward understanding its biogeochemical cycle.

Most of the CH_4 produced annually is of anthropogenic origin (from 55 to 70%). The highest source of emissions is agriculture, with rice field, and domesticated ruminants responsible for 19 to 36% of total emissions (Fung et al., 1991; Hein et al., 1997; Lelieved et al., 1998). Natural CH_4 sources are responsible for 30 to 45% of total emissions. Some of the natural sources of CH_4 emissions are wetland soils (bogs, swamps, tundra, etc), oceans, wild ruminants, and some insect species like termites with their methanogenic microbial symbionts (ASM, 2000; IPCC, 2001). The biggest sources of natural CH_4 emissions are wetlands, which emit 23 to 40% of the total annual CH_4 emissions (Fung et al., 1991; Hein et al., 1997; Lelieved et al., 1998).

Peat-forming wetlands (peatlands) are characterised by slow decomposition rates leading to an accumulation of peat; their organic soil below the water table is anoxic, and often acidic (Gorham, 1991). They represent the majority of the world's wetlands, and are therefore globally an important CH_4 source. Between 10 and 20% of total CH_4 emissions originate from peatlands situated in the northern latitudes (>45°N) (Matthews and Fung, 1987; Harris, 1993; Cao et al., 1996), although they cover only 3 % of the earth surface (Kivinen and Pakarinen, 1981). Finland is the country with highest proportion of land covered by peatland. Even though large portions of land have been drained for forestry purposes (Aarnio et al., 1997), more than 29% of the country's area is still covered by peatlands (Lappalainen, 1996; Aarne, 1998).

Methane is almost exclusively produced by microbial activity (ASM, 2000). Methanogens are the unique group of microorganisms having CH_4 as a stoichiometric product of their metabolism. Methanogens belong to the domain of life *Archaea* (Woese et al., 1978), kingdom *Euryarchaeotaea* (Woese et al., 1990; Boone et al., 1993).

The continuous development of molecular tools for environmental studies of microorganisms (Head et al., 1998; Insam, 2001) has facilitated the characterisation of methanogen communities. Diversity of methanogens have been studied in, and species isolated from a wide range of environments: gut of terrestrial arthropods (Hackstein and Stumm, 1994), termite guts (Ohkuma et al., 1995; Leadbetter et al., 1998; Ohkuma et al., 1999), hydrothermal vents (Jones et al., 1983; Jeanthon et al., 1999; Takai and Horikoshi, 1999), hydrocarbon-contaminated soils (Dojka et al., 1998; Watanabe et al., 2002), ocean and lake

sediments (Falz et al., 1999; Marchesi et al., 2001; Nusslein et al., 2001; Chan et al., 2002; Earl et al., 2003), and activated sludge (Gray et al., 2002; Saiki et al., 2002). Among submerged soils, rice field soils have been extensively studied (e. g. (Grosskopf et al., 1998b; Fey et al., 2001; Lueders et al., 2001; Ramakrishnan et al., 2001). Natural wetlands have been much less investigated (Krumholz et al., 1995; Hales et al., 1996; Edwards et al., 1998; Lloyd et al., 1998; McDonald et al., 1999; Nercessian et al., 1999; Upton et al., 2000; Basiliko et al., 2003; Horn et al., 2003), and, to the best of our knowledge, only one publication has described diversity of methanogens in boreal (60-69°N in Finland) peatlands (Sizova et al., 2003).

Methanogenic Archaea

Taxonomy and phylogeny

Methanogens can be separated into three main nutritional categories. (i) Hydrogenotrophs (38 species) oxidize H_2 and reduce CO_2 to form methane, and among those some are able to oxidize formate for methane formation. (ii) The second nutritional group includes methylotrophs (20 species), which utilize methyl compounds as methanol, methylamines, or dimethylsulfides to produce methane. H_2 is also here used as an external electron donor. Thirteen species are obligate methylotrophs. (iii) The last category, acetoclastic (or acetotrophic) methanogens (9 species), utilizes the methyl group of acetate to produce CH_4; only two species are obligate acetotrophs. Some species share nutritional characteristics and cannot be classified in a single group (Garcia et al., 2000).

A number of additional standards have been used to classify methanogens. They include morphology, motility, electron microscopy images, colony morphology, nutritional spectrum, growth rates, growth conditions, metabolic end-products, Gram staining, susceptibility to lysis, antigenic fingerprinting, lipid analysis, distribution of polyamines, nucleic acid hybridisation, G + C content of the DNA, 16S rRNA sequencing and sequence analysis (Boone and Whitman, 1988). According to those criteria, five orders (Fig. 1), ten families, 26 genera, and 74 valid species have been defined (Boone et al., 1993):

- Order *Methanobacteriales* contains two families. Family *Methanobacteriaceae* has four morphologically distinct genera. They include species retrieved from a variety of habitats (freshwater, bovine rumen, wood, termite guts, etc), which are able to use substrates as H_2/CO_2, 2-propanol, formate, and methanol for CH_4 production. Family *Methanothermaceae* consists of one single genus of extreme thermophile hydrogenotrophs.
- Order *Methanococcales* contains two families, *Methanococcaceae*, and *Methanocaldococcaceae*, and four genera of hydrogenotrophs from mainly marine and coastal environments. Most of the species are able to use both H_2 and formate as electron donors.
- Order *Methanomicrobiales* comprises three families and nine genera of hydrogenotrophic methanogens. Family *Methanomicrobiaceae* contains species isolated from various environments (rumen, marine sediments, etc). Family *Methanocorpusculaceae* consists of three genera utilizing H_2/CO_2 and formate as substrate. Family *Methanospirillaceae* is a new family with one single genus; members have been reported from various habitats, and are able to use different electron donors for methanogenesis from CO_2.

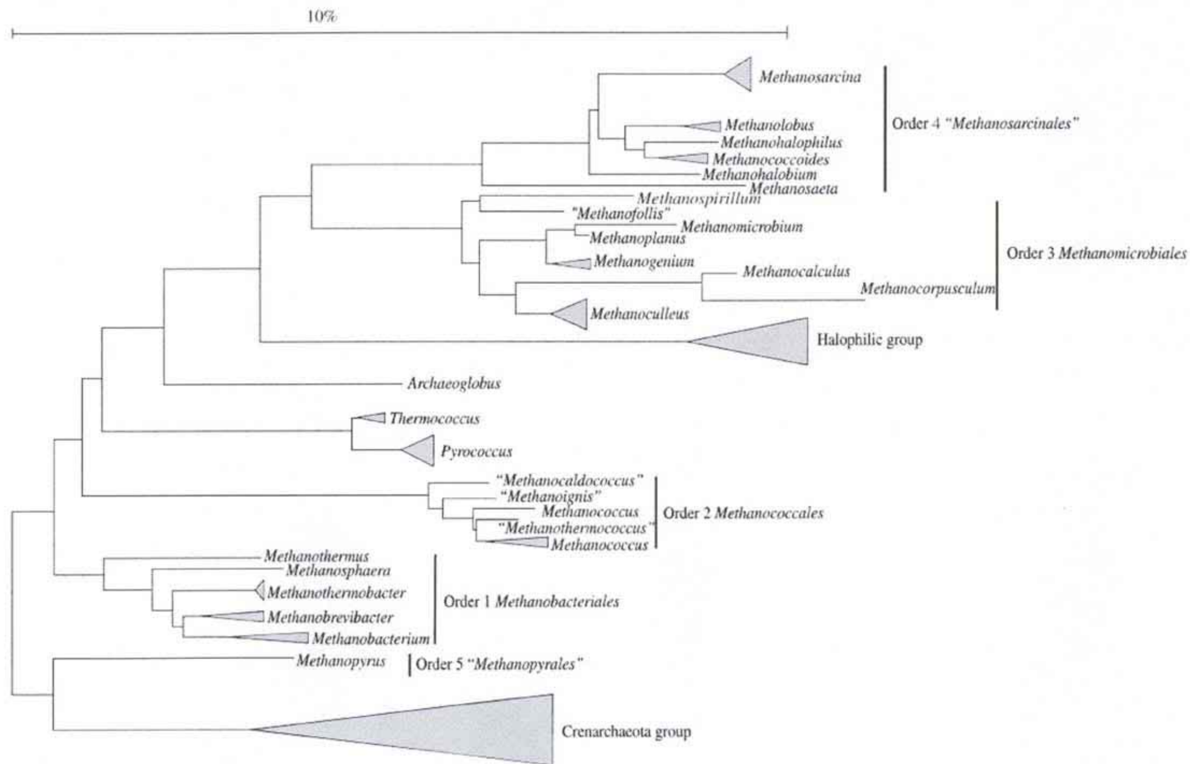

Figure 1. Phylogeny of methanogens, domain *Archaea.* Non-methanogens are indicated by their group names (large triangles) (Garcia et al., 2000).

- Order *Methanosarcinales* has newly been proposed by Boone (Boone et al., 1993). It regroups all acetotrophic, and/or methylotrophic methanogens into two families. Family *Methanosarcinaceae* contains six genera of very versatile methanogens retrieved from various habitats, and able to use H_2/CO_2, acetate, or methyl compounds as substrate. Family *Methanosaetaceae* includes one genus of obligate acetotrophic methanogens.
- Order *Methanopyrales* is a new order of hyperthermophilic methanogens, which are unrelated to all other known methanogens. The order comprises one single family *Methanopyraceae*, and one species *Methanopyrus kandleri*.

Ecological diversity

They are three different methanogenic ecosystems (Garcia et al., 2000):

(i) In wetlands, lacustrine or marine sediments, sludges, and digesters the organic matter is degraded to CH_4 and CO_2. The most studied representative of those ecosystems is rice field soil, in which diversity, and structure of methanogen communities have been examined with both ribosomal (16SrRNA) and functional (*mcr*A) molecular markers (e. g. Grosskopf et al., 1998a; Lueders et al., 2001). Methanogen populations have also been characterized in lake (Falz et al., 1999; Nusslein et al., 2001; Chan et al., 2002; Earl et al., 2003) and sea sediments (Marchesi et al., 2001; Purdy et al., 2002) from a variety of geographical regions. In many paddy soils and lake sediments, acetotrophy has been shown to be the main pathway for methanogenesis (Winfrey and Zeikus, 1979; Kuivila et al., 1989; Rothfuss and Conrad, 1993). In those habitats, the proportion of CH_4 originating from acetogenesis is close to 67%, the theoretical percentage of CH_4 produced from acetate in methanogenic soils and sediments (Conrad, 1999).

(ii) Within animal digestive tracts, methanogens are directly involved in the digestive processes. In ruminants, most of the products from fermentation are reabsorbed into the blood stream to serve as nutrition. In those ecosystems, CH_4 is therefore mainly a product originating from methanogens using H_2/CO_2 as substrate. Within living organisms, communities have been described in habitats such as rumen (Miller et al., 1986; Sharp et al., 1998; Wright et al., 2004), and termite guts (Ohkuma et al., 1995; Ohkuma et al., 1999).

(iii) In extreme environments where organic matter is absent, CH_4 is produced from geochemical hydrogen only. Hydrothermal vents represent such ecosystems. Hyperthermophilic methanogens have been isolated from those extreme environments (Jones et al., 1983; Jeanthon et al., 1999), and molecular phylogenetic analyses of communities have been conducted (Takai and Horikoshi, 1999).

Methanogenesis in peatlands

Peatlands

Peatlands are botanically defined as sites supporting a peat-producing plant community. Ecologically, they are defined as ecosystems with high water table where, due to anoxic conditions, partially decomposed organic matter accumulate as peat (Laine and Vasander , 1996). Peatland waters are typically rich in DOC, with concentrations of 10–50 mg/l; humic substances account for 70-90% of this DOC (Steinmann and Shotyk, 1997). Depending on

their hydrology and nutrient status, peatlands are generally classified into two broad groups. Bogs are ombrotrophic (nutrient poor) peatlands, which receive water and chemical elements from atmospheric precipitations only. They are consequently groundwater recharge zones. Ombrotrophic peatlands are characterized by low pH values (< 4), and low nutrient availability. Minerotrophic peatlands, called fens (treeless or sparsely treed), or swamps (forested), are fed by water enriched with mineral elements after contact with the mineral soil (Eurola et al., 1984). They are discharge zones for groundwater. Depending on the regions, the telluric water can originate from melt-water, groundwater, or limnic surface-water. Minerotrophic peatlands are divided into three nutrient level groups: oligotrophic (pH ≈ 4,5), mesotrophic (pH ≈ 5,5), and eutrophic (pH 5,5-7,5). The wettest sites, with average water table depth comprised between 0 and –5 cm, are called flarks or hollows. The other flat surfaces, with average water level 5 to 20 cm below the peat surface, are defined as lawn. The third type of microsite is called hummocks; they rise above their surroundings, and their average water level is more than 20 cm below the peat surface. Average water tables, and nutritional statuses influence the occurrence of specific plant species. Those plant species are in turn used to classify peatland sites (Eurola et al., 1984).

Substrate for methanogenesis

Anoxic degradation of organic matter

Wetlands are characterized by the anoxic conditions prevailing in the water-saturated layers of the soil. Slow diffusion of oxygen in water, and consumption of oxygen by roots of higher plants and chemoorganotrophic microorganisms create anoxic conditions (Svensson and Sundh, 1992).

Between 50 and 80% of wetland plant biomass consists of lignocellulose with a lignin-to-polysaccharide ratio ranging from 1:2 to 1:7 (Maccubbin and Hodson, 1980; Hodson et al., 1982). Plant detritus derived from wetland vegetation contain consequently primarily biopolymers including polysaccharides such as cellulose, hemicellulose, and lignin. Organic matter is mainly degraded aerobically in peatlands, but some plant debris enters the anoxic peat layers where decomposition occurs at a much slower rate (Clymo, 1984; Clymo and Pearce, 1995). Lignified materials are almost totally recalcitrant to anaerobic degradation (Zeikus, 1981), and are therefore an insignificant energy or carbon source for microorganisms in the anoxic peat layers. Some components of this polymer may, however, be degraded into precursors, as methanol, which could serve as substrate for methanogenesis (Benner et al., 1984).

Carbohydrates entering the waterlogged peat layers will be decomposed to the final products CH_4 and CO_2. The decomposition process includes different steps involving a complex community of hydrolytic, fermenting, homoacetogenic, syntrophic, and finally methanogenic microorganisms (Fig. 2) (Zehnder, 1978; Laanbroek and Veldkamp, 1982; Zinder, 1993; Stams, 1994; Schink, 1997). Methane produced in the anoxic peat layers escapes to the atmosphere through three possible mechanisms: diffusion along a concentration gradient, ebullition (bubble formation) when the pore-water is supersaturated with CH_4, or transportation via roots and tissues (aerenchyma) of vascular plants.

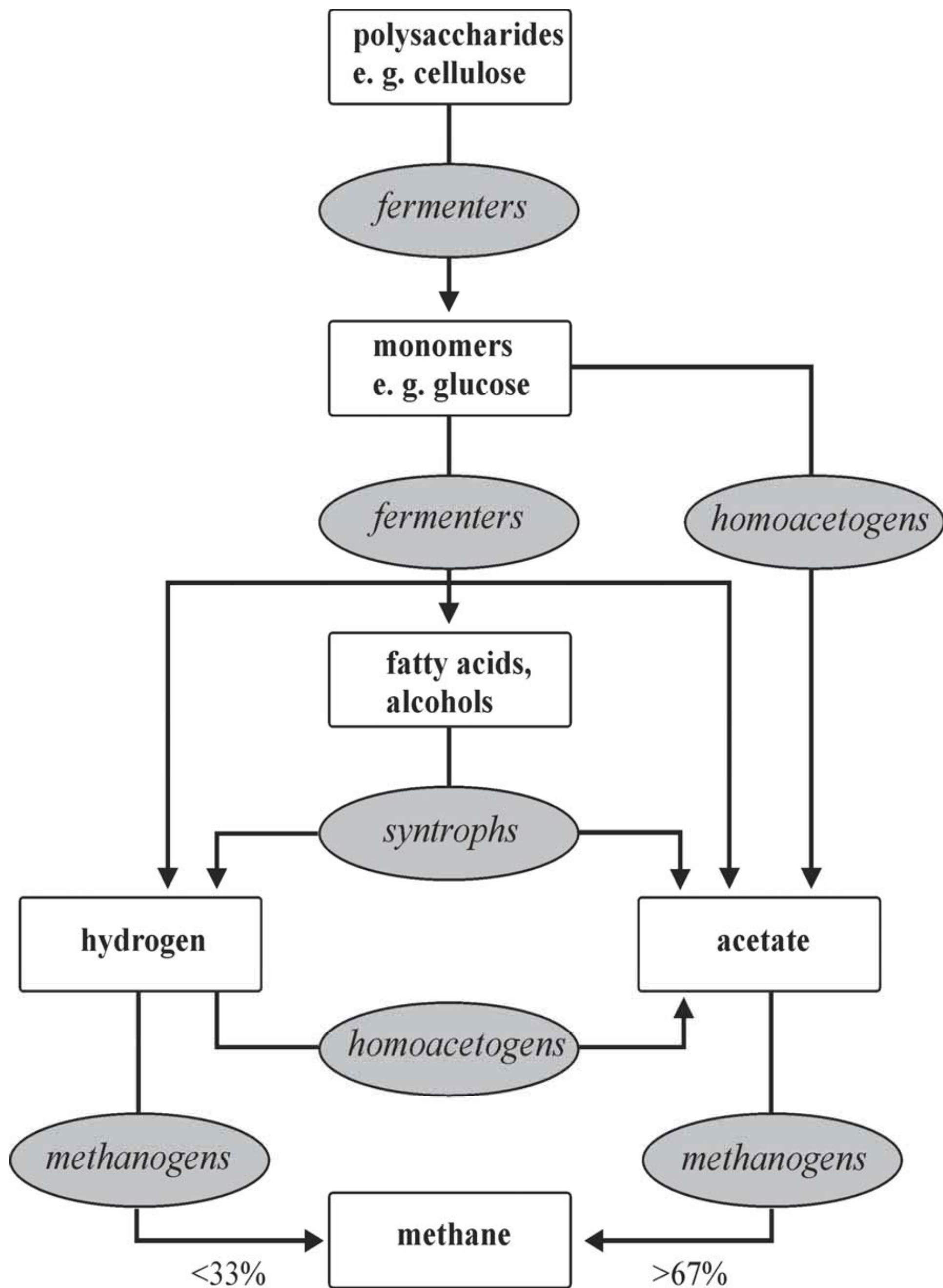

Figure 2. Generalized flow diagram of anaerobic decomposition of organic matter, and generation of methane. Modified from Conrad (1999).

The first step of anaerobic decomposition of organic matter is hydrolysis. Extracellular enzymes produced, and excreted by fermenting bacteria cleave polymers to monomers. At that stage, cellulose, which is the most common polysaccharide in plant community, will be hydrolysed to glucose molecules.

Fermentation is the next step. Monomers like glucose serve as substrate for primary fermenters, which, as end products, release simple compounds such as fatty acids, hydrogen, CO_2, and alcohols. Monomers can alternatively be catabolized to acetate by homoacetogenic bacteria. Acetate can then serve directly as substrate for CH_4 production by acetotrophic methanogens.

Syntrophic bacteria degrade simple compounds, produced during primary fermentation, to acetate, H_2, and CO_2. They live in close relationship with methanogens, which can use directly H_2 as electron donor for CO_2 reduction. Direct utilisation of H_2 for methanogenesis allows its partial concentration in the soil to remain low. Low partial H_2 pressure is necessary for syntrophic bacteria's metabolism; it allows their fermentative energy producing reaction to be exergonic, and therefore thermodynamically favourable (Schink, 1997).

Methanogenesis is the last step of the anaerobic degradation of organic matter. As discussed before, methanogens use mainly H_2 (alternatively formate), and CO_2, or acetate as substrate for methane production. One mole of glucose produces anaerobically two moles of acetate, and four moles of H_2, and the production of one CH_4 requires four H_2, but only one acetate. Consequently, when polysaccharides are degraded, methanogenesis from acetate, and H_2/CO_2 contribute theoretically respectively for $>67\%$ and $<33\%$ to total CH_4 formation (Conrad, 1999).

Vascular plants below-ground production

Wetland plants annual cycles of production, and decomposition are primary sources of organic matter, which in turn results in the fermentative production of H_2, and acetate (Chanton and Dacey, 1991; Schutz et al., 1991; Nilsson and Bohlin, 1993). Carbon input from below ground plant tissues could, however, also be an important substrate source for methanogens in peatland environments (Jackson and Caldwell, 1992; Whiting and Chanton, 1992; Whiting and Chanton, 1993; Chanton et al., 1995; Joabsson et al., 1999; Strom et al., 2003). Most of the carbon assimilated by peatland vegetation is allocated to belowground tissues (Saarinen et al., 1992), which are potential sources of labile carbon. Organic material such as exudates, dead cells, and material from root turn over are released by plant roots, and are exploited by fermentative bacteria, or directly by methanogens for biosynthesis and energy production (van Veen et al., 1989).

Some vascular plant species develop a cortical oxygen-transporting gas space (aerenchyma) as an adaptation to wetland conditions; it transports oxygen (O_2) to the root situated in O_2 depleted layers of soil (Koncalova, 1990; Armstrong et al., 1991). The root systems of those vascular plants tend to be less shallow than the ones from non-aerenchymatous peatland plants. Thus, roots penetrate into anoxic layers, and allow the entrance of potential carbon substrate to the deeper peat layers (Malmer et al., 1994). Acetate was, for example, shown to be the main labile carbon exudation from roots of the sedge *Eriophorum scheuchzeri* in an arctic wetland, and acetoclastic methanogenesis was consequently the dominant reaction in the vicinity of that sedge (Strom et al., 2003).

Vascular plants influence significantly methane production, but also affect the methane fluxes between peatlands and atmosphere. Vascular plants can promote the transport of CH_4 from the production layers to the atmosphere via their aerenchyma (Schimel, 1995; Shannon et al., 1996; King et al., 1998; Bellisario et al., 1999; Greenup et al., 2000), and O_2 entering the rhizosphere can inhibit the activity of strictly anaerobic methanogens (Chanton and Dacey, 1991; Watson et al., 1997).

Methanogens in peatlands

Methanogenic activity

Activity of methanogens in peatlands can easily be detected because methanogenic metabolic activity is directly related to the amount of CH_4 produced in peat. The quantities of CH_4 emitted from peatlands are, however, not always proportional to the activity of methanogens, since emission rates represent the quantity of CH_4 produced in anoxic layers minus the amount of CH_4 oxidized by methanotrophs in aerobic layers. Methanogen activity is more precisely correlated to potential methane production. It is a direct measure of the CH_4 produced in the anoxic peat layers; peat samples are incubated anaerobically, and the accumulation of CH_4 is monitored (e. g. Hall et al., 1996; Kettunen et al., 1999).

Measurements of potential methane production show that methanogenic activity is restricted to the waterlogged layers of the peat. The result is expected since methanogens are strictly anaerobic microorganisms. Rates of potential methane production are higher in the upper anoxic peat layers (from 0 to 20 cm below water table) than in the deeper ones (Bergman et al., 1998). Some studies on boreal, and temperate mire complex have detected an area of maximum potential CH_4 production situated around 20 cm below water table (Roulet et al., 1993; Sundh et al., 1994; Krumholz et al., 1995; Kettunen et al., 1999), while measurements from English bog samples have revealed highest production just below the water table (Edwards et al., 1998; McDonald et al., 1999). Available substrate is the main limiting factor for methanogenesis (Bergman et al., 1998). Consequently, the maximum CH_4 production in peat profile takes place at the depth where most of the anaerobic degradation occurs. The low potential CH_4 production detected in the deeper layers of peat is probably reflecting the lack of suitable substrate, or the presence of less favourable substrate at those depths (Svensson and Sundh, 1992; Valentine et al., 1994).

Methanogen communities

During the past decade, new methods including PCR, cloning, DNA fingerprinting, sequencing, probing, etc, have been used to uncover the diversity of methanogen communities. The most recent publications describing methanogen populations in peatlands, with molecular tools, are presented in Table 1. Methanogens have been studied in very few peatland types. Almost all research has been conducted on bog samples (ombrotrophic, nutrient poor sites), and restricted to a small number of sites; one single study has examined methanogen communities in swamp. Despite the low number of studied environments, representatives from four out of the five methanogen orders have been detected in peatlands. Members of the orders *Methanosarcinales*, and *Methanobacteriales* were retrieved from all studied sites, and representatives from the order *Methanococcales* were found additionally at an English, and Scottish bog site (McDonald et al., 1999; Upton et al., 2000). Sequences belonging to the order *Methanomicrobiales* were retrieved from a German, and a Scottish site only (Upton et al., 2000; Horn et al., 2003).

Table 1. Overview of publications describing methanogenic communities with molecular methods in different peatland ecosystems.

Sites type and name	Sample depth	Molecular marker/Methods	Phylogenetic affiliation	References
Bog Moorhouse, northern England (54°65'N, 2°45´W)	0 to 30 cm below surface	16SrRNA-*mcr*A / PCR, cloning, sequencing	Two new clusters of methanogens, one related to order *Methanosarcinales*, family *Methanosarcinaceae* and the other to order *Methanomicrobiales*, family *Methanospirillaceae*	(Hales et al., 1996; Edwards et al., 1998)
		16SrRNA-*mcr*A / PCR, oligonucleotide probing	Representatives from families *Methanosarcinaceae* *Methanococcaceae* *Methanobacteriaceae*	(McDonald et al., 1999)
Bog Ellergower Moss, Scotland	0 to 30 cm below surface	16SrRNA-*mcr*A / PCR, oligonucleotide probing, CLSM	Representatives from families *Methanosarcinaceae* *Methanococcaceae* *Methanobacteriaceae* *Methanomicrobiales*	(Upton et al., 2000)
		16SrRNA / PCR, CLSM	No phylogenetic analysis	(Lloyd et al., 1998)
Bog Moorhouse and Ellergower Moss	0 to 30 cm below surface	*mcr*A / PCR, cloning, sequencing	At both sites, new clusters of methanogens related to order *Methanosarcinales* family *Methanosarcinaceae*	(Nercessian et al., 1999)
Bog Fichtelgebirge, east-central Germany (50°08'N, 11°51'E)	5 to 10 cm below surface	16SrRNA / PCR, cloning, DGGE, sequencing, F*IS*H	Order *Methanomicrobiales* Families *Methanosarcinaceae* and *Methanobacteriaceae*	(Horn et al., 2003)
Swamp and Bog Labrador Hollow and McLean bog, north-east USA (42°45'N, 76°01'W)	10 cm below surface (water table depth)	16SrRNA / PCR, cloning, ARDRA, sequencing	Both sites with representatives from families *Methanosarcinaceae*, *Methanosaetaceae*, and until now uncultured methanogens. Additionally in bog site, representative from family *Methanobacteriaceae*	(Basiliko et al., 2003)
Bog Bakchar Bog, West Siberia, Russia (57°N, 82°E)	20-25 cm below surface	16SrRNA / enrichment, PCR, cloning, RFLP, FISH	Isolated consortia with representatives from Rice cluster-I, and orders *Methanomicrobiales*, *Methanobacteriales*	(Sizova et al., 2003)

Sequences from until now uncultured methanogens were detected at all studied sites. Those novel groups of sequences clustered with members of the order *Methanomicrobiale*s (Basiliko et al., 2003), or *Methanosarcinales* (Nercessian et al., 1999), or both (Hales et al., 1996; Edwards et al., 1998). Members from the Rice cluster I, which represent uncultured H_2+CO_2 utilizing methanogens (Grosskopf et al., 1998b; Lueders and Friedrich, 2000; Lueders et al., 2001), were only retrieved from a West Siberian bog (Sizova et al., 2003). In the same study, the authors isolated methanogenic consortia belonging to the order *Methanomicrobiales* and *Methanobacteriales*.

Pathways for methanogenesis

The most commonly used methods to study methanogenic pathways are incubation experiments with radioactive precursors for methanogenesis, or calculation of $\delta^{13}C$ values for produced CH_4. Values for $\delta^{13}C$ between –65 and -50‰ indicate acetoclastic methanogenesis, and values of –110 to –60‰ are indicative of CH_4 produced by CO_2 reduction (Whiticar et al., 1986). Use of phylogenetic sequence analysis may also give some information about pathways used for CH_4 production. Pathways remain, however, often unknown since members from the same family may be able to use different substrates, and since novel sequences, belonging to organisms unrelated to any known groups of methanogens, are often present in peatland soil.

Methanogens utilize a limited number of substrates, and the most important ones are acetate and H_2/CO_2 (or formate) (Zinder, 1993). In peat-accumulating wetlands, CO_2 reduction is thought to be the main pathway for methanogenesis (Whiticar et al., 1986; Lansdown et al., 1992; Hornibrook et al., 1997; Horn et al., 2003); hydrogenotrophy might be such a dominating process that other potential precursors for methanogenesis, as acetate, may even accumulate (Hines and Duddleston, 2001). In fen ecosystems, however, acetoclastic methanogenesis is often predominant in upper peat layers. Input of labile carbon sources from the rhizosphere of vascular plants (e. g. *Carex* or *Eriophorum* sedges) may promote the acetotrophic pathway in upper minerotrophic layers. In the deeper fen layers, where mostly recalcitrant old peat is present, hydrogenotrophy is the dominating pathway (Kelley et al., 1992; Popp and Chanton, 1999; Chasar et al., 2000). In bogs, vegetation is often restricted to non-aerenchymous plants (e. g. *Sphagnum* mosses), and hydrogenotrophy may be the dominant reaction at all depths (Chasar et al., 2000; Horn et al., 2003). The association of peatland types with methanogenic pathways remains, however, difficult to establish. For instance, Avery and co-workers (Avery et al., 1999) suggested that pathways change with season according to rates of primary production. In the studied bog, methane production changed from hydrogenotrophy during winter to acetotrophy during late spring, following vegetation growth.

Molecular markers

Traditional methods in microbiology, based on cultivation, were shown to underestimate dramatically the diversity of microorganisms in ecosystems. In soil, for example, cultivation methods can retrieve less than 1% of all present microorganisms (Torsvik et al., 1990; Ward and Weller, 1990; Amann et al., 1995). A more precise description of microbial communities, and a better understanding of their function in natural ecosystems is now possible with the recent development of molecular biology methods in microbial ecology. Those molecular tools appear especially well suited for the study of organisms known to be difficult to

cultivate. Methanogens are such a kind of organism: they are obligate anaerobes, some require long incubation periods, they are difficult to separate from their syntrophic partners (Garcia et al., 2000), and require low reducing potential (<-330 mV, (Sowers, 1995)).

The classical set up for molecular ecology studies of microorganisms is: (i) extraction of DNA from environmental samples by different methods including chemical, or mechanical cell lysis, (ii) amplification by PCR of the targeted organisms DNA with specific primers, and (iii) characterisation of microbial communities by fingerprinting methods like RFLP, ARDRA, T-RFLP, DGGE, and/or sequence analysis (Theron and Cloete, 2000). A crucial step in molecular analysis is the selection of adequate primers to amplify the selected molecular marker. This molecular marker is usually either a portion of the 16SrRNA, or a part of a functional gene.

Methyl-coenzyme M reductase gene

A molecular marker widely used for studies of methanogen communities is the gene coding for methyl-coenzyme M reductase (MCR), a key enzyme for methanogenesis. The enzyme enters the final steps of CH_4 synthesis (Ermler et al., 1997), and catalyses the reduction of a methyl group, attached to coenzyme M, with release of methane (Ellermann et al., 1988). The *mcr* transcription unit is composed of five open reading frames, *mcrBDCGA* (Fig. 3). Genes *mcr*A, *mcr*B, and *mcr*G encode the three polypeptide α, β, γ subunits of the MCR coenzyme (Allmansberger et al., 1986; Bokranz and Klein, 1987; Cram et al., 1987; Bokranz et al., 1988; Weil et al., 1988). The functions of the two additional open reading frame, *mcr*C and *mcr*D, remain unknown. Some members of the order *Methanobacteriales*, and *Methanococcales* additionally contain an isoenzyme, the methyl-coenzyme M reductase two (MCR-II) (Lehmacher and Klenk, 1994; Pihl et al., 1994; Bult et al., 1996).

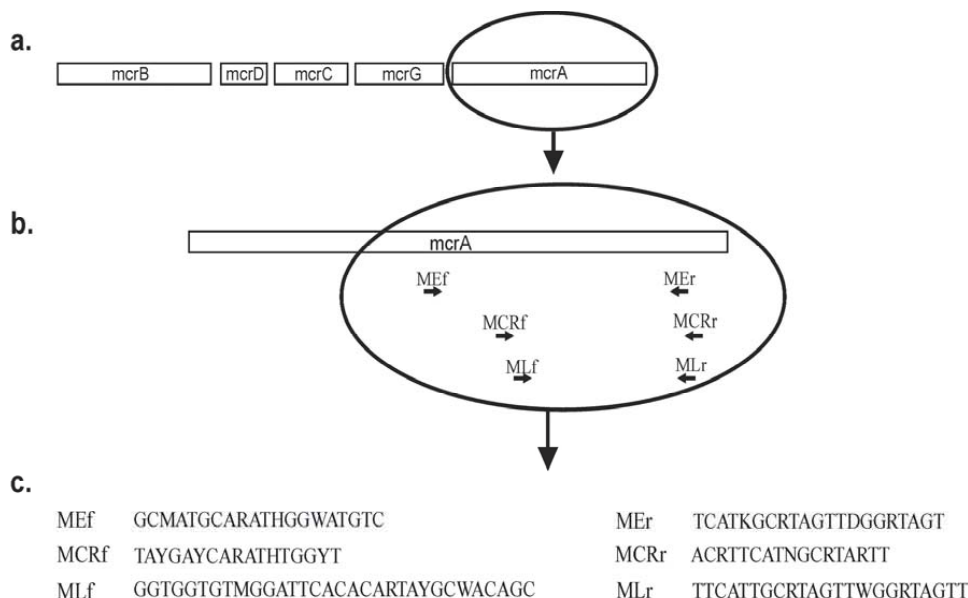

Figure 3. Diagram representing (a.) the five *mcr* open reading frames, *mcrBDCGA*; (b.) the positions of three different primer pairs (ME, MCR and ML) on the gene *mcr*A, and (c.) the primer sequences.

Mcr is thought to be a good molecular marker because it appears to have evolved from common ancestral gene. In addition, it is unique to, and ubiquitous in methanogens (Thauer, 1998). Its detection guaranties the presence of methanogens, which is not the case with the 16SrRNA marker. Finally, it has conserved sequence segments well suited for primer design (Weil et al., 1988; Weil et al., 1989; Springer et al., 1995; Nolling et al., 1996). On the other hand, phylogenetic analysis of *mcr* may remain imprecise due to the lower number of *mcr* sequences present in databanks. The A subunit of the *mcr* gene has been suggested as a useful genetic marker (Weil et al., 1988), and different primers targeting the *mcr*A gene have been designed (Table 2, Fig. 3).

Small subunit 16S ribosomal RNA (16SrRNA)

The comparative analysis of ribosomal RNA (rRNA) sequences, which allowed the definition of the three domains of life (Woese et al., 1990), stressed the importance of rRNA as a phylogenetic marker. At the same period, works which used molecular biology methods in combination with the new rRNA phylogeny revealed the importance of rRNA as tool for analysing natural microbial populations (Olsen et al., 1986; Pace et al., 1986). Among the three existing ribosomal RNA (5S, 16S/18S, and 23S/28S), the 16SrRNA became the most widely used marker (Head et al., 1998).

The 16SrRNA molecule is an excellent marker to infer phylogeny because it is found in all cellular life form. It comprises highly conserved regions interspersed with more variable ones. The variable regions allow the comparison of sequences, and some of the conserved portions can be recognized as signature sequences for the domains Archaea, Bacteria, or Eukarya (see Head et al., 1998). Conserved regions also allow the development of useful primers or probes (Giovannoni et al., 1988; Stahl and Amann, 1991), which enable the amplification or identification of sequences down to species level. Primers for the study of methanogen populations have been designed to amplify various regions of the 16SrRNA. Some primers target the domain *Archaea* generally, other are specific for methanogenic 16SrRNA (Table 2).

Table 2. PCR primers for study of methanogenic communities.

Targeted gene	Name	Primer sequences[a] (conserved amino acid region)	Fragment length (≈)	Reference
*mcr*A	MLf MLr	5’-GGTGGTGTMGGATTCACACARTAYGCWACAGC (GGVGFTQYATA) 5’-TTCATTGCRTAGTTWGGRTAGTT (NYPNYAMN)	470 bp	(Luton et al., 2002)
	MCRf MCRr	5’-TAYGAYCARATHTGGYT (YDQIWL) 5’-ACRTTCATNGCRTARTT (NYAMNV)	490 bp	(Springer et al., 1995)
		5’-GCNATGCARATHGGNATG (AMQIGM) 5’-GCNCCRCAYTGRTCYTG (QDQCGA)	700 bp	(Ohkuma et al., 1995)
	MEf MEr	5’-GCMATGCARATHGGWATGTC (AMQIGM) 5’-TCATKGCRTAGTTDGGRTAGT (YPNYAM)	760 bp	(Hales et al., 1996)
Archaeal 16SrRNA	A109f A934r	5’-ACKGCTCAGTAACACGT 5’-GTGCTCCCCCGCCAATTCCT	825 bp	(Stahl and Amann, 1991; Grosskopf et al., 1998a)
	A109f A912rt	5’-ACKGCTCAGTAACACGT 5’-GTGCTCCCCCGCCAATTCCTTTA	803 bp	(Grosskopf et al., 1998a; Lueders and Friedrich, 2002)
	1Af 1100Ar	5’-TCYGKTTGATCCYGSCRGAG 5’-TGGGTCTCGCTCGTTG	1100 bp	(Embley et al., 1992)
Methanogen 16SrRNA	146f 1324r	5’-GGSATAACCYCGGGAAAC 5’-GCGAGTTACAGCCCWCRA	1178 bp	(Marchesi et al., 2001)

[a] R (A or G), Y (C or T), M (A or C), K (G or T), W (A or T), H (A or C or T), N (G or A or T or C)

AIMS OF THE STUDY

Methanogens have been studied in a wide range of habitats, but the composition of communities in peatlands remains poorly known. Previous population studies were mainly restricted to bogs; methanogens had, to the best of our knowledge, never been described in fen ecosystems, even though these are the most active peatland ecosystems in methane production and release. The general aim of the study was to characterize the methanogenic *Archaea* communities in a natural boreal minerotrophic peatland (fen), and in an ombrotrophic peatland (bog) transformed for forestry purposes. The specific aims of this study were to:

- Describe methanogen communities in peatlands by culture independent molecular methods (I, II, and III).

- Determine possible effect of depth on methanogen communities, and their activity in an undisturbed fen (I).

- Investigate if spatial heterogeneity within a fen ecosystem, represented by different microsites, correlates with a change of methanogen populations (II).

- Estimate possible effects of forestry practices, such as drainage and ash fertilization, on methanogenic activity and populations at different depths of peat (III).

MATERIAL AND METHODS

Sites and sampling

Salmisuo site

Peat samples were collected in June 2001 from the natural Salmisuo mire complex in eastern Finland (62°47'N, 30°56'E). The site is a minerogenic, oligotrophic low-sedge *Sphagnum papillosum* pine fen. Four different types of microsites have been described at the Salmisuo fen: *Eriophorum* lawn, Flark, hummock, and *Carex* lawn. Samples were taken from two different microsites: *Eriophorum* lawns (I and II), and hummocks (II). The *Eriophorum* lawn is the most common microsite covering 86% of the mire surface. At this site the water level is mainly 5-20 cm below the peat surface, and *Sphagnum* mosses, *Eriophorum vaginatum* L., *Vaccinium oxycoccos* and *Andromeda polifolia* dominate the vegetation. Hummocks rise above their surroundings and their average water table is >20 cm below the peat surface. The most typical hummock species is *Sphagnum fuscum* (Scimp.) Klinggr. *S. angustifolium* (Russow) C. Jens., and *E. vaginatum* are found in the lower parts of the hummocks (for more details, (Saarnio et al., 1997; Saarnio et al., 2000)). Three parallel peat profiles, distant of approximately 20 cm from each other, were collected for each analysis with a box sampler (8×8×100 cm). Soil samples for DNA analyses, and potential methane production measurement were taken each 10 cm (±2 cm) of the core.

Muhos site

The experimental field was located in Muhos, Pelso, Resula (64°30'N, 26°18'E). The site is within the boreal coniferous zone classified as a Cotton grass pine bog. Ditching was done in the 1930's and continued in 1994 and 1997. The bog is in a transforming state where the typical bog vegetation is changing to forest vegetation as a result of drainage. Peat depth averages 100 cm. Pines (*Pinus sylvestris*) 1-7 m height and 20 – 60 year of age are growing in the bog. They are intermixed with slowly growing birches (*Betula pendula*). Three replicate plots (30 m x 30 m) were treated with 15,000 kg/ha of fly ash in June 1997. Another three replicate plots of the same size were kept unfertilized as control. One peat core was taken from each replicate plot with a box sampler (4x6.8x100 cm). In May 2002, after snow melting, peat samples for potential methane production measurements and for DNA extraction were taken from 10 cm (+-2 cm) depth intervals in the anoxic parts of the peat cores (III).

Methods

The experimental methods used in this study are described in detail in the original publications, and are summarized in Table 3.

Table 3. Methods used in this study.

Analysis	Method	Used and described in Study	Reference
Potential CH_4 production	Incubation experiment	I, II and III	(Kettunen et al., 1999)
pH measurement		II and III	(Galand et al., 2003)
DNA extraction from soil	Soil DNA Isolation Kit	I, II and III	Mo Bio Laboratories, Inc., Solana Beach, Calif., USA
PCR	16SrRNA primers	I, II and III	(Marchesi et al., 2001)
	MCR primers	II	(Hales et al., 1996)
RFLP	Restriction enzyme: *Msp* I	I and III	(Galand et al., 2002)
	Hinf I	II	(Galand et al., 2003)
DGGE fingerprinting	Denaturing gradient: 45 to 56%.	I	(Galand et al., 2002)
	45 to 57%.	II	(Galand et al., 2003)
	45 to 70%.	III	(Galand et al., 2004)
Cloning	pGEM-T vector / JM109 competent cells	I, II and III	Promega, Mannheim, Germany
Plasmid extraction	Wizard Plus Minipreps	I, II and III	Promega
Phylogenetic analysis	PROTDIST / FITCH	I and III	(Felsenstein, 1993)
	SEQBOOT / DNADIST / FITCH	II	
Statistical analysis	Shannon diversity index / coverage of clone libraries	I, II and III	(Shannon and Weaver, 1963)
	Evenness indices / Abundance models	II	(Fisher et al., 1943; Simpson, 1949)
	Richness	II and III	(Krebs, 1989)
	Distance matrix for RFLP and DGGE data	II and III	(Jaccard, 1901; Cavalli-Sforza and Edwards, 1967)

RESULTS AND DISCUSSION

The results, and the corresponding discussions of all three studies included in the thesis are presented in detail in each particular article. In this chapter, a synthesis of the results is presented, and selected parts are discussed.

Potential methane production

Measurement of potential CH_4 production in incubated peat samples always revealed highest CH_4 production in the upper waterlogged peat layers, and a decrease of rates with depth. At all sampled sites, the highest potential production was measured in samples taken between 10 and 20 cm below the water table. The minimum rate of CH_4 production was detected at the depth of 40 cm, with values close to, or like zero (I and II, Fig. 1; III, Table 1). This trend is typical for peatlands, and has been shown in various studies (Edwards et al., 1998; Kettunen et al., 1999; McDonald et al., 1999).

The highest rates of potential CH_4 production correspond to a depth, below the water table, where the oxygen concentration in the peat is low enough to enable methanogenic activity, and where there is sufficient amount of substrate. The highest concentrations of carbon sources are localised in the upper peat layers where most of the degradation of organic matter occurs, and where roots from aerenchymous plants are concentrated. The low potential methane production in the deeper layers of the peat can be explained by the lack of suitable substrate, or the presence of less favourable substrate for methanogenesis at those depths (Svensson and Sundh, 1992; Valentine et al., 1994).

At the depth of maximum CH_4 production, and when the total production for each site was compared, there were no significant differences in potential CH_4 production between hummock and *Eriophorum* lawn. In a previous study, *Eriophorum* lawn had higher emissions of CH_4 than hummock (Saarnio et al., 1997). Those results were, however, based on measured flux on site, and not on potential production measured in the laboratory.

At the drained Pelso-Resula bog, the peat samples taken at the beginning of the growing season in May showed low potential rates of methane production (III, Table 1). The low potential CH_4 production suggests a general lack of adequate substrate for methanogenesis. Drainage causes typical mire vegetation to change toward forest vegetation (Laine et al., 1995). Primary production increases fast aboveground, while the major rooting zone concentrates organic matter in the aerobic first 30 cm below the peat surface. Consequently, less carbon is allocated to anaerobic peat layers (Laiho and Laine, 1997; Laiho et al., 2003). Additionally, with lowered water table, recently produced organic matter is efficiently degraded in the increased aerobic layers, limiting potential substrate for methanogenesis to reach anaerobic layers of the peat (e. g. Svensson and Sundh, 1992). No significant difference in CH_4 production was observed between control and ash fertilized plots.

Fen cluster, a new group of uncultured methanogens

Phylogenetic analysis of portions of the *mcr*A gene, retrieved from the *Eriophorum* lawn of the Salmisuo fen, revealed a novel group of methanogenic sequences, which formed a cluster distinct from all known orders of methanogens (I, Fig. 4). The new cluster was named Fen

cluster, and was divided into four sub-clusters: Fen cluster-I to IV. Fen cluster sequences were clearly distinct from other published sequences. They had closest phylogenetic connection to sequences belonging to members of the order *Methanomicrobiales*, with maximum amino acids sequence similarity ranging from ≈85% (sequence Fen-D) to ≈87% (sequence Fen-K) to the closest described species, *Methanospirillum hungatei*. Members of the order *Methanomicrobiales* are hydrogenotroph methanogens, and *Methanospirillum hungatei* is a mesophilic methanogen utilising H_2/CO_2 and formate for methanogenesis (Garcia et al., 2000). The branching between representatives of the Fen cluster, and members of *Methanomicrobiales* is, however, deep, and any attempt to associate members of the Fen cluster with a particular methanogenic pathway will therefore remain speculative.

Several of the sequences retrieved from the drained Pelso-Resula bog belonged to the new Fen cluster. Those sequences were, however, rare in the clone libraries (III, Fig. 2 and 4), indicating that representatives of the Fen cluster were not the dominant methanogens in the bog ecosystem. Interestingly, members of the Fen cluster-II were detected only in the plots of the bog, which had been fertilized with ash. The presence of this group of sequences could represent a gradual change of the methanogen community induced by ash fertilization. Members of the Fen Cluster-II may have responded and adapted to new physico-chemical peat conditions, such as the observed increase of peat pH.

Phylogenetic analysis of the 16SrRNA gene, amplified from different sites and depths of the Salmisuo fen, also revealed a novel group of methanogens (II, Fig. 6). The novel sequences grouped with members of the order *Methanosarcinales* and *Methanomicrobiales* (similarity values ≈76% for both), but were not directly related to any known methanogens. The new phylogenetic cluster included sequences retrieved from various environments such as forested wetland, rice field, and oil contaminated soils (II, Fig. 6).

The *Eriophorum* lawn methanogen community was analysed with the two sets of primers, 16SrRNA, and functional *mcr*A, which both revealed a substantial presence of until now uncultured methanogens. In addition, the 16SrRNA and *mcr*A methanogenic phylogenetic tree both displayed a similar positioning of the novel sequences, related to *Methanosarcinales* and *Methanomicrobiales*. We can therefore assume that the novel 16SrRNA sequences retrieved from the fen also originated from members of the new Fen cluster.

Depth related variations of communities

mcrA analysis

In both fen and bog ecosystems, community analysis showed that methanogen populations changed with depth. Depth related variation of methanogen communities has never been shown before. A few works have described methanogen populations at different peat depths in bogs, but none of them have observed a shift of community with depth (McDonald et al., 1999; Nercessian et al., 1999; Upton et al., 2000).

In the Salmisuo fen, both RFLP and DGGE analysis revealed a clear difference in the occurrence of methanogenic *mcr*A gene between depths representing high and low methane production (10 and 40 cm below water table) (I, Fig. 2 and 3). The sequence diversity was lower in the upper layers than in the deeper ones, with values for Shannon's diversity index of 1,25 and 2,43 respectively. Additionally, the most common types of sequences amplified from

the upper peat layers were different from the ones retrieved from the deeper layers. Finally, three of the novel Fen sub-clusters grouped sequences according to the depth they were retrieved from. Fen cluster-I contained only sequences from the deeper layers, Fen cluster-II contained sequences retrieved from the upper layers of the fen, and the single sequence belonging to Fen cluster-IV was from the upper part of the fen (I, Fig. 4).

Sequences characterising the upper layers of the *Eriophorum* lawn all grouped within the new Fen cluster (I, Fig. 3). The function of Fen cluster members remains unknown, and we can only speculate that the cluster's phylogenetic relation to the order *Methanomicrobiales* indicates that the group includes hydrogenotroph methanogens. Further studies, focusing on the pathway used by those newly discovered fen methanogens, are needed in order to determine the nutritional status of those organisms.

The main *mcr*A sequences from the deeper parts of the *Eriophorum* lawn were closely related to Rice cluster-I (I, Fig. 4). Members of the Rice cluster-I have been earlier selectively enriched with H_2/CO_2 as energy source (Lueders et al., 2001; Sizova et al., 2003), indicating that the group includes hydrogenotrophs. Methanogens from the deeper layers of the fen could therefore belong to a family forming methane by oxidizing H_2 and reducing CO_2.

In the Pelso-Resula drained bog, the upper layers methanogen communities also differed from the deeper layers ones (III, Fig. 2). The major *mcr*A sequences from both depths were, however, all similar to sequences retrieved from the deep layers of the natural Salmisuo fen, and grouped consequently within a same cluster related to the H_2/CO_2 utilizing Rice cluster-I (III, Fig. 4). Hydrogenotroph methanogens may therefore be well represented within communities from all layers of the bog. The result would be in accordance with previous findings, which indicated that hydrogenotrophy is the dominant pathway in bogs (Chasar et al., 2000; Horn et al., 2003). One should, however, be careful in making any definitive conclusions, since ME primers have previously failed to amplify obligatory acetotrophic methanogens (family *Methanosaetaceae*) (Lueders et al., 2001). Further phylogenetic analysis with additional sets of primers will allow a more precise description of the methanogen community in the bog.

16SrRNA analysis

16SrRNA analysis of the upper *Eriophorum* lawn layers revealed some sequences related to until now uncultured methanogens, other which belonged to the order *Methanomicrobiales*, and a high proportion of sequences which grouped with *Methanosarcinales*, genus *Methanosarcina* (II, Fig. 6). *Methanosarcina* represents the acetotrophic methanogens (Garcia et al., 2000), and acetoclastic methanogenesis may consequently be the reaction dominating the fen's upper *Eriophorum* lawn layers. The lawn's vegetation is dominated by *E. vaginatum*, a vascular plant with roots penetrating the anoxic layers of the peat. The aerenchyma of this vascular plant is known to influence CH_4 production in peatlands. The roots exudate labile carbon substrates, such as acetate, which can promote growth of acetotrophic methanogens (Strom et al., 2003). Our results confirm findings from previous works indicating that acetoclastic methanogenesis is the main pathway in upper layers of fen covered with aerenchymous vascular plants (Kelley et al., 1992; Popp and Chanton, 1999; Chasar et al., 2000). ME primers used in Study I probably failed to amplify sequences belonging to *Methanosarcinales*, since *mcr*A analysis of upper *Eriophorum* lawn layers revealed sequences belonging exclusively to the novel Fen cluster.

The deeper layers of the lawn were dominated by 16SrRNA sequences grouping with uncultured methanogens in a novel cluster (II, Fig. 2 and 6). According to its phylogenetic position, this cluster may be similar to the *mcr*A Fen cluster, which members have unknown pathway for methanogenesis. Surprisingly, 16SrRNA primers did not detect sequences related to Rice cluster-I, even though the *mcr*A analysis revealed deeper lawn layer communities dominated by members of Rice cluster-I.

Site related variations of communities

We investigated a possible correlation between the methanogen communities, and the microsites representing the spatial heterogeneity of the Salmisuo fen. 16SrRNA analysis revealed a site related change of methanogen populations between the hummock and *Eriophorum* lawn. Interestingly, the community shift was restricted to the upper peat layers. The observed variation is certainly correlated to the vegetation cover, and level of water table characterising the sites, which again influence nutrient availability in the peat surface. Remarkably, the community structure was very similar between replicate samples within the same site, indicating that the composition of methanogen communities was homogenous within the fen microsites.

Phylogenetic 16SrRNA analyses indicated that sequences dominating the upper layers of hummock clustered with members of *Methanomicrobiales*, known to include hydrogen utilising methanogens (II, Fig. 2 and 6). The hummocks near surface community could therefore be composed of microorganisms oxidising H_2 and reducing CO_2 to form CH_4. Typical hummock plant communities such as dwarf shrubs are adapted to dry conditions: they don't posses aerenchyma (Thomas et al., 1996), and their root systems concentrate in the uppermost aerobic part of the peat (Wallén, 1992). The transport effect of carbon compounds to the waterlogged peat layers is therefore limited, and substrate for methanogenesis originates mainly from the anoxic decomposition of litter. The amount of litter entering the anaerobic layers is, however, restricted because the low water table and large aerobic layer defining hummocks promote oxic decomposition of organic matter (Svensson and Sundh, 1992).

Sequences retrieved from the upper parts of the *Eriophorum* lawn were, on the other hand, related to members of the order *Methanosarcinales*, genus *Methanosarcina*, known to regroup acetotrophic methanogens (Garcia et al., 2000). As described above, in the *Eriophorum* lawn, the nutrient availability is influenced by the presence of vascular aerenchymous plants, like *E. vaginatum*, with roots providing easily degradable carbon to the anaerobic parts of the fen.

In the deeper parts of the fen, no differences in methanogen communities were observed between microsites. It illustrates that the physico-chemical conditions, at those depths of the peat, are stabile horizontally. The influence of the covering vegetation and water table level is low; the nutrient types and amount are probably similar across sites resulting in the presence of similar communities. Phylogenetic analyses of methanogenic 16SrRNA revealed deeper layers community composed of novel methanogens, certainly belonging to Fen cluster. *Mcr*A phylogeny showed that the majority of deep layers sequences grouped with representatives of Rice cluster-I, known to be hydrogenotrophs. We can therefore expect H_2-dependent CH_4 formation to be the main reaction in the deeper layers of the Salmisuo fen. This result would confirm findings from earlier works, presenting hydrogenotrophy as dominant pathway in

deeper fen layers, where more recalcitrant old peat is present (Kelley et al., 1992; Popp and Chanton, 1999; Chasar et al., 2000).

Primer efficiency

The study was conducted with two different sets of primers: ME (Hales et al., 1996) targeting the *mcr*A gene, and 146f-1324r (Marchesi et al., 2001) amplifying methanogenic 16SrRNA.

In comparison with other primers targeting the *mcr*A gene, ME amplify a relatively long portion of the gene (760 bp, Table 2), and therefore reveal more genetic information for phylogenetic analysis. It was suggested, however, that ME primers failed to amplify sequences belonging to members of the family *Methanosaetaceae* (Lueders et al., 2001). *Mcr*A analysis of upper *Eriophorum* lawn layers revealed sequences belonging exclusively to the novel Fen cluster, while 16SrRNA analysis additionally showed sequences grouping with the order *Methanosarcinales*, genus *Methanosarcina*. It indicates that, in study I, ME primers did not amplify representatives from *Methanosarcina*. This is intriguing since members of *Methanosarcinaceae* were earlier successfully recovered from rice field soil (Lueders et al., 2001). In the drained bog, however, ME amplified one sequence grouping with *Methanosarcina*, but few Fen cluster sequences were retrieved. The lack of amplification of *Methanosarcina* representatives in the fen may therefore be due to PCR bias, amplifying the Fen cluster preferentially.

Methanogen specific 16SrRNA primers amplified representatives from the family *Methanosarcinaceae* but failed to amplify sequences related to Rice cluster-I. The presence of Rice cluster-I associated sequences in the fen was confirmed by ME primer analysis. Even though 146f-1324r 16SrRNA primer pairs were shown to amplify representatives from all five known orders of methanogens (Marchesi et al., 2001), this study indicates that they fail to amplify sequences from members of the Rice cluster-I.

Both functional and ribosomal primer sets used in this study failed to amplify some groups of methanogenic sequences. It confirms that the use of “universal” primers introduces bias in PCR based analysis (Wintzingerode et al., 1997), and stresses the need to use multiple sets of primers to uncover the total diversity of a community. Failure to detect 16SrRNA from members of the important Rice cluster-I indicates that methanogenic 146f-1324r primers may not alone be well suited for diversity studies. *Archaea* specific primers (e. g. Table 2) may be more adequate for analysis of methanogen communities.

SUMMARY AND CONCLUDING REMARKS

This thesis revealed the structure, composition, and activity of methanogen communities in some boreal peatlands, and presents results, which can be used as starting points for further research on methanogenic pathways in those habitats.

We showed that in different fen microsites, and in a bog, the methanogen communities changed with depths of peat. At both sites, and at all depths, we retrieved sequences from until now uncultured methanogens, forming a new phylogenetic cluster. The new cluster was named the Fen cluster. Its members had closest relation to the order *Methanomicrobiales*, but their function remains unknown.

The structure of the methanogen populations was homogenous within each microsite but varied significantly between sites. The upper layers of the *Eriophorum* lawn was characterised by some sequences grouping within the new Fen cluster, and others clustering with members of the family *Methanosarcinales*. The presence of a high number of sequences related to *Methanosarcinales* may indicate that acetotrophy is the dominating pathway in upper *Eriophorum* layers, where roots from aerenchymous plants provide the anoxic peat layers with labile carbon substrate.

Upper peat layers of hummock were dominated by sequences related to members of the order *Methanomicrobiales*. *Methanomicrobiales* is an order grouping hydrogenotrophic methanogens, indicating that in hummock upper layers the main reaction may be H_2-dependent CH_4 production. The presence of plants adapted to dry conditions, and the low water table of hummocks influence the quality of substrate entering anaerobic layers, and determine consequently the methanogenic pathway characterising the site.

Methanogen communities in the deeper layers of the fen were homogenous through the different microsites, and were represented by a majority of sequences grouping within the new Fen cluster. Even though the function of Fen cluster methanogens remains unknown, we may speculate that they include hydrogenotrophs, since H_2/CO_2 has been pointed out as the main substrate for methanogenesis in deeper fen layers.

The main pathway for methanogenesis in the drained bog is probably hydrogenotrophy; the majority of the sequences amplified from the bog belonged to Rice cluster-I, representing H_2 utilising methanogens. Even though ash fertilisation increased the peat pH, it had no significant effect on the composition or activity of the major methanogen communities. However, some sequences belonging to the Fen cluster were retrieved from ash treated sites only, and may represent an adaptation of certain group of methanogens to new physico-chemical conditions in the bog. Further works involving radiotracer, or stable isotope experiments would help to uncover the pathways used by members of the novel fen cluster.

ACKNOWLEDGMENTS

This work was carried out at the Department of Biological and Environmental Sciences, General Microbiology, University of Helsinki. This study was financed by the Academy of Finland.

I am very grateful to my supervisor Docent Kim Yrjälä for accepting me as Ph.D. student, and for always taking the time to discuss the development of my research work. My deepest gratitude goes to my co-authors and collaborators, especially to Docent Hannu Fritze for his guidance, and to Heli Juottonen for her support in the lab. I warmly thank Professor Pertti Martikainen and Michael Schloter for reviewing my thesis.

My deep appreciation goes to colleague at YMBO for providing a pleasant working environment, to the staff for their technical support, and in particular to Anita for good administrative help.

You my friends, thank you for being my friends.

Maria and Selma thank you.

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