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Henri M.P. Siljanen

Activity and Diversity of Methanotrophs in a Littoral Wetland of an Eutrophic Boreal Freshwater Lake

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ABSTRACT

Most of methane (CH_4) emitted from lakes may be derived from their littoral wetlands. In the surface layers of wetlands, aerobic CH_4 oxidizing bacteria, known as methanotrophs, consume a large part of the CH_4 produced in deeper anoxic layers, having thus major atmospheric importance. However, the characteristics of the methanotroph community and its capacity to withstand environmental changes in boreal littoral wetlands are poorly understood.

The aim of the present work which was a part of the European Science Foundation project METHECO, was to study activity and structure of a methanotroph community, and its sensitivity against environmental stresses in a littoral wetland of an eutrophicated lake in Eastern Finland. The function and community composition of methanotrophs were studied with CH_4 oxidation assays and a mono-oxygenase gene (*pmoA*) specific microarray, quantitative PCR and clone libraries. Spatial and seasonal variation of methanotrophs as well as sensitivity and recovery of the community against *in situ* nitrogen loading were examined. In addition, the reproducibility of methanotroph detecting tools in ring analysis for shared joint samples in five European laboratories was investigated.

The hydrological conditions in the wetland affected the community composition and activity of methanotrophs. Wet conditions supported growth and activity of type I methanotrophs, whereas in dry conditions, type II methanotrophs were dominant and the overall CH_4 oxidation capacity of the methanotroph community was reduced. There was only minor seasonal variation in the activity, in contrast to the diversity of methanotrophs. A higher water table, typical feature in spring, caused succession of type Ib methanotrophs in the dry area which did not harbour these methanotrophs during seasons with a lower water table. In addition, a low temperature supported growth and activity of type II methanotrophs. Ammonium nitrate loading did not affect the overall CH_4 oxidation or CH_4 fluxes in the littoral wetland but increased *pmoA* transcription of type I methanotrophs and decreased the relative abundance of type II methanotrophs. Ring analysis suggested that DNA extraction is a sensitive step in the molecular detection of methanotrophs and results between laboratories could be better compared by determining the ratio of type I to type II methanotroph abundance than simply describing the numbers of methanotrophs.

The results of the present work demonstrate that hydrology is a key factor for diversity and activity of methanotrophs in littoral wetlands. One could predict that climate change will likely alter hydrological conditions in littoral wetlands and thereby activity and diversity of methanotrophs and also CH_4 emissions. Methanotroph species inhabiting wetlands possess different strategies against inorganic nitrogen. Finally, this diverse methanotroph community is well suited to tolerate the nitrogen load leached from the catchment.

Universal Decimal Classification: 502.51, 547.211, 574.5, 579.266, 579.68

CAB Thesaurus: water microbiology; microbial activities; microbial ecology; aquatic environment; wetlands; freshwater lakes; eutrophication; methane; oxidation; Bacteria; spatial variation; seasonal variation; nitrogen; ammonium nitrate; hydrology; water table; laboratories; comparisons; variation

Yleinen suomalainen asiasanasto: ympäristömikrobiologia; mikrobiekologia; järvet; kosteikot; rannat; ranta-alueet; rehevöityminen; metaani; hapettuminen; hapetus; bakteerit; alueelliset erot; vuodenajat; vaihtelu; typpi; ammoniumnitraatti; hydrologia; laboratoriot; vertailu

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Kuopio 30th January 2012.

Henri Siljanen

LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal RNA gene
cDNA	Complementary DNA
CH_4	Methane
$CH_3\cdot$	Methyl radical
DNA	Deoxyribonucleic acid
MMO	Methane mono-oxygenase enzyme
OH	Hydroxyl
OH·	Hydroxyl radical
PCR	Polymerase chain reaction
pMMO	Particulate MMO
pmoA	Gene encoding 27 kDa fragment of methane mono-oxygenase enzyme
SIP	Stable isotope probing
sMMO	Soluble MMO
qPCR	Quantitative PCR
RNA	Ribonucleic acid

LIST OF ORIGINAL ARTICLES

Chapter 2. Henri MP Siljanen, Anne Saari, Sascha Krause, Anssi Lensu, Guy CJ Abell, Levente Bodrossy, Paul LE Bodelier, Pertti J Martikainen. 2011. Hydrology is reflected in the functioning and community composition of methanotrophs in the littoral wetland of a boreal lake. *FEMS Microbiol Ecol* **75:** 430-445.

Chapter 3. Henri MP Siljanen, Anne Saari, Levente Bodrossy, Pertti J Martikainen. 2011. Seasonal variation in the function and diversity of methanotrophs in the littoral wetland of a boreal eutrophic lake. *FEMS Microbiol Ecol,* in press.

Chapter 4. Henri MP Siljanen, Anne Saari, Levente Bodrossy, Pertti J Martikainen. 2011. The effect of nitrogen load on the functioning and diversity methanotrophs in the littoral wetland of a boreal lake. *Front Microbiol* **3:** 39 doi: 10.3389/fmicb.2012.00039.

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AUTHOR'S CONTRIBUTION

Chapter 2. The author, Anne Saari and Pertti Martikainen planned the experiments. The author and Anne Saari performed the field and laboratory work. The author contributed to planning and performing the statistical and phylogenetic analyses, and wrote the first draft of the manuscript. All co-authors contributed to the subsequent writing process.

Chapter 3. The author, Anne Saari and Pertti Martikainen planned the experiments. The author and Anne Saari were responsible for the field and laboratory work. The author performed the statistical analyses and wrote the first draft of the manuscript. The following modification of the manuscript was done by all co-authors.

Chapter 4. The author, Anne Saari and Pertti Martikainen planned the experiments. The author and Anne Saari performed the field and laboratory work. The author performed the statistical analyses and wrote the first draft of the manuscript. All co-authors contributed to the subsequent writing process.

Chapter 5. The author contributed to the planning of the experiments, participated in the laboratory work and contributed to planning and performing the statistical analyses. The author contributed writing of the manuscript.

Contents

Chapter 1: General introduction

1.1 ATMOSPHERIC CH_4

Methane (CH_4) is second most abundant greenhouse gas in the atmosphere after carbon dioxide. Methane is a 25 times more efficient as a greenhouse gas than carbon dioxide with a time horizon of 100 years (Global Warming Potential, Forster et al. 2007) and it accounts for about 20% of the radiative forcing (warming effect) of the atmosphere (Forster et al. 2007).

Pre-industrial atmospheric CH_4 (600-700ppb) is assumed to have originated largely from the expanding peatlands 5000 years ago and onwards (Korhola et al. 2010). Due to the increased anthropogenic CH_4 emissions (e.g. rice fields, landfills, eutrophication of lakes) and decreased consumption of CH_4 in soil resulting from land-use changes (Denman et al. 2007), the concentration of CH_4 in the atmosphere has more than doubled since the preindustrial period and is currently 1774 ppb (Forster et al. 2007). The methane concentration in the atmosphere remained almost constant during the period 1998-2006 (Dlugokencky et al. 2003, Monteil et al. 2011) but began to increase again in 2007 (Dlugokencky et al. 2009, Frankenberg et al. 2011). Two explanations have been proposed for the period when there was no increase in the atmospheric CH_4 concentration i.e. either a decrease in natural wetland emissions or an increase in the anthropogenic NO_x emissions which would facilitate OH radical driven CH_4 oxidation in the troposphere and stratosphere (Karlsdóttir and Isaksen 2000, Monteil et al. 2011).

1.2 PRODUCTION AND OXIDATION OF CH_4

The total global CH_4 emission is estimated to be 500-600 Tg $year^{-1}$ (Table 1)(Denman et al. 2007, Conrad 2009). Methane originates from biomass burning, fossil fuel production/usage, as well as from CH_4 production by methanogenic archaea when organic matter becomes decomposed under anaerobic conditions in ruminants, termites, wetlands, rice fields, landfills, oceans and sewage treatment. Wetlands are the most important natural CH_4 source contributing a quarter of the total natural CH_4 emissions (Table 1). Methane has been reported to be emitted also from chemical reactions in plants, i.e. pectin is a potential source of CH_4 (Bruhn et al.

2009, Wishkermann et al. 2011). In some studies, chemical reactions in plants have been estimated to contribute 4-6% to the total CH_4 emissions (Keppler et al. 2006, Conrad 2009, Mukhin and Voronin 2011). However, other reports do not support the proposal that plants would be significant direct sources of CH_4 (Nisbet et al. 2009, Rice et al. 2010).

Methane is oxidized chemically in the atmosphere whereas in soils, sediments and waters the oxidation reactions are performed by microorganisms (Lelieveld et al. 1998). Oxidation of CH_4 in the troposphere is mediated by OH radicals: $CH_4 + OH\cdot \rightarrow CH_3\cdot + H_2O$ (see examples Crutzen and Zimmermann 1991). Some CH_4 is removed in the stratosphere also by charged oxygen which is produced by photodissociation reactions with chlorine and ozone (Crutzen 1991).

Table 1. *Global sources of CH_4 to the atmosphere (% of the total emissions of 500-600 Tg per year, Conrad 2009).*

Source	**% of the total emissions**	
	Natural	Anthropogenic
Wetlands	23	
Plants	6	
Termites	3	
Ocean	3	
Gas hydrates	2	
Rice fields		10
Ruminants		17
Landfills		7
Sewage threatment		4
Biomass burning		7
Fossil fuel		18

Whether ecosystems act as a sink or a source of CH_4 is determined by the balance between CH_4 production by strictly anaerobic methanogenic archaea and CH_4 oxidation by aerobic and anaerobic microorganisms (Hanson and Hanson 1996, Hinrichs et al. 1999, Boetius et al. 2000, Raghoebarsing et al. 2006, Hu et al. 2009, Ettwig et al. 2010). In soil, aerobic methane oxidizing bacteria, i.e. methanotrophs, play a unique role in the carbon cycle because they are the only organisms using CH_4 as both an energy and a carbon source. In upland soils, methanotrophs consume about 30 Tg of CH_4 per year (Denman et al. 2007). The methane emission from wetlands is about 100 Tg per year (Table 1). Methane oxidation greatly decreases CH_4 emissions from wetlands because methanotrophs in the aerobic surface layers of wetlands can consume more than 90% of the CH_4 produced in the deeper anoxic layers (Oremland and Culbertson 1992) thus implementing an important ecosystem service.

1.3 DEVELOPMENT OF METHODS IN MICROBIAL ECOLOGY

Methanotrophs were first described in 1906 (Söhngen 1906). Attempts to isolate methanotrophs from environmental samples began over 60 years later (Whittenbury et al. 1970). The cultivation of methanotrophs is time-consuming, and the isolation of high affinity methanotrophs living in environments with low CH_4 concentration has not yet been successful. Therefore, molecular biological methods that rely on extraction of microbial nucleic acids (i.e. DNA/RNA) from environmental samples, are now the key tools to study the occurrence and diversity of methanotrophs.

The development of Sanger's chain-termination sequencing (Sanger et al. 1977) and polymerase chain reaction (PCR) (Mullis et al. 1986) have facilitated the progress of molecular studies in microbiology. The identification of bacteria and archaea is possible with clone libraries of 16S rRNA gene (Olsen et al. 1986) and functional gene markers. The number of published 16S rRNA gene sequences has approximately doubled every year since the early 1990s (Pace 2009). Currently there are over 3.1 million entries of 16S rRNA gene fragments in the NCBI (National Center for Biotechnology Information) Genbank. The overall development of microbiology and microbial ecology of methanotrophs can be estimated from the public citation database by examining at how many times "16S" and the word "methanotroph*" are found (Figs. 1a,b).

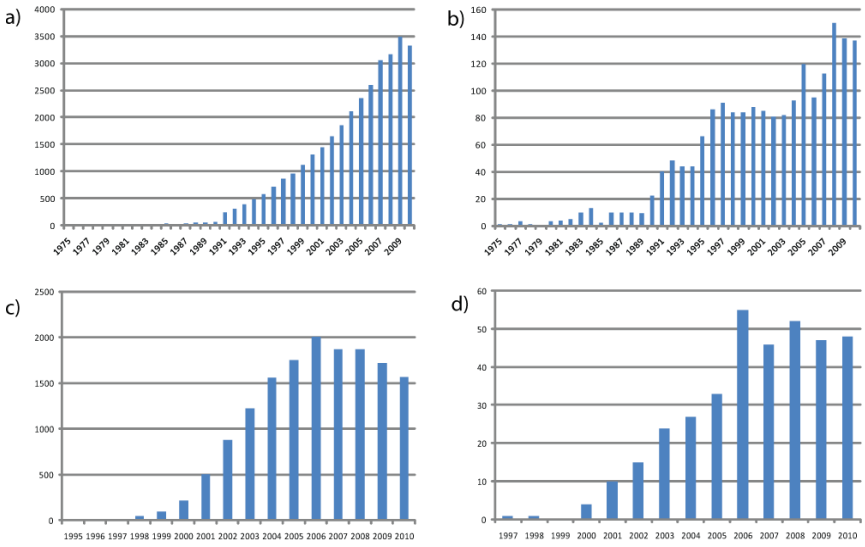

Fig. 1. *Hits from the ISI Web of Knowledge -database to describe the development of molecular microbial ecology. Shown are the numbers of "hits" from the database plotted against the year. For the development of microbiology in general, the keyword "**16S**" was used (a), for the development of microbial ecology of methanotrophs the keyword "**methanotroph***" was used (b), for the development of the microarray methods in general, the keywords "**microarray** AND **DNA**" were applied (c) and for the development of microarrays to detect microbes the keywords "**microarray** AND **DNA** AND **microbial**" were used (d).*

The development of DNA microarray technologies has helped to study the expression of many genes in parallel (Schena et al. 1995). DNA microarrays are basically glass slides which have the ability to recognize known genes in samples subjected for assay. The genetic characteristics of subject DNA/cDNA can be recognized after its hybridization on probes of microarray slide. The subject DNA hybridized is detected on slide by labelling techniques. Recently some other technologies (e.g. next generation sequencing, pyrosequencing) have started to replace microarrays in molecular biology (Fig. 1c) (Margulies et al. 2005, Bartram et al. 2011, Lemos et al. 2011). Microbial diagnostic microarrays were introduced soon after the first microarray study (Guschin et al. 1997). Since microbial diagnostic microarrays offer both rapid and inexpensive detection of various organisms within complex communities, it can be assumed that they will be applied in microbiology also in the future (Fig. 1d). Microbial communities can be profiled with methods such as Terminal restriction fragment length polymorphism (T-RFLP) and Denaturing gradient gel electrophoresis (DGGE). However, compared to these methods, methanotroph specific microarrays offer a tool to detect methanotrophs semi-quantitatively with better single species level detection ability (Fig. 2)(Bodrossy et al. 2003, Stralis-Pavese et al. 2011).

Since methanotrophs use CH_4 as their carbon source, stable isotope probing (SIP) is a technology which allows detection of active methanotrophs when they incorporate ^{13}C - derived from $^{13}CH_4$ into their DNA within the cell (Radajewski et al. 2000). The DNA-SIP approach and analysis of active methanotrophs through mRNA have both shown the active methanotroph community in soils with naturally high CH_4 concentrations (Kumaresan et al. 2011). In SIP, the concentration of CH_4 (e.g. 1%) has to be sufficiently high to attain enough label in DNA/RNA. Therefore, one limitation of DNA-SIP is that, it cannot be utilized at low CH_4 concentrations (close to the atmospheric level) which are the prevalent in well aerated soils (Bengtson et al. 2009).

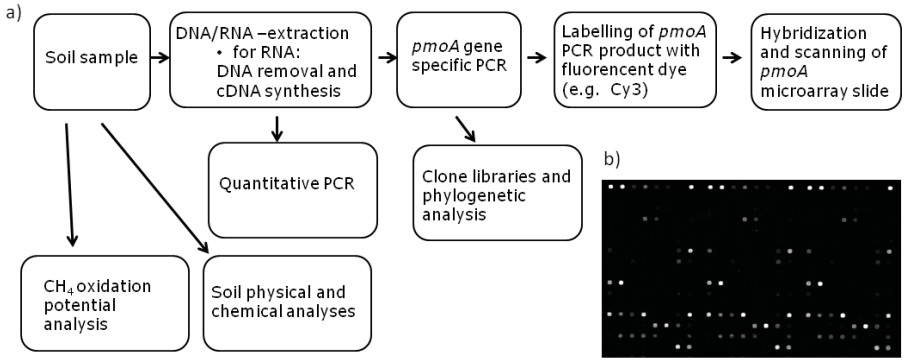

Fig. 2. *Overview of the workflow in microbial ecological studies of methanotrophs when CH_4 oxidation potential analysis, soil physical and chemical analyses, quantitative PCR, clone libraries and pmoA microarray technique is performed (a). Example of scanned tif-image of pmoA microarray (b).*

1.4 CLASSIFICATION OF METHANOTROPHS

Methane oxidizing microbes occupy both aerobic and anaerobic environments. In marine sediments, anaerobic CH_4 oxidizing archaea (ANME) oxidize CH_4 associated to sulfate reduction (Hinrichs et al. 1999, Boetius et al. 2000). Methane oxidation by sulfate reduction (ANME) has not been found to take place in soils or freshwater lakes. However, in anaerobic freshwater environments, CH_4 oxidation can be carried out by *Candidatus Methylomirabilis oxyfera* bacterium when nitrite is present. This bacterium first releases oxygen from nitrite in its cell and then uses this oxygen for CH_4 oxidation (Ettwig et al. 2010, Deutzmann and Schink 2011). It is not known if this mechanism occurs also in soil.

Methane oxidizing aerobic bacteria, or methanotrophs, can be taxonomically divided into three phylum, *Alpha-* and *Gammaproteobacteria* and *Verrucomicrobia*, based on their intracellular membrane structure, carbon assimilation pathways, PLFA patterns and phylogeny of molecular markers (Table 2)(Semrau et al. 2010). Despite the high diversity of methanotrophs and their flexibility in being able to occupy various environmental niches, they share similarities in the pathway to oxidize CH_4 to methanol by the methane mono-oxygenase enzymes (MMO). There are two different forms of oxygenases for CH_4 in methanotrophs: cytoplasmic membrane-bound particulate methane mono-oxygenase (pMMO) and soluble methane mono-oxygenase (sMMO) located in the cytoplasm.

Particulate MMO is widespread in almost all methanotrophs, including the anaerobic Candidatus *M. oxyfera*, but not *Methyloferula* and facultative *Methylocella* species (Dedysh et al. 2005, Ettwig et al. 2010, Vorobev et al. 2011). The polymorphic gene of the 27 kDa fragment of pMMO (*pmoA*) detects a broad spectrum of methanotrophs and its variation is similar to that of the 16S rRNA gene marker. Therefore, it represents a good functional marker to detect methanotrophs (McDonald et al. 2008). In contrast, sMMO has been found in *Methylocella* and recently isolated *Methyloferula*, but not from the majority of other methanotrophs. *Methylocella* species have been isolated from acidic ombrotrophic Sphagnum peat bogs and acidic forest cambisols (Dedysh et al. 2000, Dunfield et al. 2003). However, *Methylocella* has not been found in acidic forest soils in general (Kolb et al. 2005). Recently, *Methylocella* phylotypes have also been detected in alkaline conditions, but their functions and role in the CH_4 cycle in these kinds of ecosystems are unknown (Rahman et al. 2010).

Proteobacteria methanotrophs have been studied for decades, but *Verrucomicrobia* methanotrophs have been found only recently (Dunfield et al. 2007). *Verrucomicrobia*

methanotrophs have been detected from geothermal areas having extremely acidic conditions (Op den Camp et al. 2009). Aerobic *Proteobacteria* methanotrophs are grouped into three families in two phylum: *Methylocystaceae* and *Beijerinckaceae* in the *Alphaproteobacteria* phylum, and *Methylococcaceae* in the *Gammaproteobacteria* phylum. There are 17 methanotroph genera within these families. The *Methylocystacaea* family has two genera, i.e. *Methylosinus* and *Methylocystis,* whereas the *Beijerinckaceae* family consists of *Methylocapsa*, *Methyloferula* and *Methylocella* genera. The *Methylococcaceae* family consists of *Methylobacter*, *Methylococcus*, *Methylocaldum*, *Methylohalobius*, *Methylomicrobium*, *Methylomonas*, *Methylosoma*, *Methylosarcina*, *Methylosphaera*, *Methylothermus*, *Crenothrix* and *Clonothrix* genera (Semrau et al. 2010, Vorobev et al. 2011). The *Methylococcacaea* family is often referred to as type I and *Methylocystaceae* as type II methanotrophs. Type I methanotrophs can be subdivided into type Ia (*Methylobacter*, *Methylomonas* and related species) and type Ib (called before also type X) (*Methylococcus*, *Methylocaldum* and related species) subgroups (Bodrossy et al. 2003).

Methanotrophs possess a great phylogenetic variability. Since newly found genera and species (i.e. *Methyloacidphilum* and Cand. *Methylomirabilis*) seem to be moderately distantly related to the previously identified species, it can be assumed that differentiation of these species occurred a long time ago and furthermore that new species will be found (Semrau et al. 2010). There are recent findings suggesting that facultative methanotrophy is not limited only to species of *Beijerinckaceae* family (Dedysh et al. 2005, Dunfield et al. 2010), but it can also occurs in specific lineages of *Alphaproteobacteria* methanotrophs (Belova et al. 2010, Im et al. 2010, Pratscher et al. 2011).

Table 2. *General characteristics of methanotrophic families.*

Characteristic					
Phylum	*Gammaproteobacteria*	*Alphaproteobacteria*	*Alphaproteobacteria*	*Verrucomicrobia*	New methanotrophic species
Family	*Methylococcaceae*	*Methylocystaceae*	*Beijerinckceae*	*Methyloaciphilceae*	ND
Genera (candidatum species)	*Methylobacter, Methylococcus, Methylocaldum, Methylohalobius, Methylomonas, Methylosoma, Methylosarcina, Methylosphaera, Methylothermus, Crenothrix, Clonothrix*	*Methylosinus, Methylocystis*	*Methylocapsa, Methylocella, Cand. Methyloferula*	*Methyloaciphilum*	Cand. *Methylomirabilis oxyfera*
RuMP pahway	+	-	-	-	ND
Serine pathway	-	+	+	+	ND
sMMO	Varies between species	Varies between species	Varies between species	-	-
pMMO	+	+	Varies between species	+	+
Nitrogen fixation	Varies between species	Varies between species	+	Varies between species	ND
Intracytoplasmic membrane (ICM) formation	Bundles of disks perpendicular to cell periphery	Membrane stacks parallel to cell periphery	*Methylocapsa* - membrane vesicles parallel to long axis on one side of cell membrane. *Methylocella* - cytoplasmic membrane invaginations. *Methyloferula* lacks ICM.	-	ND
Facultative	-	Varies between species	Varies between species	-	ND
Anaerobic CH_4 oxidation	-	-	-	-	+

References: Ettwig et al. 2010, Semrau et al. 2010, Belova et al. 2011, Vorobev et al. 2011. ND, not determined. +, present; - absent.

1.5. FACTORS CONTROLLING THE ACTIVITY AND DIVERSITY OF METHANOTROPHS

1.5.1. Availability of CH_4

Methane oxidation is distinguished by low affinity (high CH_4 concentration environments) and high affinity (low CH_4 concentration environments, i.e atmospheric concentration or less) methanotrophic activity. Affinity types can be defined with K_m-values. Temperate and boreal forest soils harbor high-affinity methanotrophs (K_m 5-92 ppmv) (Bender and Conrad 1992, Whalen and Reeburgh 1996, Saari et al. 2004) whereas sediments and peat soils contain low-affinity methanotrophs (K_m 7900-43000 ppmv) (Lidstrom and Somers 1984, Watson et al. 1997). The substrate availability in soil affects both the activity and the diversity of the methanotrophs. In forest soils, where the CH_4 concentration is low, so called upland-soil-clusters dominate and oxidize atmospheric CH_4, i.e. they are able to take up atmospheric CH_4 (Kolb 2009, Degelmann et al. 2010). In the CH_4 emitting environments (wetlands/peatlands, river/lake sediments, rice fields, landfills) with a high CH_4 concentration, the methanotroph community is generally dominated by type II species (Henckel et al. 2000, Gebert et al. 2008, Kumaresan et al. 2009, Steenbergh et al. 2010). These environments also support fast-growing type I methanotrophs which react rapidly to any increase in the CH_4 availability. Type I methanotrophs tolerate fluctuations in environmental conditions by implementing an r-type life strategy, whereas type II methanotrophs live in more stagnant conditions maintaining a K-type life strategy (Henckel et al. 2000, Steenbergh et al. 2010).

1.5.2 Soil hydrology

The methanotrophic activity tends to reguire specific optimal moisture conditions (Semrau et al. 2010). An increase in the soil water content can either stimulate (Mosaavi & Crill 1997; West & Schmidt 1998; Kettunen et al. 1999) or inhibit the functioning of methanotrophs (Whalen & Reeburgh 1990; Bender & Conrad 1995). In upland soils, a high water content in the soil can inhibit methanotrophs by limiting diffusion of CH_4 and O_2 into the soil. On the other hand, increased water availability can activate resting cells (West & Schmidt 1998). In wetlands, a higher water table can support CH_4 oxidation via an increase in the availability of CH_4, resulting from enhanced methanogenesis (Mosaavi and Crill 1997, Kettunen et al. 1999). Drainage tends to have only a minor impact on the type II methanotrophs, whereas type I methanotroph community can become more diverse after drainage (Henckel et al. 2001). The overall mechanisms through which hydrology affects the functions and diversity of methanotrophs are poorly understood (Semrau et al. 2010). Littoral

wetlands, with their natural hydrological gradients, offer a good model system to study the effects of variable hydrological conditions on methanotrophs.

1.5.3. Soil temperature

Temperature is an universal factor controlling microbial activity. To my knowledge there is only one published study where the effects of temperature on diversity and activity of methanotrophs have been studied simultaneously (Mohanty et al. 2007). The results from that study suggest that methanotrophs with different temperature optima are present in soil. Thermophilic, mesophilic and psychrophilic species have been identified (Semrau et al. 2010). All conventional thermo/psychrophilic methanotrophs, except thermophilic *Verrucomicrobia*, belong to the phylum gammaproteobacteria (Dunfield et al. 2007, Semrau et al. 2010). Methane oxidation shows moderately low temperature dependence (Q_{10} from 1.8 to 2.9) (Whalen 2005). Methanotrophs inhabiting landfill and forest soils have been reported to be dominated by mesophiles which can be inhibited by low (< 10 ^{o}C) or high (> 40 ^{o}C) temperatures (Boeckx and Van Cleemput, 1996, Boeckx et al. 1996, Whalen and Reeburgh 1996). The effect of temperature on the function and diversity of methanotrophs in littoral wetlands is unknown.

1.5.4. Nitrogen concentration in soil

The effects of nitrogen load/fertilizers on methanotrophs have been studied for decades (Steudler et al. 1989), but the results have been contradictory. In some studies nitrogen has inhibited, in others it has stimulated functioning of methanotrophic community (Bodelier and Laanbroek 2004). There are a few studies where the effects of nitrogen on methanotrophs have been studied with high taxonomical resolution. Most of studies combining diversity and functioning of methanotrophs have been conducted under *in vitro* conditions. It has been found that nitrogen fertilizers decrease the abundance of type II methanotrophs in well-areated soils (Mohanty et al. 2006, Cebron et al. 2007). However, in rice field soil, nitrogen has stimulated methanotrophs (Bodelier et al. 2000). Subsequently it was shown by DNA-SIP that type I *Methylocaldum* methanotrophs in rice paddy soil can be stimulated by nitrogen (Noll et al. 2008). It has been postulated that the characteristics of the methanotroph community is a key factor in explaining its reaction against nitrogen loading (Mohanty et al. 2006). The effects of nitrogen on the functioning and diversity of methanotrophs in littoral wetlands are unknown.

1.6 LAKES AS SOURCES OF CH_4

Global CH_4 emissions from freshwaters have been estimated to be 103 Tg CH_4 $year^{-1}$ (Bastviken et al. 2011). Previously, CH_4 emissions from lakes have been calculated together with the emissions from wetlands, contributing 23% to the total emissions (Table 1) (Conrad 2009). A recent estimate indicated that open freshwater sources alone contributed 17-20% of the total CH_4 emissions (Bastviken et al. 2011). Freshwater CH_4 and CO_2 emissions together (expressed as CO_2 equivalent) are important because they correspond to 79% of the total global CO_2 sinks. Therefore, it has been recommended that greenhouse gas emissions from freshwater lakes need to be included into the global C models (Bastviken et al. 2011).

Aquatic ecosystems are an important element of the boreal landscape. In Finland, there are 188 000 lakes (surface area more than 500 m^2) covering about 10% of the total land area (Raatikainen and Kuusisto 1990). In the boreal and arctic regions, lakes are typically small. Small lakes (< 10 km^2) have the highest CH_4 emissions per surface unit (Bastviken et al. 2004, Juutinen et al. 2009). Small lakes account for about 60% of the global lake area (Downing et al. 2006) and thus are responsible for most of the CH_4 emitted from lakes (Bastviken et al. 2011). In Finland, about 9% of lakes are classified as being eutrophic (Mannio et al. 2000) and these lakes have the highest CH_4 emissions (Juutinen et al. 2009).

The littoral zone of lakes can contribute as much as 70% of the total CH_4 released from lakes (Juutinen et al. 2003b, Bastviken et al. 2008). The littoral wetland acts as a buffer zone between terrestrial and aquatic ecosystems. It is exposed to nutrients leached from the catchment area and to the fluctuations in the lake water level. Littoral wetlands are therefore periodically inundated (e.g. during spring flooding).

The littoral wetlands typically have, in addition to high variation in hydrology, variability also in soil quality and vegetation, and all of these factors can influence the production, oxidation, transport and release of CH_4. Changes in the water level affect both CH_4 production and CH_4 oxidation, and thus CH_4 fluxes, primarily through the availability of oxygen (e.g. Mosaavi and Crill 1997, Kettunen et al. 1999). A high water level in the littoral wetland enhances CH_4 emissions (Juutinen et al. 2001, Chapter 2). In the water-saturated littoral wetlands of lakes, high primary production (mainly by vascular plants) fuels methanogenesis by providing organic substrates, i.e. above-ground litter, root litter and root exudates. Many emergent wetland plants have large interior open spaces, termed aerenchyma, through which they transport oxygen from the atmosphere to support respiration in the roots and

release CH_4 from sediments into the atmosphere (Amstrong 1967, Bubier 1995, Bergström et al. 2007). The plant biomass correlates positively with CH_4 emissions in littoral zones (Kankaala et al. 2003, Kankaala et al. 2005). Eutrophication activates primary production in lakes and thus also increases the possibilities for CH_4 production.

1.7 METHANOTROPHS IN LAKES

Measurements of CH_4 oxidation in water column and sediments of freshwater lakes started already in early 1970s (see review Reeburg and Heggie 1977). The first studies on the ecology of methanotrophs in lake sediments were conducted in Lake Washington (Lidstrom and Somers 1984, Costello and Lidstrom 1999, Costello et al. 2002). Since then, many other freshwater lakes have been studied (Pester et al. 2004, Rahalkar and Schink 2007, Rahalkar et al. 2009, Antony et al. 2010). New methanotroph species have been discovered (Kalyuzhnaya et al. 2005, Rahalkar et al. 2007), and stable isotope probing (SIP) (Lin et al. 2004, Anthony et al. 2010, Dumont et al. 2011) as well as metagenomics (Kalyuzhnaya et al. 2008) have been employed to study the roles of methanotrophs in CH_4 oxidation. In addition to studies on the overall distribution and activity of methanotrops, also methanotrophs associated with emergent macrophytes have been investigated (King et al. 1994, Boon et al. 1996). Studies on aquatic foodwebs suggest that a large amount of carbon originated from methanotrophs is incorporated into macroinverterbrates living in the lakes (Deines et al. 2007). However, most foodweb studies have focused on profundal freshwater sediments and there is little information available from temporally flooded littoral wetlands.

The phylogenetic analyses, by functional or 16S rRNA gene markers, suggest that in profundal as well as littoral sediment, type I methanotrophs are dominant over type II methanotrophs (Costello et al. 2002, Rahalkar et al. 2009). Recently, SIP was used to determine that type I and type II methanotrophs were active CH_4 oxidizers in the oligotrophic Lake Stechlin, but type I methanotrophs dominated over type II methanotrophs (Dumont et al 2011). A study in the alkaline Lonar Lake suggested that *Methylomicrobium* methanotrophs were the dominant group in CH_4 oxidation in this lake, but there were also some previously uncultivated CH_4-utilizing methanotrophs in *Betaproteobactia*, *Deltaproteobacteria*, *Verrucomicrobia*, and *Firmicutes* phyla (Antony et al. 2010). A study from the oligotrophic Lake Stechlin detected also *Betaproteobacteria* in heavy fractions of DNA-SIP but not in RNA-SIP suggesting that the detection of *Betaproteobacteria* could be a result from cross-feeding of ^{13}C (Dumont et al. 2011). In general, the present results suggest that type I methanotrophs are likely the dominant group being responsible for oxidizing CH_4 in lake sediments.

1.8 MATERIALS AND METHODS

Table 1. *Summary of methods used in this thesis.*

Method	**Original publication**
Collection of soil samples	Chapter 2, 3, 4, 5
Methane oxidation potential measurements	Chapter 2, 3, 4
Methane flux measurements	Chapter 2, 4
In situ nitrogen manipulation	Chapter 4
Soil DNA extraction	Chapter 2, 5
Soil RNA extraction and cDNA synthesis	Chapter 3, 4
pmoA clone library generation and phylogenetic analyses	Chapter 2
pmoA microarray analysis	Chapter 2, 3, 4, 5
Quantitive PCR	Chapter 2, 5
Geostatistics	Chapter 2

1.9 AIMS OF THE STUDY

The aim of this work was to study the functioning and diversity of methanotrophs in the littoral wetland of a boreal lake and to examine reproducibility of methanotroph detection between various laboratories. More specifically the aims were:

- To analyze the activity and diversity of methanotrophs in hydrologically different sub-zones of a littoral wetland.
- To evaluate spatial and seasonal variations in function and diversity of methanotrophs in a littoral wetland.
- To determine the sensitivity of methanotrophs to withstand nitrogen load in a littoral wetland.
- To evaluate intra- and interlaboratory variations of methanotroph detection.

Chapter 6: General discussion

6.1. SPATIAL VARIATION OF THE METHANOTROPH COMMUNITY IN A LITTORAL WETLAND

There are enormous spatial and seasonal variations in the moisture content in littoral wetlands. These variable moisture conditions in different subsites of littoral wetlands affect the physico/chemical characteristics of the soil as well as the vegetation. The spatial variability of the methanotroph community in the littoral wetland was evaluated with a geostatistical approach (Chapter 2). The structure of the methanotroph community was non-homogeneous and patchy and it was modified by hydrology (Fig. 3a, Chapter 2). The structure of methanotrophs in the littoral wetland was more patchy (Chapter 2) than the methanotroph communites in other environments studied up till now (e.g. alpine meadows, landfill and rice paddy soils: Abell et al. 2009, Krause et al. 2009, Kumaresan et al. 2009). The littoral wetland had high species richness (47 OTUs, 93% similarity) compared to other environments (e.g. 26 OTUs in temperate forest soils, 93% similarity, Degelmann et al. 2010; about 35 OTUs, 90 % similarity, in rice field soils, Lüke et al. 2010). There was spatial variability also in the functioning of methanotrophs (Chapter 2). Water level is known to be a factor controlling CH_4 oxidation and CH_4 production in wetlands (Kettunen et al. 1999). When the water table was sufficiently high, methanotrophs exhibited a high capacity to oxidize CH_4 in the littoral wetland (Chapter 2). This can be explained by the increased availability of CH_4 and the greater abundance of methanotrophs in the wet conditions (Chapter 2).

The results of the present study suggest, that type I (especially uncultivated type Ib phylotypes in "freshwater-cluster") methanotrophs enjoyed a competitive advantage in soils where there was a high water content (Fig. 3a, Chapter 2). In contrast, dry conditions in littoral wetlands supported the growth of type II and type Ia methanotrophs. These findings agree with previous results on the dominance of type I methanotrophs in freshwater sediments (Costello et al. 2002, Rahalkar et al. 2009, Antony et al. 2010, Dumont et al. 2011). The concurrent increase in the number of type Ib freshwater-cluster methanotrophs with the increasing moisture represents a new insight into the ecology of methanotrophs in littoral wetlands. It has to be noted that type II methanotrophs dominated in dry areas of littoral wetland and these microorganisms are sensitive against inorganic nitrogen load (Mohanty et al. 2006, Cebron et al. 2007), e.g. to nitrogen leached from agricultural soils which could affect the overall function of the methanotroph community in the dry regions of littoral wetlands (see Chapter 6.3).

6.2 SEASONAL VARIATION IN METHANOTROPH COMMUNITY IN LITTORAL WETLAND

The methanotrophic activity has a low temperature dependency as shown by the Q_{10} values (between 1 and 2.8) (Whalen et al. 2005, Semrau et al. 2010). In the present study, functioning of methanotrophs was in general rather stable in the different seasons. However, in the dry subsite of the wetland, methanotrophs were activated during summer (Chapter 3). In spring, when there were high water levels, type I methanotrophs expanded their niche and functioning into the area which was dry during the growing season but transiently wet in spring (Fig. 3a, Chapter 3). Type I methanotrophs could also have been transported by water from the wet parts to the dry parts of the wetland when the water level rose.

Previous studies in biofilters and landfill soils have indicated that type I methanotrophs become the dominant group at low temperatures (Gebert et al. 2003, 2004, Börjesson 2004). This is supported by the findings that all psychrophilic methanotrophic strains so far indentified are *Gammaproteobacteria* (Semrau et al. 2010). Data from littoral wetlands indicate that the methanotroph community in wet conditions is more susceptible to cold conditions than the communities that are living in dry conditions (Chapter 3). However, in the studied littoral wetland, winter conditions did not increase type I methanotroph abundance, but in contrast, the relative abundance of type II increased. This suggests that littoral wetlands are not occupied by similar type I psychrophilic methanotrophs as described previously.

6.3 EFFECT OF NITROGEN LOAD ON THE ACTIVITY AND DIVERSITY OF METHANOTROPHS

The nitrogen load experiment *in situ* revealed that methanotrophs in littoral wetland displayed variable reactions against nitrogen (Chapter 4). Type II methanotrophs were susceptible to nitrogen (reduction in their relative abundance, and negative correlation with CH_4 oxidation and nitrogen content)(Fig. 3b, Chapter 4). Concurrently, there were also nitrogen tolerant type I methanotrophs (increase in their *pmoA* transcription and there was a positive correlation between type I *pmoA* transcription and CH_4 oxidation/the nitrogen content) (Fig. 3b, Chapter 4). As a result of these mixed reactions of methanotrophs against nitrogen, the nitrogen load had only minor effects on the CH_4 oxidation potential and CH_4 fluxes in the littoral wetland. These results provide new insights into the previous, conflicting results about responses of methanotrophs to nitrogen (Bodelier and Laanbroek 2004). In forest soil, in contrast to rice field soil, nitrogen has inhibited the overall functioning of methanotrophs (Steudler et al. 1989, Bodelier et al. 2000, Mohanty et al. 2006). The stimulated activity in rice field soil was later linked to activation of type I (*Methylocaldum*, *Methylomicrobium*) methanotrophs with DNA-SIP (Noll et al. 2008). The responses of methanotrophs against nitrogen have been postulated to be related to community composition since type I methanotrophs have been stimulated whereas type II methanotrophs have been inhibited by nitrogen (Mohanty et al. 2006). Our results from littoral wetlands suggest that the diverse methanotroph communities in littoral wetlands are tolerant to changes in the nitrogen load. However, climate change can alter hydrological conditions in littoral wetlands and subsequently shape methanotroph communities to be more type II methanotrophs dominated (Chapter 2) i.e. species which are more sensitive to nitrogen.

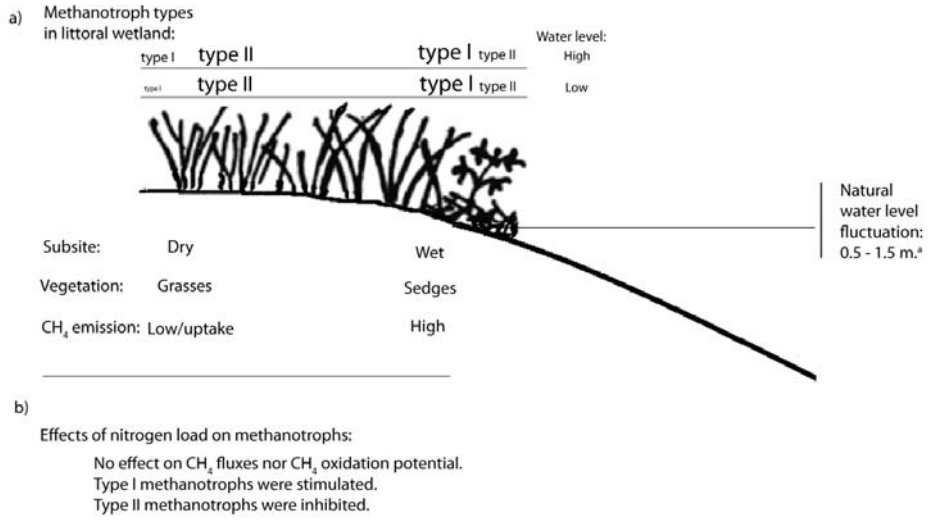

Fig. 3. *Methanotrophs in littoral wetland based on the results of the thesis. Major methanotroph types in littoral wetland in different hydrological conditions (a). The relative abundance of methanotroph types is described with size of the text. Effects of nitrogen load on methanotrophs in littoral wetland (b). Wetland description modified from Juutinen 2004. [a]Water level fluctuation from Larmola 2005.*

6.4 REPRODUCIBILITY OF METHANOTROPH DETECTION

Methanotrophs form a distinct group of bacteria which use one simple substrate (CH_4) as their energy and carbon source and are responsible for a specific biogeochemical process (CH_4 oxidation) in the environments. Therefore, they are good model organisms in microbial ecology. Methane oxidizing bacteria have been used as model organisms in an European research consortium METHECO in studies on microbial molecular ecology in various European ecosystems. The standardization of laboratory protocols for the detection of methanotrophs was one urgent need for success of this project. This standardization was done for extraction protocols of nucleic acids from environmental samples, PCR amplification of *pmoA* genes and utilization of microarray detection. Intra- and interlaboratory variations were evaluated between five European laboratories (Chapter 5). Even though there were some differences in the DNA extraction and handling techniques in the five laboratories, the data was still valid allowing basic conclusions to be drawn. Firstly, the abundance ratio of type I and type II methanotrophs was not affected by the variable laboratory practices. Secondly, the results of community composition determined for various samples within a laboratory were valid because any possible bias in the results was "constant".

6.5 SUMMARY AND CONCLUSIONS

Lakes contribute significantly to the global CH_4 emissions and littoral wetlands are important sources of CH_4 in lake ecosystems. Activity of methanotrophs is known to highly reduce methane emissions from wetlands but methanotrophs in littoral wetlands are poorly known. The results of this thesis show how the variable hydrological conditions, typical in littoral wetlands, affect functioning and diversity of methanotrophs in this environment. There is spatial variability in community composition and functioning of methanotrophs across the hydrological gradient. There are seasonal changes in wetland hydrology and the function and diversity of methanotrophs respond to these changes.

Littoral wetlands as a buffer zones receive nitrogen leached from the catchment which could inhibit the ability of methanotrophs to oxidize methane. The results from experiments carried out during one season indicate that the diverse methanotroph community in littoral wetland withstands nitrogen and nitrogen loading does not disturb methane oxidation.

In changing climate there can be changes in the amount and timing of precipitation which are reflected by hydrology, vegetation, methane dynamics and methanotroph community structure of the littoral wetlands. It is important that methanotroph community structure of an ecosystem determined in one laboratory retain similar basic characteristics when determined in another laboratory.

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Activity and Diversity of Methanotrophs in a Littoral Wetland of an Eutrophic Boreal Freshwater Lake

Littoral wetlands are significant methane sources. Methanotrophs can reduce methane emissions in wetlands, but methanotrophs in littoral wetlands are poorly known. This thesis shows how the variable hydrological conditions, typical in littoral wetlands, affect functioning and diversity of methanotrophs in this environment. It shows also that nitrogen load does not disturb methane oxidation in littoral wetlands. In addition, the effects of sample handling practices on methanotroph detection was studied in five European laboratories.

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