Methanotrophy in peat bogs

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Diana Jantina Kip

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Promotoren:

Prof. dr. ir. Mike S.M. Jetten

Prof. dr. ir. Jaap S. Sinninghe Damsté (UU)

Copromotoren:

Dr. Huub J.M. Op den Camp

Dr. Alfons J.P. Smolders

Manuscriptcommissie:

Prof. dr. ir. Nicole M. van Dam

Dr. Paul L.E. Bodelier (NIOO, Nieuwersluis)

Prof. dr. Julia A. Vorholt (ETH Zürich, Zwitserland)

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according to the decision of the Council of Deans

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at 15:30 hours

by

Diana Jantina Kip

Born on December 9, 1980

in Oostellingwerf (the Netherlands)

Supervisors:

Prof. dr. ir. Mike S.M. Jetten

Prof. dr. ir. Jaap S. Sinninghe Damsté (UU)

Co-supervisors:

Dr. Huub J.M. Op den Camp

Dr. Alfons J.P. Smolders

Doctoral Thesis committee:

Prof. dr. ir. Nicole M. van Dam

Dr. Paul L.E. Bodelier (NIOO, Nieuwersluis)

Prof. dr. Julia A. Vorholt (ETH Zürich, Switzerland)

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Aan mijn Opa de Lange

"Goed je best doen, maar ook zoveel mogelijk van het leven genieten"

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Chapter 1

Introduction and thesis outline

General Introduction

Methane is an important atmospheric trace gas contributing to climate forcing (Wuebbles and Hayhoe, 2002). The understanding of sinks and sources of green house gasses within various ecosystems is crucial to make predictions on global environmental changes, including global warming. In order to make good models and mitigation strategies it is not only necessary to know the sources and sinks, but also knowledge on direct and indirect responses of the different ecosystems to the climatic changes is indispensible. These responses will have implications on the radiative forcing of the atmosphere and the long term trend of global environmental changes, like temperature, precipitation and water table levels. Peatlands store about a third of the total terrestrial carbon (Gorham, 1991) and are important ecosystems in the global carbon cycle. Most peatlands are located in the boreal and sub-arctic Northern hemisphere where the effect of global warming is the strongest (Forster *et al.*, 2007). However the processes and their responses to global environmental changes are not yet understood since the processes and interactions within peatlands form a complex web with many positive and negative feedback loops. In the last years many studies have been performed in order to get a better understanding of the processes in peatlands and their effects on a global scale. Studies performed by scientists of different fields should be combined in order to get a good overview of all the processes, e.g. microbiological, hydrological, ecological and geochemical. This study is part of a project in which scientist of different expertise work together to increase the knowledge on the carbon recycling in peat bogs, with a special emphasis on the role of the methanotrophic symbionts of *Sphagnum* mosses. The main focus of the present study was on the role of aerobic methane oxidizing bacteria (methanotrophs) in peatlands.

Carbon cycle in peatlands

Peatlands are covering only 3% of the continental surface area of the Earth, but they contain the equivalent of at least a third of the carbon that is in the atmosphere as CO_2 (Limpens *et al.*, 2008). Decomposition of plant material in peatlands is slow, which is why the typical peat is formed by the accumulation of partially degraded plants. A substantial part of the decomposition takes place

under anaerobic conditions which leads to methane production by methanogenic archaea (Basiliko *et al.*, 2003). The natural wetlands, among which peatlands, are regarded as the single greatest methane source, accounting for 20% of the global methane emission (Wuebbles and Hayhoe 2002). Methane is the second most important green house gas in the atmosphere and its atmospheric concentration has doubled since post-industrial times. To get a better understanding of the carbon and methane cycle in peatlands, these ecosystems should be investigated more thoroughly. The net emission of methane from peatlands is the result of production of methane in the anaerobic zone by methanogenic archaea and its consumption in the aerobic zone by methanotrophic bacteria. In addition emission comes from transport of methane by diffusion, ebullition and vascular plants (Le Mer and Roger 2001). In the latter two cases methanotrophs have no time to consume methane and methane consumption can be only effective when methane diffuses in the water column. In *Sphagnum*-dominated peat bogs, the most dominant type of Northern wetlands, hardly any vascular plants are present and methanotrophs can thrive in this ecosystem. Raghoebarsing *et al.* (2005) showed that submerged *Sphagnum* mosses, in a Dutch peat bog, consume methane through symbiosis with partly endophytic methanotrophic bacteria, leading to highly effective *in situ* methane recycling and thereby reducing methane emission from *Sphagnum* dominated peat bogs (Fig. 1).

Sphagnum dominated peatbogs are ombrotrophic; meaning they are only fed by rain water, which results in low concentrations of mineral salts and rather acidic pH values (pH 3.0-5.5), which all together makes it an harsh environment for methanotrophic bacteria. Enrichments and isolations of microbes from peatlands are still a challenge since mimicking the appropriate growth conditions is very time consuming and laborious and sometimes almost an impossible task (Dedysh, 2009).

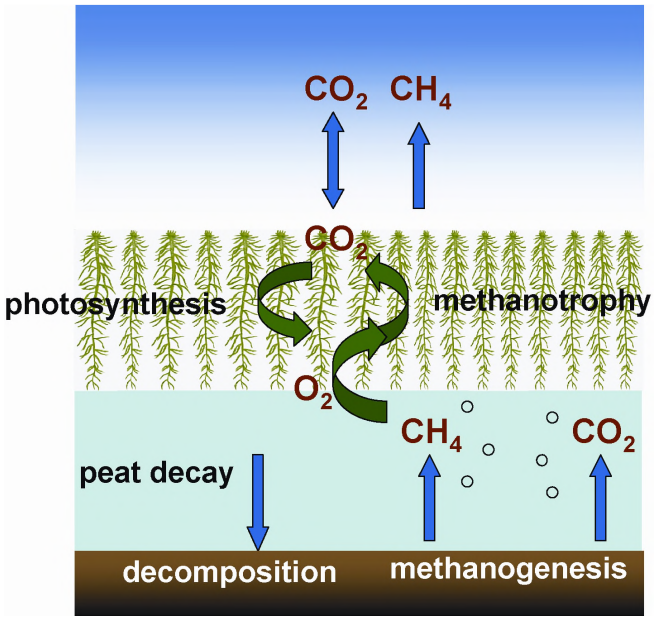

Figure 1 Schematic representation of the methane recycling in peat bogs.

Methanotrophs in peatlands

Methane oxidizing bacteria (methanotrophs) can act as a sink for methane and occur in many different ecosystems like rice paddies, soils, volcanic areas and peat bogs (Hanson and Hanson, 1996; Conrad, 2009; Op den Camp *et al.*, 2009). Methanotrophs present in peatlands can act as a filter for methane, thereby reducing methane emissions from these wetlands (Raghoebarsing *et al.*, 2005). The methane oxidation rates of these methanotrophs are of importance for emission models and it is therefore important to know their global prevalence in peat ecosystems.

Methanotrophs are a subset of Gram-negative methylotrophic bacteria that utilize methane as a carbon and energy source. Methane oxidation occurs in both aerobic and anaerobic environments. Aerobic methane oxidation is carried out by aerobic methanotrophs belonging to the *Alpha-* or *Gammaprotebacteria* or the *Verrucomicrobia* (Murrell and Jetten, 2009; Op den Camp *et al.*, 2009). Anaerobic methane oxidizers using nitrite as electron acceptor belong to the NC10 phylum (Ettwig *et al.*, 2008; Ettwig *et al.*, 2009) of which the species

Methylomirabilis oxyfera is best studied (Ettwig *et al.*, 2010). This apparent anaerobic, denitrifying bacterium encodes, expresses and transcribes the aerobic pathway for methane oxidation, in which oxygen is produced from the intermediate nitric oxide. These anaerobic methane oxidizers belonging to the NC10 phylum were enriched from two freshwater systems in The Netherlands (Raghoebarsing *et al.*, 2006; Ettwig *et al.*, 2009) and from a mixed fresh water source in Australia (Hu *et al.*, 2009). Molecular environmental surveys showed their presence in various aquatic habitats worldwide (Ettwig *et al.*, 2009). In addition archaeal anaerobic methane oxidizers were described which are living in a consortium with sulfate reducing bacteria (Boetius *et al.*, 2000). These methane oxidizing Archaea, belonging to the ANME-1, ANME-2 and ANME-3 phyla, typically occur in methane rich marine sediments, but so far none have been isolated in pure culture yet (Knittel *et al.*, 2005; Losekann *et al.*, 2007). Recently the presence of anaerobic methane oxidation has been shown in minerotrophic Northern peatlands (Smemo and Yavitt, 2007) and experiments showed that NO_3^- could have an influence, but more investigation is needed to clarify this issue. Although aerobic methanotrophs occur in all kinds of environments, three major ecosystems are investigated for several years for the presence and activity of aerobic methanotrophs: peatlands, rice paddies and soils. Environmental studies have shown the presence of yet uncultivated methanotrophs in all these different ecosystems. Rice paddies contain rice paddy cluster I and II methanotrophs that seem to be adapted to the paddy fields (Luke *et al.*, 2010). In soils, atmospheric methane oxidizers are found, some belonging to upland soil clusters, USC-alpha, USC-gamma (Knief *et al.*, 2003), JR-1, JR-2 and JR-3 (Horz *et al.*, 2005).

Here we focus on the aerobic methanotrophs living in and on the *Sphagnum* mosses in peat ecosystems. Molecular studies have shown the presence of symbiotic methanotrophs in and on the mosses and based on a 16S rRNA clone library one particular methanotrophic symbiont was found to be abundant. This symbiont forms clusters on and inside the mosses, which can be detected by fluorescence in situ hybridization and electron microscopy. It was shown to belong to the *Alphaproteobacteria* with highest homology to *Methylocella* and *Methylocapsa* spp., which are acidophilic methanotrophs isolated from peat (Raghoebarsing *et al.*, 2005). Methanotrophs isolated from peat ecosystems all

belong to the *Alphaproteobacteria*, and more specifically to the genera *Methylocella*, *Methylocapsa* and *Methylcystis*. Several molecular studies have also shown the presence of *Methylosinus* related species and gammaproteobacterial species (reviewed in Dedysh, 2009).

Aerobic methanotrophs are able to oxidize methane to carbon dioxide. The first step in this pathway is the oxidation of methane to methanol which is catalyzed by the methane monooxygenase enzyme (Hanson and Hanson, 1996). This enzyme is a defining characteristic of methanotrophs. Methane monooxygenase can appear in two forms: a particulate membrane form (pMMO) and a soluble cytoplasmic form (sMMO) (Trotsenko and Murrell, 2008). The pMMO is found in all methanotrophs except *Methylocella* spp. (Dedysh *et al.*, 2000), on the other hand sMMO is present in only a limited set of aerobic methanotrophs. The next steps in the methane oxidation pathway are the conversion of methanol to formaldehyde, and subsequently to carbon dioxide, with formate as an intermediate. The carbon assimilation pathways start from formaldehyde (Trotsenko and Murrell, 2008). Based on their cell morphology, ultra structure, phylogeny and metabolic pathways, aerobic methanotrophs can be divided into three different taxonomic groups: the *Gammaproteobacteria* (also referred to as type I), which have disc-shaped intracellular membranes (ICMs) (Fig. 2) that occur throughout the cell and use the ribulose monophosphate (RuMP) pathway to convert formaldehyde into multicarbon compounds, for cell biomass; the *Alphaproteobacteria* (also called type II) (Hanson and Hanson, 1996), which use the serine pathway for to convert formaldehyde into biomass and have ICMs parallel to the cell membrane (Fig.2) and the extremely acidophilic *Verrucomicrobia*, without any intracellular membranes of which biochemistry and physiology still needs to be resolved in detail (reviewed in (Op den Camp *et al.*, 2009).

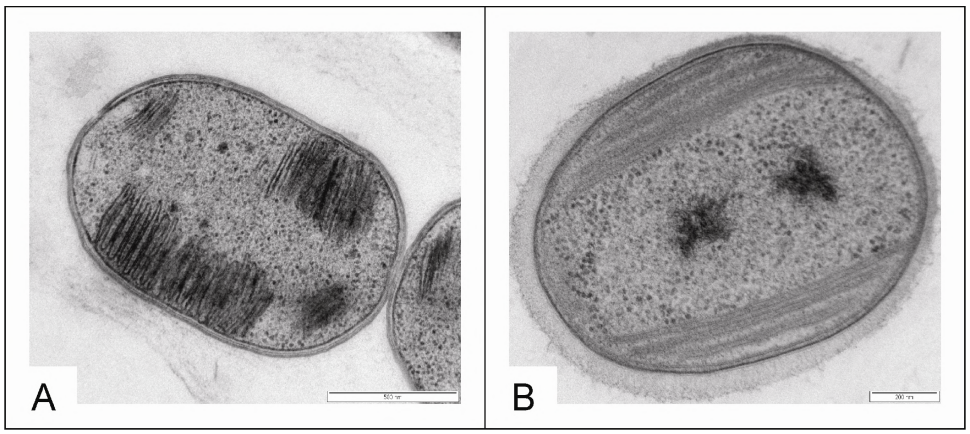

Figure 2 Electron Micrograph of methanotrophs with typical ICMs. A. type I methanotroph with disc-shaped intracellular membranes (ICMs) throughout the cell. B. type II methanotroph ICMs parallel to the cell membrane. Pictures are from isolates obtained in this study.

Thus far gammaproteobacterial methanotrophs include the genera *Methylobacter*, *Methylomonas*, *Methylomicrobium*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylohalobius*, *Methylosoma*, *Methylovulum*, *Methylococcus* and two filamentous shaped methane oxidizers *Crenothrix polyspora* and *Clonothrix fusca*. *Alphaproteobacteria* include the methanotrophic genera *Methylocystis*, *Methylosinus*, *Methylocella* and *Methylocapsa* (Dedysh, 2009) (Fig 3).

In many cases isolation of methanotrophs was successful using Whittenbury's medium (Whittenbury *et al.*, 1970a), but acidophilic methanotrophs are not able to grow on this medium. *Sphagnum* dominated peat bogs have generally pH values between pH 3.0 and 5.5 in which acidophilic or acid-tolerant methanotrophs are expected to be found. After using the very diluted oligotrophic M2 medium, several methanotrophs could be isolated from peatlands (Dedysh *et al.*, 1998; Dedysh *et al.*, 2000; Dedysh *et al.*, 2002; Dedysh *et al.*, 2004), but many remain uncultured. Several other positive attempts have been described to enrich methanotrophs from other acidic ecosystems by modifying the composition of these media (Wise *et al.*, 1999; Bussmann *et al.*, 2004; Iguchi *et al.*, 2010). Nevertheless, isolation of methanotrophs from peat ecosystems is a

challenge. Peat bogs are a harsh environment for microbes to live in, because of the low pH value and the low nutrient content. The acidophilic methanotrophs isolated from peat so far belong to the *Alphaproteobacteria*; being *Methylocella* species (Dedysh *et al.*, 2000; Dunfield *et al.*, 2003; Dedysh *et al.*, 2004), *Methylocapsa* species (Dedysh *et al.*, 2002) and *Methylocystis* species (Dedysh *et al.*, 2007).

Molecular analysis of the methanotrophic community in peat showed the presence of gammaproteobacterial methanotrophs (Vecherskaya *et al.*, 1993; Sundh *et al.*, 1995; Dedysh, 2002; Jaatinen *et al.*, 2005; Chen *et al.*, 2008), however none have been isolated so far.

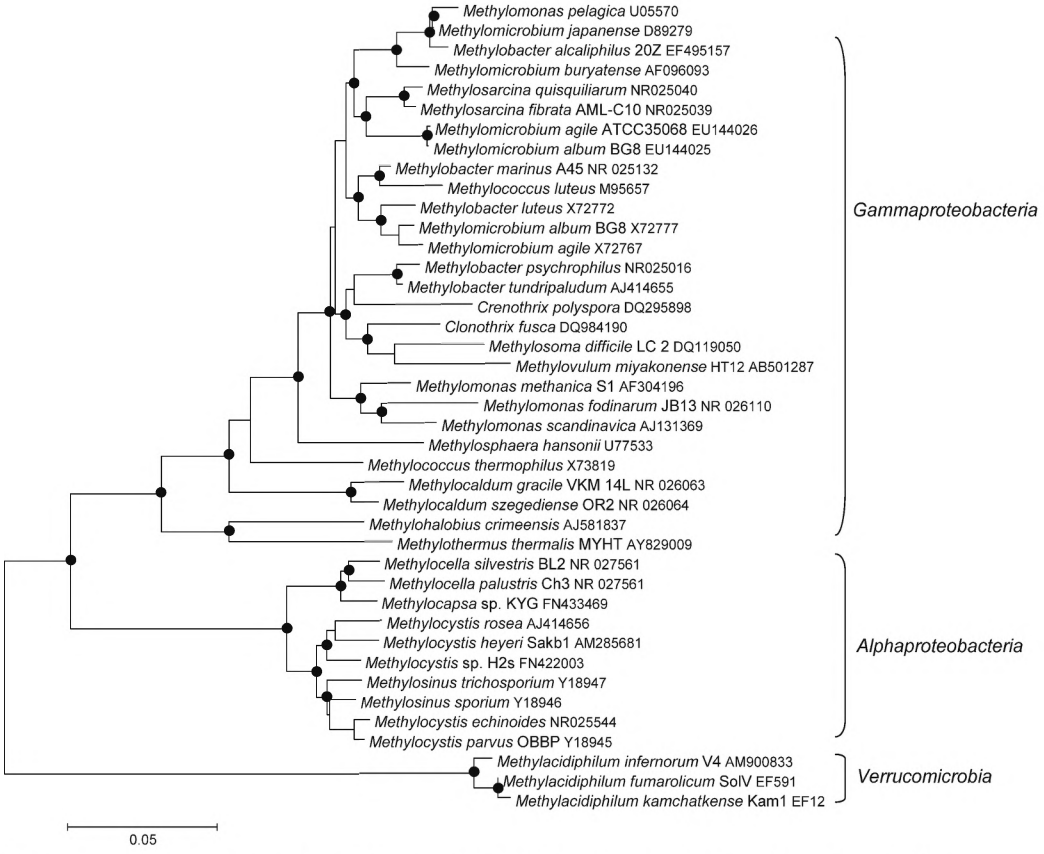

Figure 3 Phylogenetic 16S rRNA tree of cultivated methanotrophs. Dots indicate bootstrap values >60%.

In Northern peatlands permafrost underlies most of the surface. Although temperatures are below zero, there is the evidence of the presence of unfrozen water strongly bound to soil particles, which enables the metabolic activity of cold-adapted micro-organisms (D'Amico *et al.*, 2006). Within the permafrost both methanogenic Archaea, as well as methanotrophic bacteria are still viable or even active (Khmelenina *et al.*, 2002; Rivkina *et al.*, 2007). Methanotrophs are able to oxidize and assimilate methane over a wide range of temperatures and although psychophilic micro organisms are expected, members of virtually all presently known genera of methanotrophic bacteria were found in these permafrost sediments. However it is not clear whether psychrophilic/psychrotolerant methanotrophs or cold-adapted mesophilic and even thermotolerant ones are responsible for methane oxidation at low and subzero temperatures. It has been speculated that cysts, exospores or additional glycoprotein surface layers (S-layers) formed by many methanotrophs aid in their survival and viability (Trotsenko and Khmelenina, 2005).

The recently described extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum were all isolated from volcanic areas with high temperatures and extreme low pH values (below pH 1). More research is required to find out whether there are Verrucomicrobial methanotrophs in peat ecosystems. 16S rRNA clone library analysis have shown the presence of Verrucomicrobia in peat, however these are only distantly related to the verrucomicrobial methanotrophs and no isolates or methane consumption has ever been found related to these bacteria in peat ecosystems (Dedysh *et al.*, 2006; Raghoebarsing, 2006)

Detection of methanotrophs in environment

In the last decades, microbial ecologists have experienced a great leap forward in the study of microbial ecosystems independent of their ability to culture the resident microorganisms. Large-scale surveys have revealed that different ecosystems support unique microbial populations (Fierer *et al.*, 2005; Green and Bohannan, 2006; Adler and Levine, 2007; Zhou *et al.*, 2008; Luke *et al.*, 2010) giving rise to the notion that microbial populations can exhibit geographic

distribution. As culturing techniques are currently limited for most bacteria and archaea, microbial ecologists use molecular techniques that are dependent on the universal marker gene encoding for the 16S ribosomal RNA to document microbial presence at every level, from division to strain. However, sequencing of the 16S rRNA genes in soil samples is both labor intensive and expensive. Consequently, true replication and statistical characterization of microbial diversity in an environment is problematic. In addition to the structural 16S rRNA gene, several functional genes can be used as a molecular tool.

To study methanotrophic communities functional markers are based on the genes of the methane pathway, in particular methane monooxygenase. The first step in the oxidation of methane to CO_2 is the conversion of methane to methanol by the enzyme methane monooxygenase, of which two distinct types are known, the particulate pMMO and soluble sMMO (Gilbert *et al.*, 2000). The pMMO consists of three subunits, A, B and C encoded in the operon *pmo*CAB. The *pmo*A gene is strongly conserved and evolutionary related and homologous to the *amo*A gene, which encodes for a subunit of ammonium monooxygenase of ammonium oxidizers (Holmes *et al.*, 1995). This *pmo*A gene was found to be an excellent functional marker and is frequently used in the molecular ecological studies targeting methanotrophs and its phylogeny corresponds largely to the 16S rRNA gene phylogeny (Kolb *et al.*, 2003). Large *pmo*A sequence datasets from various habitats are available, which makes the *pmo*A gene an excellent proxy to study the correlation between methanotrophs, environmental factors and geographical regions. Of alphaproteobacterial and verrucomicrobial methanotrophs it is known that they can contain several sequence-divergent *pmo*CAB operons, e.g. *Methylocystis* strain CS2 (Dunfield *et al.*, 2002) has an extra pmoCAB2 operon encoding for an enzyme with a much higher methane affinity than that encoded by its pmoCAB1 operon (Baani and Liesack, 2008). In contrast, gammaproteobacterial methanotrophs encode single or multiple near-identical copies of the *pmo*CAB operon. Recently a new *pmo*A like gene was discovered this *pxm*A gene can be targeted as functional gene (Tavormina *et al.* 2010). The gene is so far only found in known gammaproteobacterial methanotrophs in the genera *Methylomonas*, *Methylobacter* and *Methylomicrobium*. It is encoded in the pxmABC operon which differs from the

known 'CAB' order for the pMMO and AMO operons. Unfortunately so far the substrate specificity is still elusive, but phylogenetically the genes cluster within a class of putatively ethane-oxidizing gammaproteobacterial methanotrophs (Tavormina *et al.*, 2010). Since only a few *pxm*A sequences are known, phylogenetic analysis only based on *pxm*A is difficult at the moment.

For a fast screening of the methanotrophic communities of an ecosystem, a *pmo*A based microarray was developed (Bodrossy *et al.*, 2003). This microarray consists of oligonucleotide probes designed and validated against the *pmo*A sequences of almost all known methanotrophs, the *amo*A sequences of ammonium oxidizers and many environmental *pmo*A or *amo*A sequences.

Besides genetic markers also lipids can be used as markers for the presence of methanotrophic bacteria. Phospholipid fatty acids (PFLA) have been used as biomarkers for certain species (Bodelier *et al.*, 2009). However, the PLFA detection is limited, since the lipids have to be present in high concentrations and so far no real specific lipid has been found for methanotrophs. Most gammaproteobacterial methanotrophs produce PLFAs with 16 carbon atoms (C16) and no PLFAs with 18 carbon atoms (C18), while alphaproteobacterial methanotrophs are known to produce high amounts of C18 and much less C16 PLFAs. The PLFA 18:1ω8 is routinely used as a biomarker for *Methylocystis/Methylosinus*, while 18:1ω7 is a biomarker for *Methylocella* and *Methylocapsa* (Dedysh, 2009).

Nucleic acid analysis is more taxonomically specific than PLFA analysis, but they do not distinguish between living and dead microorganisms due to the persistence of DNA upon cell death. These limitations can be overcome by stable isotope probing (SIP) of nucleic acid and PLFA analysis that enables the selective ^{13}C labeling of the biomass of active microbial communities (Boschker *et al.*, 1998; Bull *et al.*, 2000; Radajewski *et al.*, 2002). Through the addition of a ^{13}C labeled substrate (e.g. $^{13}CH_4$) to environmental samples, the highly enriched isotope can be tracked in bacterial biomass, DNA, RNA and other biomarkers, e.g. methanotroph specific lipids. Upon symbiosis or grazing the isotope labels

can be traced back in higher organisms for food chain analysis (Deines *et al.*, 2007; Murase and Frenzel, 2007; Maxfield *et al.*, 2008).

Using the molecular toolbox outlined above one has to bear in mind that new and unknown strains will be difficult to detect, because the methods use probes and primers that are based on our current knowledge and datasets which might be too small to understand the real bacterial diversity of peat and many other ecosystems. Isolation of new, so far unknown, strains is indispensable to expand our knowledge on the microbial communities. With new isolation techniques methanotrophic strains, that do not belong to the known methanotrophic families, could be enriched that are otherwise overlooked in molecular studies. New strains could reveal new or interesting physiology and biochemistry and can be used for complete genome sequencing. Complete genome sequencing of methanotrophs could provide a better insight into the molecular nature of methanotrophy, for example the different pathways in obligate and facultative methanotrophs. Genomic and proteomic analysis provide information for studying the biology of methane oxidation and may provide insights into how methanotrophy is regulated under different environmental conditions (Chistoserdova *et al.*, 2005; Kelly *et al.*, 2005). The use of high throughput sequencing opens many doors for environmental microbiological studies. Techniques like 454-pyrosequencing and Solexa sequencing have improved and expanded the possibilities of deep coverage revealing even low abundant community members (Ley *et al.*, 2006; Sogin *et al.*, 2006). These next generation sequencing techniques yield large amounts of data. Therefore novel bioinformatic tools are needed to be able to analyze these data. For example the sequencing techniques can be used instead of clone libraries to analyze the microbial community of an ecosystem based on PCR amplified structural of functional genes (Hong *et al.*, 2010). This will give a much better coverage and can be used quantitatively. The combinations of different tools would provide a good insight into methanotrophic communities.

In recent years our understanding of the methanotrophic world has broadened and shown that there are many undiscovered organisms and features. In order to understand an ecosystem and the processes within, joint research by

scientists from different disciplines within biology and earth sciences is needed and expected to be profitable.

Outline of this study

This study is part of a joint study on the carbon recycling in peat bogs, with a special emphasis on methanotrophic symbionts funded by the Darwin centre for Biogeosciences. This centre encourages the cooperation of scientist on the interface of biology and earth sciences. In this project microbiologists and biogeochemists work together to get a better understanding of biogeochemical processes and carbon sequestration of *Sphagnum* mosses in different ecosystems around the globe. In 2005 Raghoebarsing *et al.* showed the presence of methanotrophic symbionts in *Sphagnum* peat mosses in the Mariapeel Nature Reserve in the Netherlands. These methanotrophs act as a filter for methane diffusing from the anaerobic methanogenic zone to the atmosphere. This effective recycling prevents methane to reach the atmosphere and provides extra carbon to the plant for photosynthesis (Fig. 1). However, only one peat ecosystem was described and the most abundant methanotroph could not be isolated. Therefore this follow up study was performed to investigate this symbiosis in more detail in different peat bog ecosystems.

This thesis is showing the results obtained on the investigation of the global occurrence and diversity of methanotrophic symbionts in *Sphagnum* mosses of geographically and environmentally different peat ecosystems and enrichments and isolations of methanotrophic symbionts. The sister project of this work focused on the geochemistry part of this project, on e.g. peat bogs proxies that can be developed based on the actual occurrence of methanotrophic bacteria to get insight in palaeoecological evidence for carbon recycling, which could unravel environmental controls that have a significant influence on the functioning of the methane cycle. Stable isotope probing was used to detect label incorporation in methanotroph and *Sphagnum* specific lipids to analyze the importance of carbon recycling in peat bogs. Furthermore analysis of the lipid biochemistry of methanotrophic isolates from *Sphagnum* mosses provided biomarkers that can be traced back in contemporary and ancient peat bogs. The

data obtained in this study is divided over five chapters and a final summary and discussion.

Chapter 2 of this thesis describes the global prevalence of methanotrophic symbionts and the rather similar diversity of methanotrophic communities inside and on the *Sphagnum* mosses all over the world. ^{13}C carbon labeling studies reinforced the symbiosis between methanotrophs and *Sphagnum* mosses by showing ^{13}C methane derived labeling into moss-lipids and chlorophyll-*a*.

Chapter 3 describes the enrichment of *Gammaproteobacterial* methanotrophs from *Sphagnum* mosses on a newly designed medium. Two new strains are described, strain M5, originating from the Mariapeel (NL), shows highest homology to *Methylomonas* spp. and strain M200, originating from the Hoge Venen (B), showed homology to *Methylovulum* and *Methylosoma* sp.

In **Chapter 4** the enrichment of many alphaproteobacterial methanotrophs and the isolation and purification of several strains have been described. Two new *Methylosinus sporium* strains were isolated, which are the first described acidophilic methanotrophs belonging to this genus and several strains showing high homology to *Methylocystis* H2s were described as well.

Chapter 5 describes the study of the methane oxidizing activity of *Sphagnum* mosses from different microhabitats in Patagonian peatlands and shows methanotrophs within lawns and hummocks are active at the water level. Furthermore, the abundance of *Methylocystis* species was shown by two molecular methods. The presence of these species could explain the fast adaptation to new conditions of the methanotrophic community.

Chapter 6 shows a comparison between deep coverage 454 titanium sequencing of a *pmo*A PCR product and a *pmo*A microarray which reveals the abundance of two genera in the methanotrophic community in a Dutch peat bog.

Chapter 7 summarizes and discusses the main results of foregoing chapters.

Chapter 2

Global prevalence of methanotrophic symbionts

Nardy Kip, Julia F. van Winden, Yao Pan, Levente Bodrossy, Gert-Jan Reichart, Alfons J. P. Smolders, Mike S. M. Jetten, Jaap S. Sinninghe Damsté and Huub J. M. Op den Camp

Nature Geoscience 3: 617-621(2010)

Peat bogs store up to a third of all terrestrial carbon on Earth (Smith *et al.*, 2004), and are one of the largest natural sources of atmospheric methane (Gorham, 1991). Anaerobic degradation of submerged *Sphagnum* species, mosses that are prevalent in peat bogs across the globe, produces significant quantities of methane in these systems. However, a study on peat mosses in the Netherlands revealed that a large fraction of this methane is consumed by aerobic methane-oxidizing bacteria, known as methanotrophs (Raghoebarsing *et al.*, 2005); in return, the methanotrophs provide *Sphagnum* mosses with carbon. Here, we show that *Sphagnum* associated methane oxidation occurs ubiquitously across the globe. We collected *Sphagnum* mosses from pools, lawns and hummocks in nine *Sphagnum*-dominated peatlands across the world, and measured their capacity to oxidize methane in a series of laboratory incubations. All mosses were capable of oxidizing methane. The rate of methane oxidation increased with temperature, and was most pronounced in submerged mosses, collected from peatland pools. According to DNA microarray analyses, the methanotrophic community responsible for methane oxidation was highly diverse. ^{13}C labeling revealed that methane-derived carbon was incorporated into plant lipids when mosses were submerged, indicative of a mutually beneficial symbiosis between mosses and methanotrophs. Our findings suggest that the interaction between methanotrophs and *Sphagnum* species may play a role in carbon recycling in waterlogged *Sphagnum* vegetation, potentially reducing methane emissions.

Wetlands are among the largest natural sources of atmospheric methane (Conrad, 2009). Atmospheric methane concentrations have doubled since pre-industrial times and mitigating emissions of this important greenhouse gas has become a major global concern (Forster *et al.*, 2007). *Sphagnum*-dominated wetlands contain a third of the global terrestrial carbon as peat deposits and consequently play an important role in the biogeochemical recycling of carbon (Smith *et al.*, 2004). The anaerobic decay of peat mosses results in production of large amounts of methane, which diffuses to the atmosphere unless consumed. Aerobic methanotrophs are the largest known terrestrial methane sink and are present in a large variety of ecosystems (Hanson and Hanson, 1996). They belong to three different phylogenetic groups: *Gammaproteobacteria*, *Alphaproteobacteria* and *Verrucomicrobia* (Op den Camp *et al.*, 2009). Except

for *Methylocella* spp. all methanotrophs possess a membrane-bound methane mono-oxygenase for which the phylogenetic marker *pmo*A, encoding a subunit of this enzyme responsible for the first step in methane oxidation, can be used in combination with DNA micro-arrays (Bodrossy *et al.*, 2003). Complementary to phylogenetic markers, different types of methanotrophs have specific hopanoids, which have been used to study methane cycling in various environments (Rohmer *et al.*, 1984; Talbot *et al.*, 2001; Bodelier *et al.*, 2009). Hopanoids are relatively stable and thus provide the possibility to reconstruct past methane cycling using peat cores (Pancost *et al.*, 2002; Pancost and Sinninghe Damsté, 2003). Recently it was shown that, in a restored peat ecosystem in The Netherlands, submerged *Sphagnum* mosses consume methane through cooperation with in part endophytic methanotrophic bacteria, leading to effective *in situ* methane recycling, thereby reducing methane emission from *Sphagnum* dominated peat bogs (Raghoebarsing *et al.*, 2005). To understand the impact of these methanotrophs on methane recycling, information on a global scale is required.

We initiated a worldwide survey of *Sphagnum* ecosystems to assess distribution, activity and diversity of methanotrophic symbionts using both molecular and geochemical tools. *Sphagnum* mosses from pools, lawns and hummocks were collected from nine different *Sphagnum*-dominated peatlands, representing the most extensive peatlands around the world. Methanotrophic activity was detected in all living *Sphagnum* mosses tested (Fig. 1).

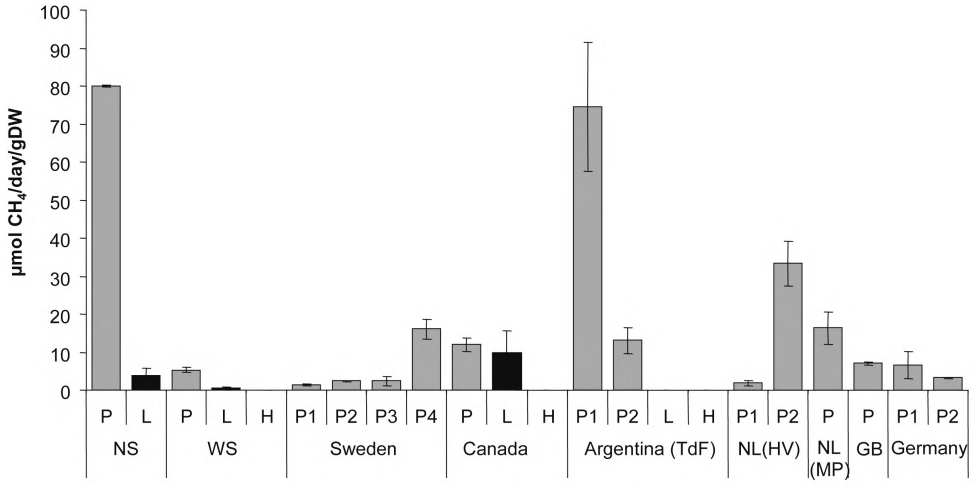

Figure 1 Initial methane oxidation rates of *Sphagnum* mosses. The mosses tested originated from different sites and methane oxidation rates shown here were measured at 20°C. NS=Northern Siberia, WS=Western Siberia, TdF= Tiera del Fuego, Argentina, NL = the Netherlands, GB = Great Britain. P = Pool (grey bars), L = Lawn (black bars), H = Hummock. P1-4 Sweden = four different *Sphagnum* species from four different pools. P1-2 Germany = two different *Sphagnum* species from two different pools. P1-2 of Argentina = two different *Sphagnum* species from the same pool. Values are means of at least 3 incubations ± S.D.

The highest methane oxidation rates were found in *Sphagnum* mosses collected from pools, where *Sphagnum* grows at or below the water table, with potential methane oxidation rates as high as 80 µmol CH_4 day^{-1}g dw^{-1}. Potential methane oxidation rates in *Sphagnum* mosses collected from lawn and hummock vegetation were typically lower. Variations in the potential methane oxidation rates are probably related to *in-situ* methane concentrations from the sites the *Sphagnum* mosses were collected, which in turn are related to the water level (Basiliko *et al.*, 2004). Moreover, methane oxidation rates were two times higher when incubated at 20 ºC compared to 10 ºC, and below detection limit at 4 ºC, except for the *Sphagnum* collected from North Siberia.

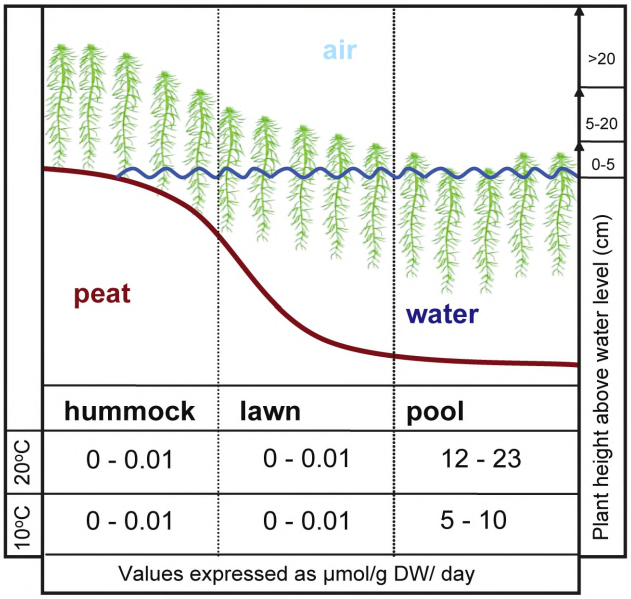

Figure 2 Methane oxidation rates at different temperatures. Schematic representation of the three different tested vegetation types, all *Sphagnum magellanicum,* with the average methane oxidation rates (in µmol/gDW/day) found in Tierra del Fuego at two different temperatures. Values are means of at least 6 incubations ± S.D.

A detailed comparison was made for a pool, lawn and hummock, containing the same *Sphagnum magelanicum* species, from Tierra del Fuego, Argentina (Fig. 2).The established temperature-relationship suggests that the predicted increase in methane production and emissions as a result of global warming (Williams and Crawford, 1984; Frenzel and Karofeld, 2000) may be counteracted by a simultaneous increase in the methane oxidation rate. To show the potential *in situ* importance of methane oxidation we measured methane emissions from *Sphagnum cuspidatum* dominated peat cores from ombrotrophic peat bogs before and after removal of the *Sphagnum* layer. The removal resulted in a 5-fold increase of methane emission, indicating that methane oxidizing bacteria in and on *Sphagnum* act as an *in situ* emission filter (Fig. 3).

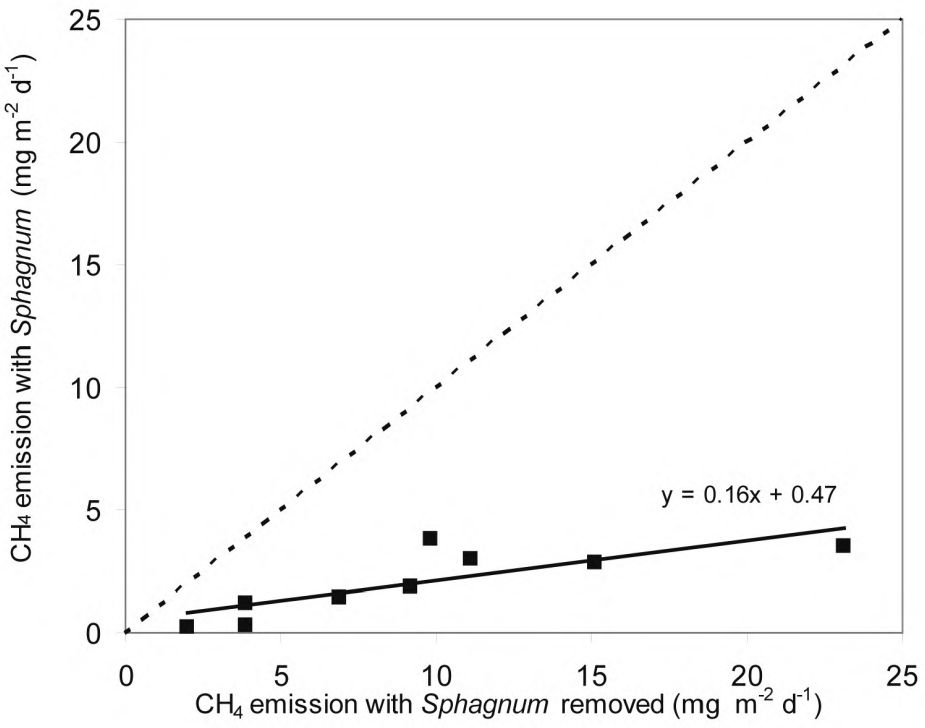

Figure 3 | Comparison of methane emission from peat cores before and after removal of the *Sphagnum* layer. Emissions were determined from 9 different cores. Emission with *Sphagnum* was plotted against the emission after removal. The dotted line indicates the trend when no changes in methane oxidation rates would have occurred.

To document the microbial nature of the methane oxidation, the specific methane monooxygenase inhibitor, acetylene, was added to the *Sphagnum* incubations, which resulted in complete inhibition of methane oxidation. In addition, no methane oxidation was observed in peat water samples, indicating the absence of significant amounts of free-living methanotrophs. Evidently, especially under wet conditions, methanotrophs thrive when living together with *Sphagnum*, probably because they profit from a favourable and stable position along the methane-gradient in the water column and from oxygen supply by the mosses.

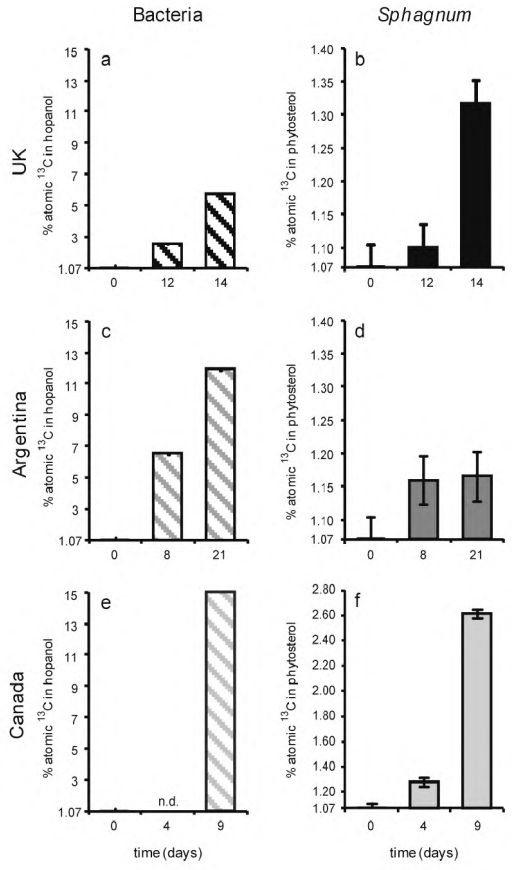

Figure 4 | Methane-derived ^{13}C incorporation into bacterial and *Sphagnum* lipids. Incorporation into bacterial lipids: hopanol (17β,21β(H)-bishomohopanol) and plant lipids: phytosterol (24-ethylcholest-5,22-dien-3-beta-ol) in *S. cuspidatum* from the UK (a-b), *S. magellanicum* from Argentina (c-d) and *S. magellanicum* from Canada (e-f). The x-axis crosses at 1.07, the natural ^{13}C isotope abundance. Note differences in scale. All samples were incubated submerged in medium. Control incubations in air, did not show methane-derived label incorporation into plant lipids. Control incubations with $^{13}CO_2$ were used to correct incorporation levels.

The flow of carbon from methane to symbiotic methanotrophs and ultimately to plant material was investigated by stable carbon (^{13}C) isotopic labelling experiments: *Sphagnum* mosses were analysed for ^{13}C incorporation into bacterial and plant (*Sphagnum*) lipids after incubation with 99% ^{13}C-methane. A strong enrichment of methane-derived ^{13}C in bacterial hopanol (detected as 17β,21β-bishomohopanol released from tetra-functionalized hopanoids using the Rohmer method[10]) was observed in these *Sphagnum* mosses (Fig. 4).

Hopanoids are exclusively produced by bacteria and have been found in relatively high amounts in pure cultures of methanotrophs (Cvejic *et al.*, 2000; Talbot *et al.*, 2001). These results illustrate that bacteria are responsible for the observed methane oxidation, in good agreement with the acetylene inhibition test.

The fact that the ^{13}C label is also incorporated into the phytosterol 24-ethylcholest-5,22-dien-3-beta-ol, which is produced by *Sphagnum* and not by bacteria, shows that methane-derived carbon is also incorporated into *Sphagnum* itself (Fig. 4b,d, f). Additionally, the uptake of methane-derived ^{13}C by *Sphagnum* was also demonstrated by MALDI-TOF mass spectrometry analysis of chlorophyll-a (Fig. 5).

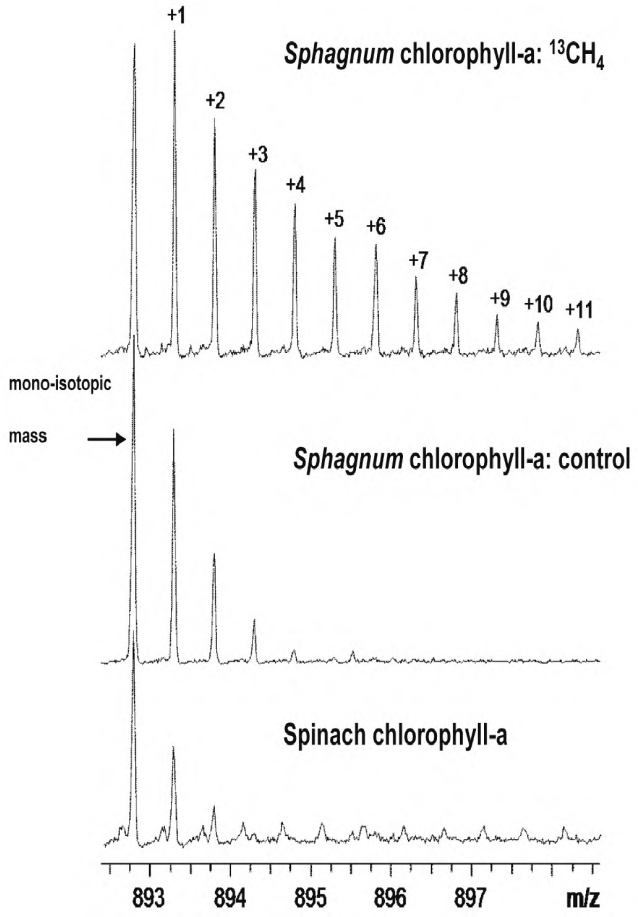

Figure 5 | 13C enrichment of chlorophyll-*a* extracted from *S. cuspidatum*. MALDI-TOF spectra of chlorophyll-a extracted from *S. cuspidatum* (Mariapeel, The Netherlands) incubated with 99% 13C-labelled methane (top), compared to natural 13C abundance in *Sphagnum* (middle) and spinach (bottom). The spectra show the relative abundance of chlorophyll-*a* with a mono-isotopic mass (100% 12C) and chlorophyll-a containing one 13C, two 13C, three 13C etc. consecutively.

Altogether this implies that *Sphagnum* mosses incorporate methane-derived carbon into their plant tissue irrespective of their geographic origin, as this was demonstrated for *Sphagnum* from the UK, Argentina, Canada and The Netherlands. The *Sphagnum* mosses were incubated while submerged in medium.

Air-incubated control experiments did not show methane-derived label incorporation into plant lipids. Whereas the air-incubated *Sphagnum* uses atmospheric CO_2, submerged *Sphagnum* is dependent on carbon fluxes from the organic sediment. Although CO_2 concentrations in pools tend to be higher than in the atmosphere diffusion of CO_2 in water, is very slow resulting in carbon limited conditions (Smolders *et al.*, 2001). Consequently, *Sphagnum* growing under submerged conditions benefits from additional methane-derived CO_2 as a carbon source.

To understand the relative importance of methane-derived CO_2, the incorporation of $^{13}CO_2$ was analysed in a parallel experiment with *S. magellanicum* from a peat bog pool in Canada. An average of 24% ^{13}C of the CO_2 resulted in 1.7% ^{13}C in phytosterol, compared to 2.6% ^{13}C after incubation with on average 51% ^{13}C labelled CH_4. Comparing these ^{13}C enrichments in phytosterol between the parallel experiments, we calculated that methane-derived carbon accounted for approximately 35% of the CO_2 assimilated by *Sphagnum* in these experiments, after correction for label build-up in the headspace and duration of the experiments. In the case of lawn or hummock mosses, the nature of the cooperation appears to be commensal, as the *Sphagnum* does not benefit from supplementary methane-derived CO_2. In pool-derived *Sphagnum,* however, the symbiosis is mutualistic, as submerged *Sphagnum* mosses, deprived from CO_2, benefit from the methane-derived CO_2 provided by the methanotrophs. Our results thus indicate that methane recycling is most profound in submerged *Sphagnum* mosses, where the methane oxidation activity is highest and the consumption of methane-derived carbon by *Sphagnum* is most pronounced. In fact, these results indicate that methane recycling is most intense in areas with high water levels (Frenzel and Karofeld, 2000; Basiliko *et al.*, 2004). Although White *et al.* (1994) suggest that $\delta^{13}C$ values of *Sphagnum* spp. could be used to create records of palaeo-atmospheric CO_2, our results indicate that the use of bulk mosses $\delta^{13}C$ as a proxy has to be re-evaluated since a substantial fraction of their carbon is derived from isotopically light methane.

Both activity and labelling experiments showed the ubiquitous occurrence of methane oxidation in *Sphagnum* mosses. Isolation of methanotrophs, however,

has been proven difficult, and only a limited number of methanotrophic species inhabiting peat ecosystems are known (Dedysh, 2009). To determine the composition of the methanotrophic community, we used a specific methane mono-oxygenase gene (*pmo*A) based PCR in combination with a micro-array[8]. DNA extracted from *Sphagnum* mosses from geographically and environmentally contrasting locations was analyzed (Fig. 6).

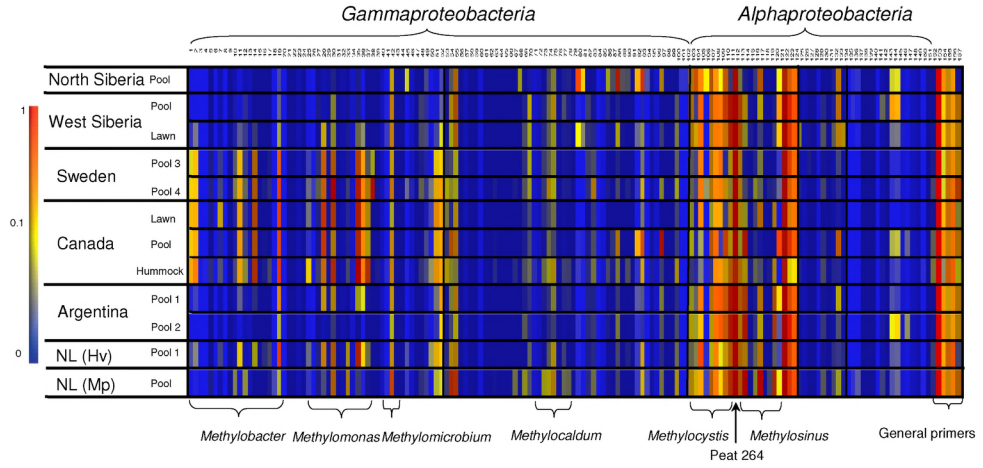

Figure 6 | Representation of the *pmo*A based microbial methanotrophic community analysis microarray. Only selected samples are shown. Color coding bar on the left side represents achievable signal for an individual probe (1 indicates maximum signal, 0.1 indicates 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). HV: Hatertse Vennen; MP: Mariapeel. A larger version of figure 6 can be found in the appendix and a list of the probes in the respective order is given in Appendix Table S1.

The methanotrophic diversity within the *Sphagnum* mosses was high compared to other studies performed on peat soils (Chen *et al.*, 2008b; Chen *et al.*, 2008c), upland soils(Cebron *et al.*, 2007) and rice fields (Vishwakarma *et al.*, 2009). The alphaproteobacterial probes showed that both *Methylocystis* sp. and *Methylosinus* sp. were present in all mosses tested. This is in agreement with previous studies (Chen *et al.*, 2008b; Chen *et al.*, 2008c) showing *Methylocystis* sp. to be abundantly present in peat ecosystems. Also, probe Peat264, targeting a group of uncultivated peat-related alphaproteobacterial methanotrophs, hybridized with PCR products from all mosses. Surprisingly, the microarray also showed a

strong signal with probes targeting the gammaproteobacterial genera *Methylomonas* and *Methylobacter* and *Methylomicrobium*, except for the Russian peatlands. Within these genera a broad diversity was detected for *Methylomonas* and *Methylobacter*. Such a high biodiversity of both alphaproteobacterial and gammaproteobacterial methanotrophs has never been observed in other wetland ecosystems (Cébron *et al.*, 2007; Chen *et al.*, 2008a; Chen *et al.*, 2008c; Vishwakarma *et al.*, 2009). No gammaproteobacterial isolates from peatlands have been reported so far. Still, our microarray results indicate that gammaproteobacterial methanotrophs are ubiquitously present and might contribute substantially to *Sphagnum*-associated methane oxidation.

Previously, *Methylocella* spp. were also shown to be abundantly present in *Sphagnum* (Dedysh *et al.*, 2000; Dedysh, 2002; Dedysh *et al.*, 2003). *Methylocella* spp. do not possess a membrane-bound methane mono-oxygenase (Dedysh *et al.*, 2000), and could therefore not be studied using a *pmoA*-based PCR. Instead, *Methyllocella* sp. do posses a soluble methane monooxygenase gene, which can be detected with PCR primers targeting one of the subunits, *mmo*X. We performed a PCR using five different *mmo*X primer combinations (McDonald *et al.*, 1995; Miguez *et al.*, 1997; Auman *et al.*, 2000) with DNA from the different peat ecosystems and the reference strain *Methylocella palustris* as a template. In only 4 out of 96 PCR's performed on DNA from the *Sphagnum* mosses we observed a very small amount of PCR product, indicating that *Methylocella*-like methanotrophs were not abundant in the ecosystems studied or that no suitable primers are available to resolve this issue. Although some indications for the presence of verrucomicrobial methanotrophs were obtained from the microarray, further research will be needed.

Apart from the natural extent of variability, in general, the same types of methanotrophs, based on genus- and family-specific probes, seem to be present in all the *Sphagnum* mosses, except for *Sphagnum* from North Siberia. Although methane oxidation activity varied between pools, lawn and hummocks, the methanotrophic communities are comparable.

Using genetic and lipid biomarkers, we were able to show both the presence and activity of methanotrophs in *Sphagnum* mosses from peatlands around the world. Methane oxidation rates increased with increasing temperature and were most pronounced in submerged mosses, indicating they could potentially counteract the expected methane production increase in Northern peatlands upon global warming. Labelling experiments illustrated the flow of methane-derived carbon into bacterial lipids and finally also into *Sphagnum* lipids and chlorophyll. Furthermore, genetic markers showed a high bacterial diversity in the methanotrophic community in *Sphagnum.* Our results provide compelling evidence for the worldwide occurrence of methanotrophs in living *Sphagnum* mosses. The interaction between methanotrophs and *Sphagnum* may play a significant role in carbon recycling in waterlogged *Sphagnum* vegetation, strongly reducing methane emissions.

Materials and Methods

Sample collection

Intact *Sphagnum* mosses were collected from nine different peatlands around the world (Table 1). Whole and alive, chlorophyll containing mosses were used. When possible, mosses from pool, lawn and hummock were sampled.

Table 1 Overview of sampling locations of collected Sphagnum mosses.

Country	Sample site	coordinates	sampling time	sample name
Netherlands	Mariapeel	51° 24' 90"N; 5° 54' 90"E	December 2006, January 2007, May 2008	Mariapeel (MP)
	Hatertse Vennen	51° 47' 4"N; 5° 48' 2"E	May 2007, May 2008	Hatertse Vennen (HV)
UK	Bodmin Moor	50° 27' 59"N; 4° 43' 32"W	January 2008	England
Russia	Northern Siberia	70° 37'N; 147° 53'E	July 2008	Northern Siberia
	Western Siberia	60° 53' 26"N; 68° 41' 20"E	July 2008	Western Siberia
Canada	La mer bleue	45° 22'N; 75° 30'W	October 2008	Canada
Germany	Hespermoor	52° 25' 60"N; 7° 10' 60"E	September 2008	Germany
Sweden	Abisko, Stordalen	68° 20'N, 19° 00'E	October 2008	Sweden
Argentina	Av peat	54° 48'S 68° 18'W	December 2007, February 2008, April 2008	Tierra del fuego

Methane oxidizing activity tests

Intact *Sphagnum* mosses were thoroughly washed 3 times with sterile demineralised water, and incubated in 120 ml serum bottles, sealed with airtight, butyl rubber stoppers. To each bottle 1 ml pure methane was added and methane concentrations were monitored daily. Incubation was performed at 4, 10, 15 and 20°C in the dark. When available, peat bog water was tested for methane oxidation. Methane was measured on a HP 5890 gas chromatograph equipped with a flame ionization detector and a Porapak Q column (100/120 mesh). Acetylene addition, irreversibly stopping methane oxidation, was performed with samples from the Mariapeel and Hatertse Vennen.

Methane emission measurements

9 Peat cores (diameter: 15 cm, length 40 cm) were taken from very wet *Sphagnum cuspidatum* dominated hollows from two different peatlands (Tuspeel (51°19'92"N; 5°88'29"E) en Haaksbergerveen (52°13'01"N; 6°77'27"E), The Netherlands). Approximately the upper 10 cm of the cores consisted of living *Sphagnum* moss. The peat cores were filled with water till the top and methane emissions were measured at 18 °C. After a month of pre-incubation at ambient light levels and 18 °C, methane emission rates were measured by means of a funnel (diameter 12.5 cm) closed with a rubber stopper, which was placed upside-down onto the water surface of the column. Accumulation of CH_4 under the funnel was measured every 2 h over a period of 12 h by carefully taking air samples with syringes. As CH_4 concentration showed a linear increase, methane emission could be calculated by simple linear regression. Afterwards the upper 6 cm of the living parts of the *Sphagnum* mosses were carefully cut away and two days later methane emissions were measured again.

$^{13}CH_4$ and $^{13}CO_2$ incorporation into lipids

Submerged labelling experiments were performed as described previously (Raghoebarsing *et al.*, 2005) on *S. cuspidatum* from the UK, *S. magellanicum* from Argentina and *S. magellanicum* from Canada. Briefly, intact *Sphagnum* mosses were thoroughly washed and incubated in 250 ml serum bottles, submerged in 150 ml medium resembling bog water, after which 99% ^{13}C-labelled methane was added to a final concentration of 200 µM. In parallel $^{13}CO_2$ experiments were performed with the Canadian mosses in the same way as described above, with non labelled methane and the addition of 4.2 mg of labelled sodium bicarbonate. Phosphoric acid was used to adjust the pH to 4.5 in all experiments. The bottles were shaken at 150 rpm at ambient conditions, and sacrificed for lipid analyses at two points in time. Control experiments, where *Sphagnum*, was incubated in

air, were performed as described in the previous section, except with 99% ^{13}C labelled methane. Measurements of CO_2 and CH_4 were performed on a GC/MS (5975C, Agilent technologies).

Total lipid extracts of freeze-dried *Sphagnum* species were obtained with an Accelerated Solvent Extractor (Dionex), using a mixture of dichloromethane (DCM):methanol (MeOH) (9:1, v/v). An aliquot of the total extract was methylated with borontrifluoride-methanol (BF_3/MeOH) at 60 °C for 20 min, after which it was separated into three increasingly polar fractions over an activated Al_2O_3 column using hexane:DCM (9:1 v/v), DCM and DCM:MeOH (1:1 v/v), respectively. The DCM:MeOH (1:1 v/v) fraction was subsequently silylated with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide (BSTFA) (1% TMS) and pyridine at 60 °C for 20 min. An aliquot of the total extract was treated with H_5IO_6 and $NaBH_4$, as described by Rohmer et al. (1984), to cleave the C-C bonds of vicinal polyols, and subsequently silylated.

Fractions were analyzed on a gas chromatograph (HP 6890) equipped with a flame ionization detector (FID) set at constant pressure (100 kPa). A fused silica column (30 m x 0.32 mm i.d., film thickness 0.1 μm) coated with CP Sil-5CB was used with helium as a carrier gas. Extracts were injected on-column at 70 °C. The temperature increased with 20 °C/min to 130 °C and 4 °C/min to 320 °C, followed by an isothermal hold for 20 min. Components were identified using a gas chromatograph–mass spectrometer (Thermo Trace GC Ultra).

Compound specific $\delta^{13}C$ values were determined by Isotope-Ratio-Monitoring Gas Chromatography Mass Spectrometry (GC-IRMS, ThermoFinnigan Delta-Plus XP). Carbon isotopic values are reported in the delta notation relative to the VPDB standard. The $\delta^{13}C$ values of the alcohols were corrected for the attached TMS groups derived from BSTFA, which were determined offline.

The extent of methane-derived CO_2 incorporation was estimated by comparing label uptake in phytosterol (measured value minus the natural isotope abundance) to the enrichment the $^{13}CO_2$ treated sample, through time. The isotope ratios of the gases were monitored through time. The experiment in which *Sphagnum* was incubated with $^{13}CH_4$ showed gradual build up of $^{13}CO_2$, up to 16%, in the headspace. This was corrected for using the effect calculated based on the parallel $^{13}CO_2$ labelling experiment. Differences in label in CO_2 and CH_4, changes therein over time, and differences in duration of experiments, were accounted for. The calculation was as follows, where $\Delta^{13}C$ stands for

the enrichment in ^{13}C in plant sterol relative to the natural isotopic abundance, in atomic percentages (corrected for % ^{13}C in the substrate and duration of the experiment): $\Delta^{13}C_{CH4}$ + $\Delta^{13}C_{CO2}$ = 100%. The $\Delta^{13}C_{CH4}$ was obtained when the measured $\Delta^{13}C_{CH4}$ was subtracted by the amount of ^{13}C incorporated as a result of build up of labeled CO_2 in the headspace derived from methanotrophic respiration: $\Delta^{13}C_{CH4} = \Delta^{13}C_{CH4\ measured} - \Delta^{13}C_{CH4\text{-}derived\ CO2}$ in which $\Delta^{13}C_{CH4\text{-}derived\ CO2} = (^{13}CO_{2\ headspace\ 13CH4\ exp} / ^{13}CO_{2\ headspace\ 13CO2\ exp})^* \Delta^{13}C_{CO2}$. The $\%^{13}C$ in CO_2 and in CH_4 was 24% and 51%, respectively, which was accounted for. Also the duration of the $^{13}CO_2$ incubation was 7 days compared to the $^{13}CH_4$ incubation of 9 days for which was corrected as well.

$^{13}CH_4$ incorporation into chlorophyll

Analyses of methane-derived ^{13}C incorporation into chlorophyll-*a* was performed with *S. cuspidatum* collected from the Mariapeel, the Netherlands. *Sphagnum* mosses were washed with demineralised water and incubated in 250 ml serum bottles. In each bottle, seven *S. cuspidatum* plants were incubated. Subsequently, 130 µmol of 99% ^{13}C-labelled methane was added after which the bottles were incubated in the light. In the control treatment the same amount of ^{12}C-labelled methane was added. After two weeks chlorophyll-*a* was extracted and purified using thin layer chromatography (TLC, on silicagel-60 Merck). Chlorophyll-a spots were scraped from the silicagel and eluted with ethanol. Isotope fractions of chlorophyll-*a* molecules were analysed with a Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass-spectrometer (type Bruker Biflex III, reflection mode) with alfa-cyano-5-hydroxy-cinnamic acid (CCA) as the matrix.

gDNA isolation

Sphagnum mosses were washed with sterile demineralised water and stored at -20°C. Frozen mosses were grinded to powder in a mortar with liquid nitrogen, of which 5 ml were transferred to a 50 ml tube. PBS buffer was added and SDS and NaCl were added to a final concentration of 2% and 1M, respectively. One volume of phenol:chloroform: isoamylalcohol (25:24:1) was added and incubated at 65°C for 2 h. After centrifugation for 20 min at 4000 rpm, the water phase was transferred into a new vial and combined with 1 volume chloroform:isoamylalcohol (24:1), after which it was mixed and centrifuged for 20 min at 4000 rpm. From the aqueous phase was, genomic DNA precipitated after adding 0.1 volume 3 M NaAc (pH 4.8) and 2 volumes of ethanol (100%). After incubation for 30 min at -20°C, the mixture was centrifuged for 20 min at 4000 rpm. The pellet was washed with 70% EtOH and dissolved in 0.5 ml MQ. The

gDNA was purified by incubating the DNA with RNase for 15 min 37°C. Humic acids were removed by adding 500 µl Sephaglass bead suspension of the FlexiPrep Kit (Pharmacia P-L Biochemicals Inc.) and incubated for 1 min. Glass beads with bound DNA were washed twice with buffer and finally with 70% ethanol. The pellet was dried and DNA was dissolved in MQ. gDNA was stored at 4°C.

Microarray

*pmo*A based microarray was performed as described previously (Bodrossy *et al.*, 2003), except the *pmo*A based PCR and an extended set of probes. Genomic DNA extracted from *Sphagnum* mosses was used as template in a touchdown PCR with the *pmo*A primer set a189/T7-A682. The PCR programme: 94°C for 5 min, 11 cycles of 1 min 94°C, 1 min 62°C with decrease of 1°C every cycle, 1 min 72°C, followed by 25 cycles of 1 min 94°C, 1 min 52°C, 1 min 72, followed by 10 min 72°C. This PCR product was diluted 100 times and used for a nested *pmo*A touchdown PCR with the A189/T7-mb661 following programme: 94 °C for 5 min, 11 cycles of 1 min 94 °C, 1 min 62°C with decrease of 1 °C every cycle, 1 min 72°C, followed by 14 cycles of 1 min 94 °C, 1 min 52°C, 1 min 72, followed by 10 min 72°C.

mmoX PCR

The *mmo*X based PCRs were performed using the primer sets mmoX1-mmoX2 (touchdown PCR 70-60 °C), f882-r1403and A-B, also called f166-r1401 and the combinations f882-B and A-r1403 (all touchdown PCR 63-53 °C) (McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000). The same PCR program as described for the first PCR for the microarray has been used.

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Chapter 3

Detection and characterization of acidophilic type I methanotrophs of *Sphagnum* peat mosses

Nardy Kip, Wenjing Ouyang, Julia van Winden, Laura van Niftrik, Arjan Pol, Yao Pan, Levente Bodrossy, Elly G. van Donselaar, Gert-Jan Reichart, Mike S.M. Jetten, Jaap S. Sinninghe Damsté & Huub J.M. Op den Camp

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Abstract

Sphagnum peatlands are important ecosystems in the methane cycle. Methane oxidizing bacteria in these ecosystems serve as a methane filter and limit methane emissions. Yet little is known about the diversity and identity of the methanotrophs present in and on *Sphagnum* mosses of peatlands and only a few isolates are known. The methanotrophic community in *Sphagnum* mosses, originating from a Dutch peat bog, was investigated using a *pmo*A microarray. A high biodiversity of both type I and type II methanotrophs was found. Using a newly designed medium based on peat water composition with *Sphagnum* mosses as inoculum, two acidophilic methanotrophs, strains M5 and M200, were isolated in pure culture through dilution series and agarose plates. Both strains grew between pH 4.1 and 6.5 with an optimum at pH 5 for strain M5, while strain M200 had its optimum growth rate at pH 5.5. The 16S rRNA, *pmo*A, *pxmA* and *mmoX* gene sequences showed that both strains belong to the *Gammaproteobacteria*. Strain M5 was affiliated to the *Methylomonas* genus and strain M200 was most closely related to the genera *Methylosoma* and *Methylovulum*. Consistently, the intracytoplasmic membrane system (ICM) of strains M5 and M200 was distributed in bundles throughout the cells, a typical feature of type I methanotrophs. Phospholipid-derived fatty acid patterns were determined and compared with literature data. These isolated strains extend the described habitat of type I gammaproteobacterial methanotrophs to acidic peatlands.

Introduction

Methane is an important greenhouse gas and its concentration is rising rapidly since industrial times (Forster *et al.*, 2007). Methanotrophs can act as a sink for methane and occur in many different ecosystems like rice paddies, soils, volcanic areas and peat bogs (Hanson and Hanson, 1996; Conrad, 2009; Op den Camp *et al.*, 2009). Acidic peat bogs are the most extensive type of wetland, occupying about 3% of the total land area and storing an enormous amount of carbon (Dedysh, 2009). Methanotrophs present in these peatlands can act as a filter for methane, thereby reducing its emissions from these wetlands (Raghoebarsing *et*

al., 2005). The biodiversity of methanotrophic communities can be investigated using PCR with primers targeting the 16S rRNA gene or functional genes like the methane monooxygenase genes, *pmo*A, *pxm*A and *mmo*X (Conrad, 2007; Tavormina *et al.*, 2010). For the detection of the *pmo*A gene a microarray technique has been developed (Bodrossy *et al.*, 2003). The primers used in this technique are based on currently available cultivated and some uncultivated *pmo*A sequences and therefore culture dependent studies, e.g. isolation of new strains, remain important to expand our knowledge on the microbial methanotrophic communities.

Aerobic methanotrophs occur in two monophyletic lineages within *Alpha-* and *Gammaproteobacteria* and one lineage within the *Verrucomicrobia* (Conrad, 2009; Op den Camp *et al.*, 2009). The gammaproteobacterial representatives are called type I methanotrophs. They use the ribulose monophosphate pathway for carbon fixation and possess disc-shaped intracellular membranes (ICMs) throughout the cell. The type II methanotrophs belong to the *Alphaproteobacteria* and use the serine pathway for carbon fixation and have ICMs parallel to the cell membrane (Hanson & Hanson, 1996). The recently described extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum isolated from volcanic areas do not show intracellular membrane structures and their biochemistry and physiology still needs to be resolved in detail (Hou *et al.*, 2008; Op den Camp *et al.*, 2009; Khadem *et al.*, 2010). Currently used primers for the functional gene *pmo*A are not able to detect these new methanotrophs, which demonstrates the need of culture-dependent studies in updating our understanding of the methanotrophic world.

Isolation of methanotrophs from peat ecosystems is a challenge. Peat bogs are a harsh environment for microbes to live because of the low pH, around 4.5, and the low nutrient content. Several methanotrophs have been isolated from peatlands using strongly diluted oligotrophic media, (Dedysh *et al.*, 1998b; 2000; 2002; 2004), but molecular analysis of peat soils indicate that many methane oxidizing bacteria remain uncultured. The acidophilic methanotrophs isolated from peat so far all belong to the *Alphaproteobacteria*; *Methylocella* species (Dedysh *et al.*, 2000; 2004), *Methylocapsa* species (Dedysh *et al.*, 2002) and

Methylocystis species (Dedysh *et al.*, 2007). No methanotrophic *Gammaproteobacteria* have been isolated from peat ecosystems yet and none of the known gammaproteobacterial methanotrophic isolates are acidophilic, i.e. capable of growth below pH 5. It has been shown that methanotrophs are present on and can live inside *Sphagnum* mosses (Raghoebarsing *et al.*, 2005). So far, no methanotrophs have been isolated directly from the *Sphagnum* peat mosses, since the acidophilic *Alphaproteobacteria* were all isolated from peat soils.

The present study uses a *pmo*A based microarray to study methanotrophic biodiversity in *Sphagnum* mosses. Since both type I and type II methanotrophs appeared to be abundant in the investigated ecosystem, we initiated enrichments targeting type I methanotrophs using a new medium based on peat water composition. This resulted in the isolation of strain M5 from a *Sphagnum* peat bog in Mariapeel, The Netherlands and strain M200 from a *Sphagnum* peat ecosystem, in Haute Fagne in Belgium. Both strains represent, to the best of our knowledge, new acidophilic gammaproteobacterial methanotrophs. They could be detected in the microarray using general probes but thus far no specific probes exist.

Results and discussion

Methane oxidations rates and methanotrophic community analysis

Sphagnum mosses from the Mariapeel showed high initial methane oxidation rates 60 ± 16 µmol CH_4 per day per g dry weight and the mosses of the Haute Fagne showed low initial methane oxidation rates of 2 ± 0.9 µmol CH_4 per day per g dry weight, which is within the range reported previously (Raghoebarsing *et al.*, 2005). Peat water controls showed no activity confirming the presence of methanotrophic bacteria inside and/or attached to the *Sphagnum* mosses. DNA was isolated from the mosses from both peat bogs. A *pmo*A based PCR was performed using a combination of general primers and these products were used for microarray hybridization. Since the Haute Fagne sample did not yield sufficient DNA only the Mariapeel sample was used for the *pmo*A microarray analyses (Bodrossy *et al.*, 2003). The *pmoA* gene, encoding for the 27 kDa subunit of the membrane bound methane monooxygenase (pMMO), is generally

used as a genetic marker for methanotrophs. All known methanotrophs contain this gene, except *Methylocella* spp. which only possess a cytoplasmic soluble methane monooxygenase (sMMO) (Dedysh *et al.*,2004; Dunfield *et al.*,2003; Dedysh *et al.*, 2000). The *pmo*A diversity within DNA extracted from the *Sphagnum* mosses was very high (Fig. 1) compared to other studies using the same microarray on peat soils (Chen *et al.* 2008a), alpine meadow soils (Abell *et al.*, 2009), upland soils (Cebron *et al.,* 2007) and rice fields (Vishwakarma *et al.,* 2009).

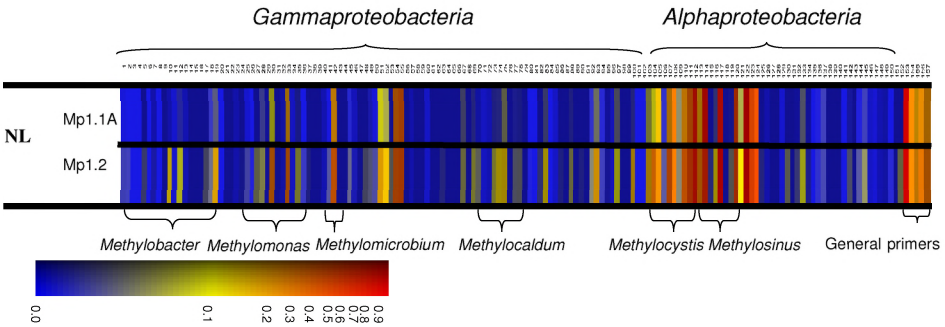

Figure 1 Representation of the results for the *pmo*A based microbial methanotrophic community analysis microarray. Colour coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). A larger version of figure 6 can be found in the appendix and a list of the probes in the respective order is given in Appendix Table S1.

The microarray results revealed the presence of both type I and type II methanotrophs. The type II probes showed that both *Methylocystis* spp. and *Methylosinus* spp. were present in the *Sphagnum* mosses. Furthermore, a probe targeting a group of uncultivated type II methanotrophs from a peatland, Peat264, hybridized with the PCR product (I.R. McDonald and J.C. Murrell, unpublished data). Surprisingly, the microarray also showed a strong signal with type Ia probes, which target the gammaproteobacterial genera *Methylomonas*, *Methylobacter* and *Methylomicrobium*. A broad diversity of *Methylomonas* and *Methylobacter* was detected. Probes based on sequences obtained from the soil-water interface of rice fields (SWI1-375, SWI1-377, (Ferrando and Tarlera, 2009)

also showed a strong signal. The type Ib specific probes, which target the thermotolerant and thermophilic gammaproteobacterial genera *Methylococcus*, *Methylothermus*, *Methylocaldum* and related, uncultivated clades, showed a weaker signal, but still indicated a diverse type Ib community, especially the *Methylocaldum* probes. Type II methanotrophs are commonly found in peat- and other ecosystems and several acidophilic methanotrophs have been isolated (Dedysh, 2009). Studies using the same microarray (Chen *et al.*, 2008a; Chen *et al.*, 2008b) showed the abundance of *Methylocystis* sp. in peat soils which was also found in the *Sphagnum* mosses in this study. However the high diversity of both type I and type II methanotrophs has never been observed in other wetland ecosystems (Chen *et al.*, 2008a; Vishwakarma *et al.*, 2009). The presence of type I methanotrophs in peatlands has been reported before (McDonald *et al.*, 1996; McDonald *et al.*, 1999; Dedysh *et al.*, 2001), but it is new that such an abundance and diversity of type I methanotrophs is observed in these ecosystems. *Methylocella* sp., which are commonly found in Northern peatlands (Dedysh *et al.*, 2000; Dedysh *et al.*, 2004) are the only known methanotrophs that do not have a *pmo*A gene and are therefore not detected in this microarray. In addition, the PCR primers used are not able to amplify verrucomicrobial *pmo*A genes. Nevertheless the microarray results indicated that gammaproteobacterial methanotrophs are indeed present and might contribute to *Sphagnum*-associated methane oxidation, although thus far no isolates from peatlands have been reported.

Enrichment and isolation of methanotrophs on a newly designed medium

Thus far methanotrophs isolated from peat ecosystems belong to the alphaproteobacterial type II group. They were isolated using a strongly diluted medium, M2 (Dedysh *et al.*, 1998a). In order to isolate methanotrophs from *Sphagnum*, the mosses were incubated with methane to enrich the endophytic and epiphytic methanotrophs. Enrichments on M2 medium only resulted in type II methanotrophs (data not shown). Several attempts have been described to enrich methanotrophs by modifying the medium composition (Wise *et al.*, 1999; Bussmann *et al.*, 2004; Iguchi *et al.*, 2010). Another widely used and modified

medium is Whittenbury's medium (Whittenbury *et al.*, 1970a), but acidophilic methanotrophs are not able to grow on this medium.

To isolate the type I methanotrophs, a new medium was designed based on the water composition of the Mariapeel and the M2 medium, designated medium N (Table 1). This medium was used for enrichments of possible methanotrophs from *Sphagnum* mosses.

Table 1 Comparison of the concentrations of the different elements in the peat water, medium N and medium M2 (Dedysh *et al.*, 1998a) in µmol/L.

element	Peat water	medium N	medium M2
PO_4^{3-}	1.24	10.00	293.92
NO_3^-	11.60	10.00	1978.04
NH_4^+	51.88	50.00	
Na^+	237.02	220.00	0.02
K^+	40.45	20.00	2271.97
Cl^-	343.39	426.09	54.96
Ca_2^+	124.60	120.00	27.21
Mg_2^+	39.75	40.00	81.14
Mn_2^+	0.60	0.30	0.03
Fe_2^+	14.55	14.39	1.44
Si_2^+	20.96	20.00	
Zn_2^+	1.01	0.70	0.07
Al_3^+	6.47	10.00	
Cu		0.96	0.09
Co		1.68	0.17
Ni		0.17	0.02
SO_4	55.06	56.04	82.65
Mo		0.25	0.02

The methane oxidizing culture enriched from the Mariapeel *Sphagnum* moss was shown to consist of at least two different species and a dilution series was made on medium N agarose plates to separate the two species. 16S rRNA analysis revealed the enrichment culture to consist of a methanotrophic *Gammaproteobacterium* showing highest homology to *Methylomonas* sp. and a

non-methanotrophic *Betaproteobacterium* that showed highest homology to *Burkholderia* sp. Belova *et al.* (2006) showed the presence of *Betaproteobacteria* of the genus *Burkholderia* in *Sphagnum* bogs in Canada, Russia and Estonia and these types of bacteria are often encountered in methanotrophic isolations. The methanotrophic culture was designated strain M5.

Although medium N proved to be a good isolation medium, M2 medium was used for further culturing of strain M5 since it resulted in better growth. Medium M2 contains more nitrate, phosphate and potassium and less trace elements, sodium, chloride and calcium compared to medium N (Table 1). On agarose plates, one-week-old colonies of strain M5 had a mucous-like appearance.

The methanotrophic culture originating from the Haute Fagne *Sphagnum* mosses showed pink slimy colonies on agarose plates and methane oxidizing pink flocs in liquid culture. This culture was designated M200 and 16S rRNA analysis showed this isolate also belonged to the *Gammaproteobacteria* of the *Methylococcaceae* family. Gammaproteobacterial methanotrophs can be found in a broad range of environments and comprise a large variety of methanotrophs, including the moderately thermophilic *Methylococcus* sp. and *Methylocaldum* sp. (Bodrossy *et al.*, 1997), the thermophilic *Methylothermus* (Bodrossy *et al.*, 1999), the halophilic *Methylohalobium (Heyer et al., 2005)* and the filamentous bacteria *Crenothrix* and *Clonothrix (Stoecker et al., 2006; Vigliotta et al., 2007)*, but no acidophilic methanotrophs have been described. So far, proteobacterial acidophilic methanotrophs all belong to the *Alphaproteobacteria* and therefore these new gammaproteobacterial isolates were further characterized. Strain purity was confirmed by repetitive plating on selective media and (transmission electron) microscopy. No growth occurred on LB, 10-fold diluted LB and M2 supplemented with 0.1% glucose agar plates.

Phylogenetic analysis

Both isolates were shown to contain a *pmo*A gene and the putative *pmo*A gene, *pxm*A (Tavormina *et al.*, 2010) and strain M5 also possessed a *mmoX* gene.

Phylogenetic analysis based on the 16S rRNA genes, *pmo*A genes, *pxm*A genes and *mmoX* gene of strains M5 and M200 showed that both strains belong to the *Gammaproteobacteria* (Fig. 2, 3 and 4).

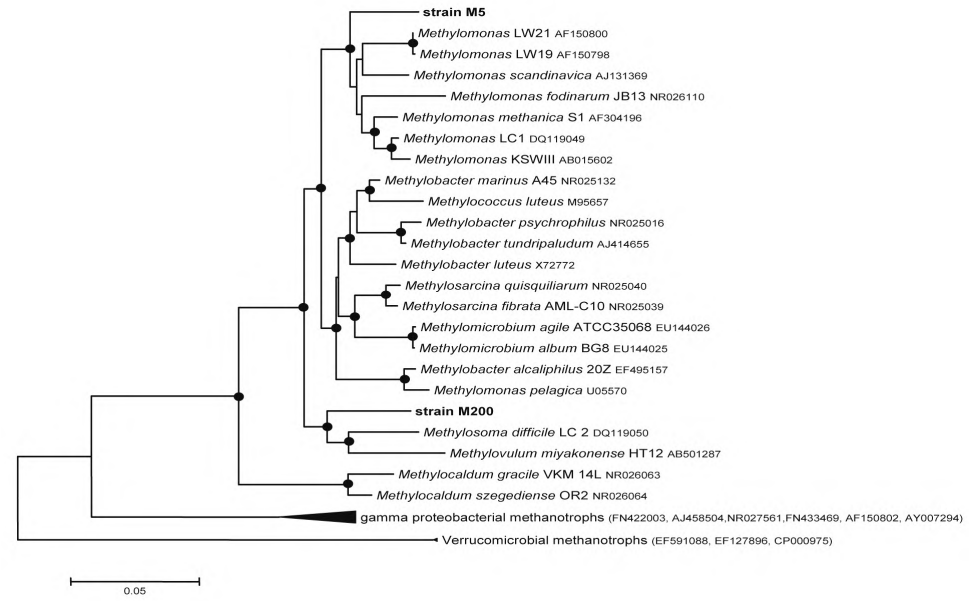

Figure 2 16S rRNA gene phylogenetic tree showing the relationship of strain M5 and M200 to selected methanotrophs. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

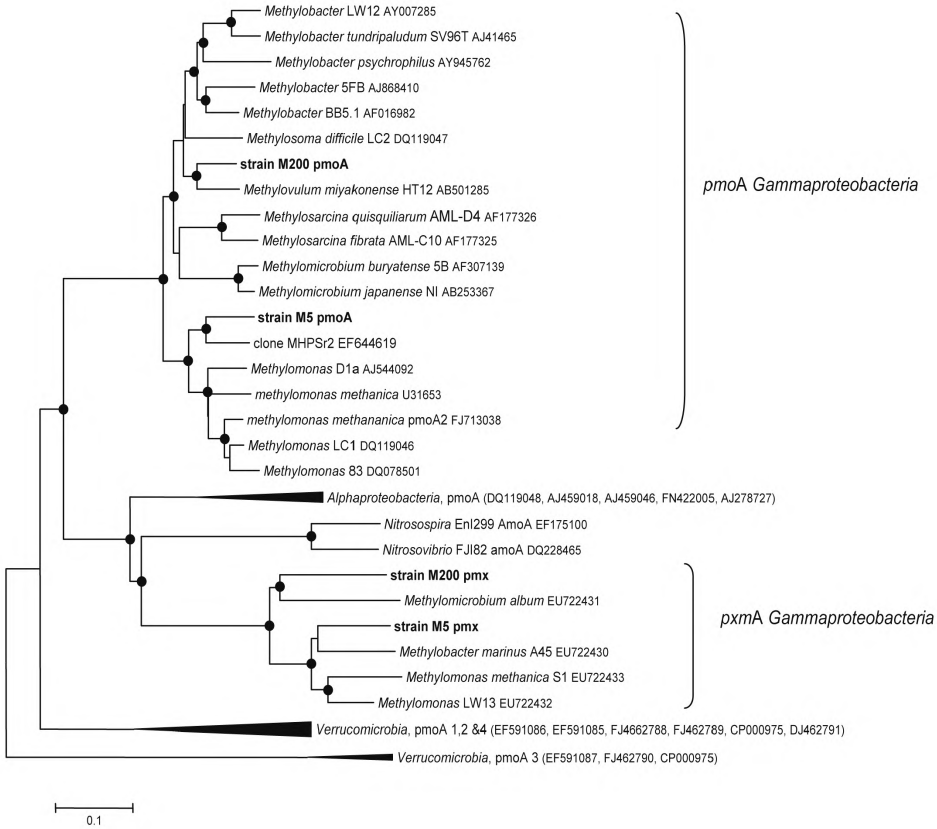

Figure 3 *pmo*A and *pxm*A gene phylogenetic tree based on DNA showing the relationship of strain M5 and M200 to selected methanotrophs. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

The *mmo*X gene of strain M5 was obtained with the primer set *mmo*XA-*mmo*XB (Auman *et al.*, 2000). No *mmo*X gene could be amplified with DNA from strain M200 as a template with all possible combinations of the general primer sets tested.

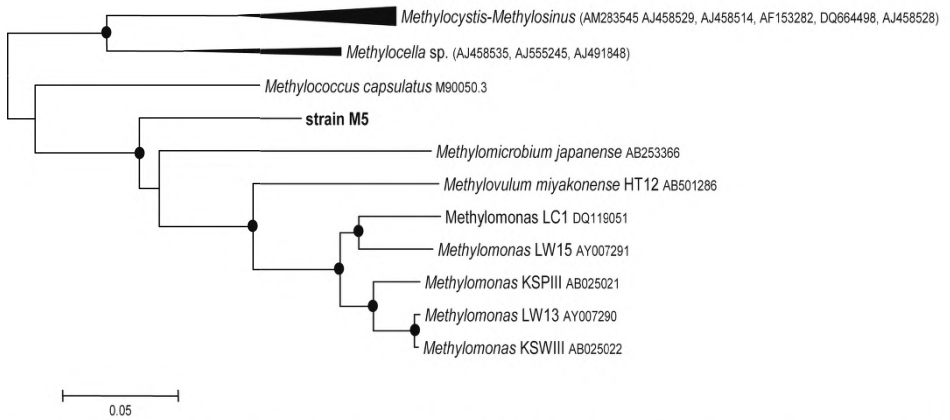

Figure 4 *mmo*X gene phylogenetic tree based on DNA showing the relationship of strain M5. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

The 16S rRNA gene sequence of strain M5 showed 95% similarity to *Methylomonas* strains LW 21 (AF150800), LW19 (AF150798) and LC1 (DQ119049) and *Methylomonas scandinavica* (AJ131369). In the Neighbour-Joining tree (Fig. 2), strain M5 clustered with the genus *Methylomonas* and this topology was confirmed with Minimum Evolution and Maximum Parsimony trees. Based on *pmo*A, *pxm*A and *mmo*X genes strain M5 was also most similar to *Methylomonas* species (Fig. 3 & 4), these genes showed 80 to 87% sequence homology to different *Methylomonas* species. The *pmo*A and *mmo*X genes were 84-87% homologous to *Methylomonas* sp. LC1 (DQ119046) and other *Methylomonas* strains. On amino acid level the similarities were 91-98% to those strains. Interestingly the *pmo*A sequence showed homology to the *Methylomonas* related clade MHPSr2, representing clones originating from *Sphagnum* covered soils in Moor house peat reserve, UK (Chen *et al.*, 2008a). The *pmo*A sequence of strain M5 was 88 and 92% identical to the MHPSr2 clones on DNA and protein level respectively and together formed a separate clade, with high bootstrap value, within the *Methylomonas* genus. This shows that similar *Methylomonas* spp. have been detected in other *Sphagnum* dominated peatlands. The *pxm*A gene of strain M5 is related to the *pxm*A gene of *Methylobacter marinus* (81%, EU722430), *Methylomonas methanica* strain S1 (81%, EU722433) and *Methylomonas* LW13

(79%, EU722432). On amino acid level this homology was 94 to 96 %. However, so far the *pxm*A gene dataset is not extensive (Tavormina *et al.*, 2010). All 16S rRNA, *pmo*A, *pxm*A and *mmo*X genes of strain M5 indicated it is most closely related to the *Methylomonas* genus and it would represent a new species within this genus. *Methylomonas* species have been isolated from various ecosystems like marine ecosystems (Sieburth *et al.*, 1987; Lidstrom, 1988), groundwater (Kalyuzhnaya *et al.*, 1999), soil, mud, sludge and wastewater (Bowman *et al.*, 1993). Many environmental *pmo*A sequences related to the *Methylomonas* genus have also been found in rice paddies (Luke *et al.*, 2010).
Strain M200 showed, based on the 16S rRNA gene, highest homology to many uncultured bacteria from different ecosystems among which many wetlands. The 16S rRNA of strain M200 showed 92 to 93% homology to *Methylovulum miyakonense* HT12 (AB501287), *Methylosoma difficile* LC2 (DQ119050), *Methylobacter tundripaludum* (AJ414655), *Methylobacter psychrophilus* (NR025016) and *Methylosarcina quisquiliarum* (NR025040). Phylogenetic analysis based on Neighbour-Joining (Fig. 2) and Maximum Parsimony revealed that strain M200 had homology to *Methylobacter, Methylovulum* and *Methylosoma* genera.

The *pmo*A gene sequence of strain M200 was clustering between *Methylosoma difficile*, *Methylovulum miyakonense* and *Methylobacter* spp. (Fig. 3). The closest relatives of the *pmo*A of strain M200 were *Methylovulum miyakonense* (89%), *Methylosoma difficile* (86%, DQ119047) and *Methylobacter* sp BB5.1, sp5FB (both 85%, AF016982, AJ868410, respectively). Amino acid sequences revealed the same results and all above mentioned relatives showed a 90 to 96% homology. Based on the *pxm*A gene and PxmA protein of M200 the closest relatives were *Methylobacter marinus* (EU722430) and *Methylomicrobium album* (EU722431) having 71-72% homology on DNA level and 79-82 % on amino acid level (Fig 3.).

The 16S rRNA, *pmo*A and *pxm* genes of strain M200 in different tree topologies did not produce a consistent affiliation to a particular genus and would therefore need further investigation. Nevertheless highest homology to *Methylobacter*, *Methylovulum miyakonense* and *Methylosoma* species was found.

Several *Methylobacter* species have been isolated from environments like polar tundra (Omelchenko *et al.*, 1996; Tourova *et al.*, 1999), soda lakes (Khmelenina *et al.*, 1997) and Artic wetland soil (Wartiainen *et al.*, 2006), but are also commonly found in soil, sewage and mud (Bowman *et al.*, 1993). *Methylosoma difficile* strain LC2, which is related to *Methylobacter*, was isolated from littoral sediment of Lake Constance (Rahalkar *et al.*, 2007). *Methylovulum miyakonense* HT12 was recently isolated from forest soil in Japan (Iguchi *et al.*, 2010).

Physiological properties

Since phylogenetic analysis suggested the isolated pure methanotroph cultures represent new species, they were further characterized. Strain M5 showed growth and methane oxidation between pH 4.1 and 6.5, with an optimum at pH 5 and between 10 and 30 °C with an optimum around 20°C. Growth in liquid M2 medium with methanol occurred, but not with formate or acetate. Strain M200 showed growth and methane oxidation between pH 4.1 and 6.5 with an optimum at pH 5.5 and between 4 and 30 °C. M200 was able to grow on methanol, but not on formate or acetate. Within *Sphagnum* dominated peat bogs the natural pH ranges between 4 and 5. Also, there is a wide temperature-range of the sites sampled, but the general temperature range would be between 0 and 15 °C which demands flexibility and adaptation of the micro-organisms.

Nitrogen fixation was tested by growing the cultures on nitrogen-free M2 medium under various oxygen conditions and analyzing ethylene formation upon addition of acetylene. Strain M5 and strain M200 showed growth and ethylene production on nitrogen-free M2 medium at 1, 2 and 5% oxygen. No growth occurred in nitrogen-free medium at higher oxygen concentrations. *Sphagnum* dominated peat bogs are limited in all nutrients, including fixed nitrogen.

Transmission electron microscopy (TEM) showed that both M5 and M200 cultures consisted of single cells that showed a cell wall typical of Gram-negative bacteria with each having a polysaccharide-like matrix around them (Fig. 5 and 6).

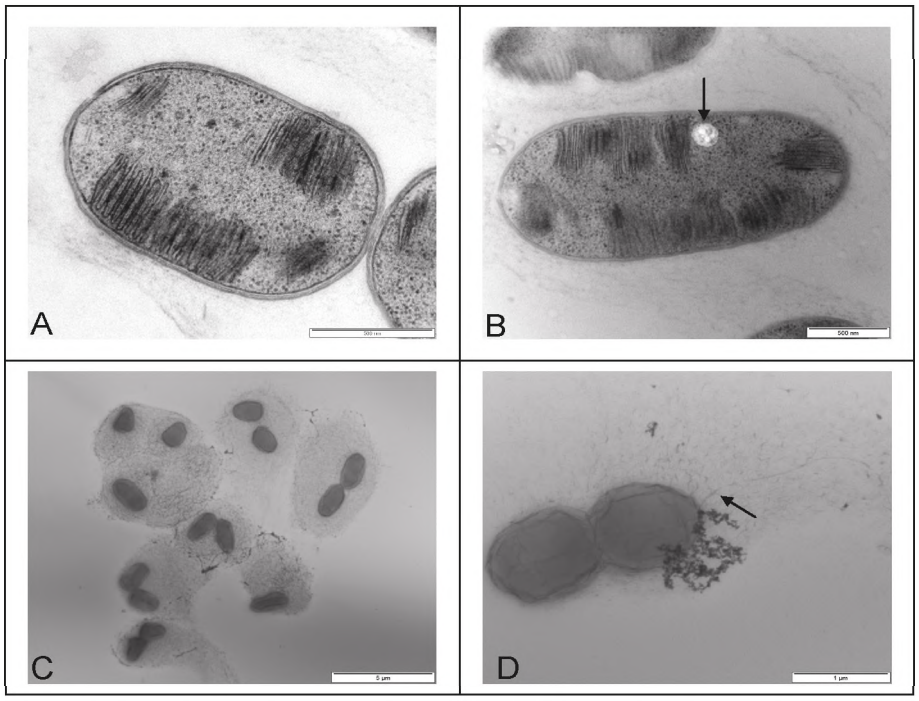

Figure 5 Transmission electron micrographs of high-pressure frozen, freeze-substituted and Epon-embedded cells (A-B) and negatively stained cells (C-D) of strain M5. **A:** cell with typical internal membranes. **B:** cell with a storage particle, indicated with an arrow. **C:** negative staining of several single cells of strain M5. **D:** negative staining of cell(s) of strain M5 where the arrow indicates the putative flagellum.

Negative staining of strain M5 where the uranyl acetate had bound to the cells instead of to the grid showed that some cells contained a singular flagellum (Fig. 5D).

Cells of both strain M5 and M200 showed intracytoplasmic membranes in bundles distributed throughout the cells, which is a typical feature of type I methanotrophs (Fig. 5 and 6). Both TEM and phase contrast microscopy of strain M5 and M200 confirmed culture purity and showed indications of intracellular storage particles. Both strains did not survive storage in glycerol at -80 °C, freeze drying and dessication, indicating there is no production of spores and most probably no cysts production either (Whittenbury *et al.*, 1970b). No spores or cysts were observed with TEM either.

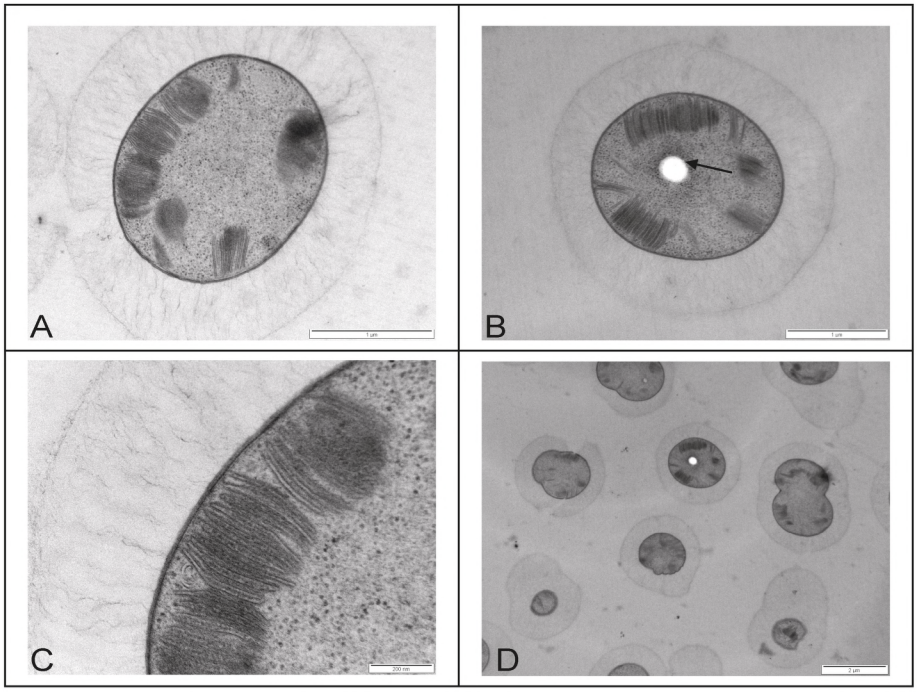

Figure 6 Transmission electron micrographs of high-pressure frozen, freeze-substituted and Epon-embedded cells of strain M200. **A:** cell with typical internal membranes. **B:** cell with a storage particle, indicated with an arrow. **C:** the cell periphery with the Gram-negative cell wall: the outer (bilayer) membrane, one thick layer of peptidoglycan and the cytoplasmic (bilayer) membrane and the slime layer around the cell. **D:** several single cells of strain M200.

Strain M5 follows the description of the *Methylomonas* genus as published in Bowman et al. (1993, 1995). Some *Methylomonas* spp. are able to fix nitrogen and most have a single polar flagellum. Inclusions of poly-P-hydroxybutyrate have been found in *Methylomonas* sp. and they produce capsules and surface pellicle and desiccation-sensitive *Azotobacter* type cysts. Some properties of strain M200 are similar to the genera *Methylovulum* (Iguchi *et al.*, 2010) and *Methylosoma* (Rahalkar *et al.*, 2007), but others resemble the *Methylobacter* genus as described by Bowman *et al.* (1993). Strain M200 is able to fix nitrogen, as are *Methylosoma* spp., but so far no *Methylobacter* and/or *Methylovulum* sp. are reported to fix nitrogen. The pink strain M200 resembles the pink *Methylosoma* spp., but none of the known *Methylobacter* and

Methylovulum sp. is reported to produce carotenoids. Both *Methylobacter* and *Methylosoma* species are able to grow between pH 5.5-9.0 while the pH range of strain M200 is pH 4.1-6.5 and *Methylovulum miyakonense* grows between pH 6.0-7.5.

Phospholipid-derived fatty acids

The proteobacterial methanotrophs possess distinct patterns of phospholipid ester-linked fatty acids (PLFA). Type I methanotrophs mainly contain C16 PLFA and type II methanotrophs mainly C18 PLFA. Methanotrophic biomarkers are C16:1ω8 and C16:1ω5 for type I and C18:1ω8 for type II methanotrophs (Bodelier *et al.*, 2009).

The major fatty acids of strain M5 and strain M200 were C16:1ω8, C16:1ω7, C16:1ω6, C16:1ω5, C14:0 and C16:0 (Table 2).

The abundance of C16 PLFA and absence of C18 PLFA in both isolates is in accordance with their other type I features. Strain M5 and M200 both showed highest abundance of C16:1ω8 and C16:1ω5 and also C16:1ω7 and C16:0 were relatively high. The PLFA distribution of both M5 and M200 were quite distinct from PLFA patterns of other isolated methanotrophs (Table 2), but showed highest similarity to some *Methylobacter, Methylomonas* and *Methylomicrobium* species. Strain M5 fits within the PLFA pattern of all the different *Methylomonas* sp. and strain M200 fits best with *Methylobacter tundripalundum, Methylomicrobium album* and also with *Methylosoma difficile*, although this genus misses C16:1ω8 and C16:1ω5 which are highly abundant in strain M200. Phylogenetic analysis of M200 revealed homology to *Methylovulum miyakonense*, but based on PLFA no similarity was found since this genus only possesses C14:0, C15:0 and C16:0 FLFA (Iguchi *et al.*, 2010).

Table 2 PLFA comparison of strain M200, M5 and other methanotrophic genera. *: combination of c and t measured. a: this article, b: Rahalkar *et al.*, 2007, c: Iguchi *et al.*, 2010, d: Bowman *et al.*, 1991, e: Wartiainen *et al.*, 2006, f: Bowman *et al.*, 1993, g: Wise *et al.*, 2001 , h: Eshinimaev *et al.*, 2004, i: Dedysh *et al.*, 2000, j: Dedysh *et al.*, 2002, k: Dedysh *et al.*, 2004, l: Dunfield *et al.*, 2007, m: Op den Camp *et al.*, 2009

	strain M200	Strain M5	*Methylosoma difficile*	*Methylovulum miyakonense HT12*	*Methylobacter luteus*	*Methylobacter tudripaludum*	*Methylomonas pelagica*	*Methylomonas methanica*	*Methylomonas aurantiaca*	*Methylomicrobium agile*	*Methylomicrobium album*	*Methylosarcina quisquiliarum*	*Methylosarcina lacus*	*Methylococcus capsulatus Bath*	*Methylocaldum* sp. H-11	*Methylocella palustris* K	*Methylocapsa acidiphila* B2	*Methylosinus trichosporium*	*Methylocystis heyerii* SAKB1	*Methylacidiphilum infernorum*	*Methylacidiphilum fumariolicum* SolV
	a	a	b	c	d	e	f	d	d	d	d	g	g	f	h	i	j	d	k	l	m
C14:0	7.5	3.9	8.6	34.2	7.4		1.2	19.9	18.9	1.8	0.7	1.2	1.1	4.3	3.1				0.6	12.6	2.1
C15:0	0.7	0.5	0.8	3.0					0.4	7.3	0.2			0.6	2.5	0.1	0.0		0.4	2.5	-
C16:1ω8*	37.4	42.9	0.0	0.0		34.9	12.1	41.3	3.0	14.3	19.0	15.9	33.6						25.1		
C16:1ω7*	14.9	12.7	60.0	0.0	57.4	23.4	19.9	9.8	14:0	14.1	17.7	32.2	18.2	1.6	43.4	12.6	4.7	14.2	11.4	0.1	-
C16:1ω6*	9.4	5.5	15.0	0.0	4.9	8.2	5.9	5.0	4.5	14.4	7.8	9.1	8.3	3.9		0.0	0.0		0.4		
C16:1ω5*	26	24.3	0.0	0.0	9.4	26.3	11.2	16.6	18.2	35.6	28.6	19.6	26.9	8.6	0.2	0.1	0.1	0.0	0.6	0.0	0
C16:0	4.1	10.3	8.5	46.9	8.4		18.1	5.0	8.7	12.5	11.3	18.3	9.9	56.0	43.6	5.9	7.3	0.7	6.9	14.4	10.5
C18:1ω7*	0	0	0.0		3.1		26.5	1:0	1.6		12.5				0.2	78.6	78.3	17.7	12.9	0.2	-
C18:0	0	0	0.0				2.8	0.1	0.1		1.9			0.5	0.2	0.9	7.6	0.0	1.0	38.8	42.1

Detection in the environment

As described above the methanotrophic community of the Mariapeel was investigated using a *pmo*A microarray. There are no probes on this microarray that specifically detect *Methylosoma* or *Methylovulum* spp. Based on its *pmo*A sequence, strain M5 has a 100% sequence match with the general type Ia probe Ia575 and strain M200 with Ia575 and the *Methylobacter* related Mb271 probe. The general type Ia probe Ia193 has one mismatch with strain M5 and three with strain M200. To confirm this, the *pmo*A PCR product of both strains was used for microarray hybridization and both showed indeed hybridization with the general methanotrophic probes and the general type Ia probe Ia Ia575, but only strain M5 hybridized with Ia193 (Fig. 7).

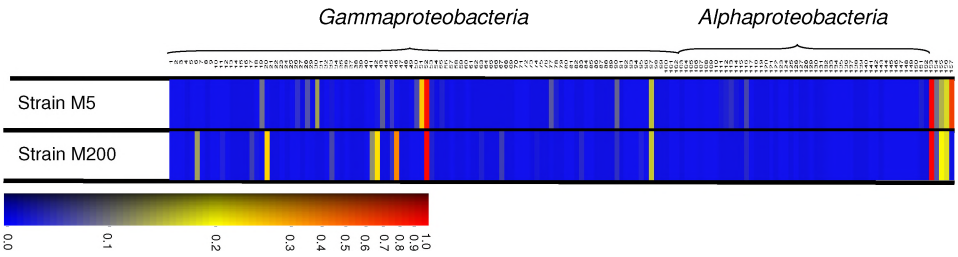

Figure 7 Representation of the results for the *pmo*A based microarray analysis of the *pmo*A PCR product of strain M5 and M200. Colour coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). A larger version of figure 6 can be found in the appendix and a list of the probes in the respective order is given in Appendix Table S1.

Both strains show a hybridization pattern in accordance with the phylogenetic analysis. Strain M5 hybridized with *Methylomonas* related probes and strain M200 hybridized with *Methylobacter* related probes. The hybridization with the Mha-500 probe of both isolates is an unspecific cross hybridization. Almost all these probes showed hybridization in the microarray of the Mariapeel indicating both isolates could have been detected in the Mariapeel *Sphagnum*.

In conclusion, this study showed that the methanotrophic community of the Mariapeel peatland is much more diverse then other previously investigated peatlands and the newly discovered gammaproteobacterial methanotrophs could also play an important role in peat ecosystems. Both acidophilic methanotrophic isolates belong to the type I methanotrophs within the family of *Gammaproteobacteria*. Strain M5 would be a new strain belonging to the genus *Methylomonas*. Strain M200 most probably represents a new strain belonging to the genus *Methylosoma* or strain M200 even represents a novel genus. These new isolates expand our knowledge on methanotrophs and can help us to update the microarray and future molecular surveys.

Materials and methods

Medium N

Medium N is a newly developed medium based on the peat water composition analysis of the Mariapeel (NL). The medium contained per litre: 1.75 mg KH_2PO_4, 1.01 mg KNO_3, 8.02 mg NH_4Cl, 2.92 mg NaCl, 17.6 mg $CaCl_2$.$2H_20$, 9.86 mg $MgSO_4$.$7H_20$, 2.44 mg Na_2SiO_3, 1.33 mg $AlCl_3$ and 0.2% of trace element solution containing per litre: 5 g EDTA, 2 g $FeSO_4.7H_2O$, 0.1 g $ZnSO_4$.$7H_2O$, 0.03 g $MnCl_2$.$4H_2O$, 0.2 g $CoCl_2.6H_2O$, 0.12 g $CuSO_4.5H_2O$, 0.02 g $NiCl_2.6H_2O$ and 0.03 g Na_2MoO_4.

Sampling and culture conditions

Samples were collected from an acidic *Sphagnum* peat bog at the Mariapeel nature reserve (The Netherlands; 51°24'28.4"N; 5°55'8"E) and the nature reserve Parc Naturel Haute Fagne (Belgium; 50°27'0.6"N; 5°55'39"E). *Sphagnum* mosses were thoroughly washed and incubated in 120 ml bottles with 1 ml of methane to determine methane oxidation rates. Incubations were performed in triplicate. After incubation the mosses from the Mariapeel were transferred to a Fernbach flask with 100 ml filter sterile peat water from the Mariapeel. The flasks were closed with a red butyl rubber septum and a screw cap. Incubations were performed with 5% (v/v) methane, at room temperature on a table shaker at 90 rpm while methane concentration and turbidity were monitored daily. The *Sphagnum* from Haute Fagne was incubated in a 250 ml bottle with 100 ml medium N, 5% methane and 5% carbon dioxide to obtain a pH of 4.5. Upon methane consumption of both enrichments dilution series were made after crushing the *Sphagnum* moss with a mortar. Dilutions (10^1 to 10^9 fold) were made in 120 ml or 60 ml serum bottles containing

6 or 3 ml of medium N and 5% CO_2 / 1% CH_4 in the headspace. The bottles were sealed with grey butyl rubber septa and alumina crimp seals. Methane and carbon dioxide were added before autoclaving. Bottles were incubated at room temperature and methane concentrations were measured when the cultures became turbid. Growth and methane consumption was found up to the 10^7-fold dilution and aliquots of this dilution were transferred to agarose plates. After colonies appeared, these were restreaked to obtain single colonies and pure cultures, which were used for colony PCR and further culturing.

Isolation of strains M5 and M200

One of the isolates from the Mariapeel *Sphagnum* enrichment showed slimy growth on agarose plates. This culture will be referred to as strain M5. The methanotrophic culture, growing as slimy, mucous-like colonies on plates, was used for further analysis and referred to as strain M5.

One of the isolates from the Belgian *Sphagnum* enrichment showed pink slimy growth on agarose plates. This culture will be referred to as strain M200.

gDNA isolation

Sphagnum mosses were washed with sterile demineralized water after sampling and kept frozen at -20 °C. Frozen mosses were grinded to powder in a mortar with liquid nitrogen. Five ml of powder was transferred to a 50 ml tube, 5 ml of phosphate buffered saline was added together with sodium dodecyl sulphate (SDS) and NaCl to final concentrations of 2% and 1 M, respectively. One volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v) was added and the mixture was incubated at 65 °C for 2 h. After centrifugation (20 min at 4000 rpm) the water phase was transferred to a new tube and the extraction was repeated by adding one volume of chloroform-isoamylalcohol (24:1, v/v), mixing and centrifuging for 20 min at 4000 rpm. The aqueous phase was transferred to a new tube and the genomic DNA was precipitated by adding 0.1 volume of 3 M NaAc (pH 4.8) and 2 volumes of 100% ethanol. After incubation for 30 min at -20°C and centrifugation (20 min at 4000 rpm) the pellet was washed with 70% ethanol and dissolved in 0.5 ml MilliQ water. The gDNA was purified by incubating the DNA with RNase for 15 min at 37 °C. Humic acids were removed by adding 500 µl Sephaglass beads suspension of the FlexiPrep Kit (Pharmacia P-L Biochemicals Inc.) and incubated for 1 min. Glass beads with bound DNA were washed with washing buffer twice and finally with 70% ethanol. The pellet was dried and DNA was dissolved in MilliQ water. Genomic DNA was stored at 4 °C.

Microarray

The *pmo*A based microarray was performed as described before (Bodrossy *et al.*, 2003) except for the *pmo*A based PCR. Genomic DNA extracted from *Sphagnum* mosses was used as template in a touchdown PCR with the *pmo*A primer set A189/T7-A682 using the following PCR programme: 94 °C for 5 min, 11 cycles of 1 min 94 °C, 1 min 62°C with decrease of 1 °C every cycle, 1 min 72 °C, followed by 25 cycles of 1 min 94 °C, 1 min 52 °C, 1 min 72, followed by 10 min 72 °C. The PCR product obtained was diluted 100 times and used in a nested *pmo*A touchdown PCR with the primer set A189/T7-mb661 and the following programme: 94 °C for 5 min, 11 cycles of 1 min 94 °C, 1 min 62 °C with decrease of 1 °C every cycle, 1 min 72 °C, followed by 14 cycles of 1 min 94 °C, 1 min 52 °C, 1 min 72, followed by 10 min 72 °C. To perform the microarray 300 ng of PCR product was needed.

PCR amplification of the 16S rRNA gene, the pmoA, pxmA and mmoX genes

Single colonies were picked with a sterile toothpick and transferred to 20 µl sterile MilliQ. The inoculated MQ was used as a template for PCR. The 16S rRNA gene was amplified with general bacterial primers 616F (Juretschko *et al.*, 1998) and 1492 (Lane, 1991). Both the *pmoA* and the *mmo*X genes were amplified with general primers using the recommended annealing temperatures (Holmes *et al.*, 1995; McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000). *pxm*A genes were amplified using the primers *pxm*A230F and *pxm*A732R (Tavormina *et al.*, 2010) and a gradient from 50-60 °C.

All PCR products were purified using the QIAquick PCR Purifications Kit (Qiagen). DNA sequencing was performed with the primers used in the PCR and for the 16S rRNA gene also the universal bacterial primer 612R (Ehrmann *et al.*, 2003) was used. DNA sequencing was performed at the sequencing facility of the UMC Sint Radboud, Nijmegen. Phylogenetic analyses were conducted with the MEGA4 package (Tamura *et al.*, 2007). Phylogenetic dendrograms were constructed using the Neighbour-Joining and Maximum Parsimony algorithms as implemented in the MEGA software. Evolutionary distances between sequences were calculated using the DNA and deduced amino acid sequences.

Physiological tests

The pH and temperature optimum tests were performed with M2 medium (Dedysh *et al.*, 1998a). The medium was adjusted to pH 3.5 to 7 with 0.1 M solutions of H_3PO_4, KH_2PO_4,

and K_2HPO_4. Serum bottles (120 ml) containing 12 ml of medium were inoculated with an active pre-culture of the isolate. Bottles contained 1-5% (v/v) methane and were sealed with butyl rubber septa and an aluminum cap. After 5-8 days of incubation at room temperature, culture turbidity and methane consumption were analyzed. When no growth occurred within 8 days, the culture was incubated up to 40 days to check for growth. Temperature optimum test was performed at pH 4.7 at 4, 10, 15, 20, 25, 30 and 37°C. Cultures of different incubation periods were tested for resistance to freeze drying, desiccation and storage in glycerol at -80 °C. Viability of the cultures was tested by growth on solid and liquid M2 medium. Cultures were centrifuged for 1 min at 13,000 rpm. Desiccation resistance was tested by spreading the culture on sterile glass slides and let them dry and cultures were stored in 20% glycerol at -80 °C. All stored cultures were tested for viability of the cells at different time intervals of growth (1, 2, and 4 weeks of growth) and storage (1, 2, 8 and 24 weeks of storage).

Carbon sources

Isolates were tested for growth and methane consumption on M2 medium with methanol (0.5% v/v), acetate (0.1% (w/v) and formate (0.1% w/v) both in liquid medium and on agarose plates. Cultures were incubated for 1 month and monitored daily. Methane consumption and growth by optical density (OD600) were monitored.

Nitrogen fixation

Cultures were tested for growth and methane consumption on nitrogen-free M2 medium with varying headspace oxygen concentrations (20, 10, 5, 2 and 1%) and a methane concentration of 1%. Upon growth on nitrogen-free medium with methane as energy source the cultures were transferred to nitrogen-free medium with 0.1% methanol and the nitrogenase activity assay was performed by adding 2% of acetylene and measuring ethylene production as described before (Khadem *et al.*2010).

Analytical techniques

Methane was measured on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh). Turbidity as a measure of growth was analyzed on an Ultraspect K spectrophotometer at 600 nm.

Phospholipid fatty acid (PLFA) determination

Total lipids were obtained from 0.5-5 mg freeze-dried cell material using a modified Bligh and Dyer extraction procedure (Boschker *et al.*, 1998; Bodelier *et al.*, 2009), after which it

was separated into three increasingly polar fractions over an activated silicic acid column using chloroform, acetone and methanol respectively. The methanol fraction, containing the phospholipids was subjected to mild-alkaline methanolysis, after which an authentic C19:0 fatty acid standard was added. The fatty acids, released from the phopholipids, were methylated using diazomethane. An aliquot of this fraction was used for dimethyl disulfide (DMDS) adduction, as described by Nichols et al. (1986) to determine double bond positions of the mono-unsaturated fatty acids. Quantification was performed on a gas chromatograph (HP 6890) equipped with a flame ionization detector (FID) set at constant pressure (100 kPa) using relative retention times. A fused silica column (50 m x 0.32 mm i.d., film thickness 0.1 μm) coated with CP Sil-5CB was used with helium as a carrier gas. Extracts were injected on-column at 70 °C. The temperature increased with 20 °C/min to 130 °C and 4 °C/min to 320 °C followed by an isothermal hold for 20 min. Components were identified using a gas chromatograph–mass spectrometer (Thermo Trace GC Ultra).

Sample preparation for transmission electron microscopy

Cells were cryofixed by high-pressure freezing, freeze-substituted in anhydrous acetone containing 2% osmium tetroxide, 0.2% uranyl acetate and 1% H_2O or anhydrous acetone containing 2% osmium tetroxide, embedded in Epon resin, sectioned, post-stained and imaged as described previously (van Niftrik *et al.*, 2008). Additionally, strain M5 was imaged by negative staining. For negative staining, cells were first adsorbed to carbon-formvar-coated grids (copper, 100 mesh, hexagonal) for 10 minutes. After incubation, excess liquid was removed with filter paper and cells were negatively stained by incubating for five minutes on a drop of 1.8% methyl cellulose containing 0.4% aqueous uranyl acetate on ice after which they were air dried and imaged in a transmission electron microscope (Tecnai12, FEI Company, Eindhoven, The Netherlands). Images were recorded using a CCD camera (MegaView II, AnalySis).

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Chapter 4

Isolation and characterization of alphaproteobacterial methanotrophs from *Sphagnum* peat mosses

Nardy Kip, Ashna Raghoebarsing, Julia van Winden, Laura van Niftrik, Arjan Pol, Yao Pan, Levente Bodrossy, Elly G. van Donselaar, Gert-Jan Reichart, Mike S.M. Jetten, Jaap S. Sinninghe Damsté & Huub J.M. Op den Camp

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Abstract

Sphagnum dominated peat ecosystems are important players in the methane and carbon cycle. Methanotrophs in these ecosystems serve as a methane filter and thereby reduce methane emissions. Yet only a few methanotrophs have been isolated from peat soil and only two have been isolated from *Sphagnum* mosses of an acidic peatland. Here we describe the enrichment and isolation of methanotrophs from *Sphagnum* mosses, originating from two Dutch and a Belgian peat bog. Using the *Sphagnum* material as inoculum and the M2 medium, developed to isolate acidophilic methanotrophs, many different enrichments were obtained via dilution series and agarose plates. However, only a few methanotrophs, all belonging to the *Alphaproteobaceria*, were isolated in pure culture. Many isolates appeared to be non-methanotrophs. The 16S rRNA, *pmo*A, and *mmoX* gene sequences showed that the isolates were affiliated to the *Methylocystis* genus and others to the *Methylosinus* genus. Several isolates were further charachterized ans showed the isolated *Methylosinus spp.* were capable to grow below pH 5.0. So far no acid-tolerant members of the *Methylosinus* group were known, but molecular surveys indicate they may be widespread in other acidic peatlands. The enrichment of only alphaproteobacterial methanotrophs, while the presence of gammaproteobacterial methanotrophs was also shown in the Dutch peatbogs using molecular methods, leads to the assumption that the M2 medium used may be more specific for acidophilic methanotrophs belonging to the *Alphaproteobacteria.*

Introduction

Sphagnum dominated peat ecosystems are important players in the methane and carbon cycle because they store up to one third of the total global carbon (Gorham, 1991).Within *Sphagnum* dominated peat bogs methane is produced by methanogens in anoxic zones and on its way to the atmosphere by diffusion and ebullition consumption by partly endophytic methanotrophs takes place. Methane is recycled and the *Sphagnum* mosses are provided with extra carbon dioxide by the methanotrophs in and on the *Sphagnum* mosses (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010). Methane emissions from wetlands contribute to the greenhouse

effect, but not all players in the methane cycle are known and more information is needed for a better understanding of the key players in the methane and carbon cycle. Methanotrophic communities can be investigated using the general 16S rRNA gene or functional genes like the methane monooxygenase genes, *pmo*A, *mmo*X (Conrad, 2007) and the *pmo*A related gene *pxm*A (Tavormina *et al.*, 2010). A *pmo*A based microarray was developed to quickly screen the methanotrophic community of an ecosystem (Bodrossy *et al.*, 2003). The *pmoA* gene, encoding the 27 kDa subunit of the membrane bound methane monooxygenase (pMMO), is present in all known methanotrophs, except *Methylocella* spp. which only possess a cytoplasmic soluble methane monooxygenase (sMMO, Dedysh *et al.*, 2000). However using these molecular techniques no information will be found about uncultivated and unknown methanotrophs. Isolation of new strains is therefore important to expand our knowledge on the microbial communities. Although cultivation has been proven to be difficult it is essential to extent the molecular tools to screen methanotrophic communities.

So far isolated methanotrophs belong to three different families within the *Proteobacteria* (*Methylocystaceae, Beijerinkiaceae* and *Methylococceae*) and one within the *Verrucomicrobia* (*Methylacidiphilaceae*) (Dedysh, 2009; Op den Camp, 2009). The families differ in their carbon assimilation pathway and the arrangement of intracellular membranes (ICM) (Hanson and Hanson, 1996; Dedysh, 2009). The *Methylococceae* belong to the *Gammaproteobacteria* or type-I methanotrophs which use the ribulose monophosphate pathway for formaldehyde fixation and have disc shaped ICMs that occur throughout the cell. The *Methylocystaceae* and *Beijerinkiaceae* families belong to the *Alphaproteobacteria* or type-II methanotrophs which use the serine pathway for formaldehyde fixation and have ICMs parallel to the cell membrane. These families include the *Methylocystis-Methylosinus* genera and the genera *Methylocella* and *Methylocapsa*. Recently, extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum have been isolated from volcanic area's (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008) and still need to be further characterized (Op den Camp *et al.*, 2009). Currently used primers for functional genes for methanotrophs are not able to detect these new

verrucomicrobial methanotrophs, again demonstrating the power of isolations of new strains in updating our understanding of the methanotrophic world.

Peat bogs are a harsh environment because of their low pH and low nutrient content. Members of the *Methylocella/Methylocapsa* group and *Methylocystis heyeri* (Dedysh et al., 2007) are so far the only proteobacterial methanotrophs that have been shown to grow at a pH lower than 5.5 and have been isolated from acidic peat bogs and acidic forest soils. The *pmo*A microarray was previously used to analyse the methanotrophic communities in *Sphagnum* mosses from different peatlands from all over the world and showed the presence of both alpha- and gammaproteobacterial methanotrophs (Kip *et al.*, 2010). Type II methanotrophs are commonly found in peat- and other ecosystems and several acidophilic methanotrophs have been isolated (Dedysh, 2009), however molecular surveys indicate to the presence of many uncultured methanotrophs. The isolation of these methanotrophs was possible by using new enrichment media with low salt content, called medium M2 (Dedysh *et al.*, 1998). There are some reports describing the occurrence of type-II methanotrophs; *Methylocystis* species have been detected by fluorescence *in situ* hybridisation (FISH) (Dedysh et al., 2003), molecular surveys using *pmoA* micro arrays and *pmoA* clone libraries (Chen *et al.*, 2008) showed the presence in peat soils of *Methylocystis/Methylosinus* species and the same genera were found to be present and still viable in permafrost sediments (Khmelenina *et al.*, 2002) and Russian subartic tundra (Kalyuzhnaya *et al.*, 2002). *Methylosinus* spp. have been detected in artic soils as well (Wartiainen *et al.*, 2003). One neutrophilic and one moderately acidic strain of *Methylocystis* have been isolated from a *Sphagnum* peat bog (Heyer *et al.*, 2002; Dedysh *et al.*, 2007). Heyer and Suckow (1985) also described the isolation of a neutrophilic bacterium of the genus *Methylosinus* from an acidic peat lake. This isolate was obtained using neutral pH medium, but the optimum pH for growth was not determined. Since molecular ecological methods were unavailable at that time, the characterization of this bacterium was based on morphological features alone. A possible explanation for the isolation of a *Methylosinus* bacterium from an acid environment in neutral medium is that *Methylosinus* species are able to form exospores (Hanson, 1991; Dedysh *et al.*, 1998) which can survive for a long time under unfavourable conditions. Since there was no

evidence of *in situ* activity, the bacterium described might have survived in the environment as exospores and was able to grow at only neutral pH.

The present study describes the enrichment of methanotrophs from different *Sphagnum* mosses, where the presence of both alpha- and gammaproteobacterial methanotrophs have been shown with molecular tools (Kip *et al.*, 2010). The use of medium M2 resulted in the enrichments resulted in the isolation of several acid tolerant methanotrophs from *Sphagnum* peat bogs in the Netherlands and Belgium. As far as we know, strain 29 is the first acid-tolerant *Methylosinus* species, only distantly related to the presently known genera of acidophilic methanotrophic proteobacteria.

Results and discussion

Methanotrophs can be found in various ecosystems and to get a better insight into the methanotrophic communities within these ecosystems the use of classical and molecular microbiology is necessary (Donachie *et al.*, 2007). Cultivation has proven to be difficult, but it is essential to extent the molecular tools to screen methanotrophic communities. In this study symbiotic methanotrophs from inside and on *Sphagnum* mosses were enriched.

Enrichment and isolation of methanotrophs

Methane oxidizing *Sphagnum* mosses from the Dutch peatlands, Hatertse Vennen and Mariapeel, of our previous study (Kip *et al.*, 2010) and a Belgian peat bog, Haute Fagne, were used as inoculum to try to enrich methanotrophs. Isolated acidophilic methanotrophs from peat ecosystems reported before, were isolated using a strongly diluted medium M2, which was also used in this study. In order to isolate methanotrophs from *Sphagnum*, the mosses were thoroughly washed and incubated with methane to enrich the endophytic and epiphytic methanotrophs. Enrichments resulted in increasing methane oxidizing activity, but the liquid medium always stayed clear and transparent, indicating an enrichment of methanotrophs inside the mosses. Thereafter, *Sphagnum* mosses were crushed with a mortar and used for dilution series. The methanotrophic cultures obtained from the dilution series were transferred to agarose plates and

subsequently streaked until single colonies and pure cultures were obtained. The colonies were transferred to liquid culture to test methane oxidizing activity and were used for colony PCR. In the end many cultures were not pure and also several would only grow on plates. Phylogenetic analysis based on the 16S rRNA genes showed most of the isolates to belong to the *Alfaproteobacteria, Gammaproteobacteria* or *Betaproteobacteria* (Fig. 1). All isolates with methane oxidizing activity belonged to the *Alfaproteobacteria*. The other isolates showed good growth on plates with methane, but no growth was observed when they were transferred to liquid medium with methane as the sole carbon source, neither methane oxidizing activity was found. Among these isolates the betaproteobacterial ones were closely related to the *Burkholderiales* and the gammaproteobacterial isolates were all belonging to the *Pseudomonaceae*. Representatives of the *Pseudomonaceae* family are commonly found in peatlands (Rydin *et al.*, 2006). Members of the order *Burkholderiales* (*Betaproteobacteria*) are most probably methylotrophs (Belova *et al.*, 2006), which hamper the isolation of pure methanotrophic cultures since they tend to spread all over the plates in a hardly visible transparent layer. Belova *et al.* (2006) showed the presence of *Betaproteobacteria* in *Sphagnum* bogs in Canada, Russia and Estonia and these bacteria are a known problem in isolations of methanotrophs. Finally, several cultures were obtained and used for further experiments.

Six pure cultures were showing high methanotrophic activity and growth in liquid medium M2 and these were designated strain 29, M162, M169, M175, M212 and M242. Strain M242 originates from a Belgian peat ecosystem, strain M169 from the Hatertse Vennen and the rest from the Mariapeel. All cultures showed white, creamy colonies on agarose plates. Strain purity was confirmed by repetitive plating on selective media and microscopy. On LB and ten times diluted LB and M2 with 0.1% glucose agar plates no growth occurred.

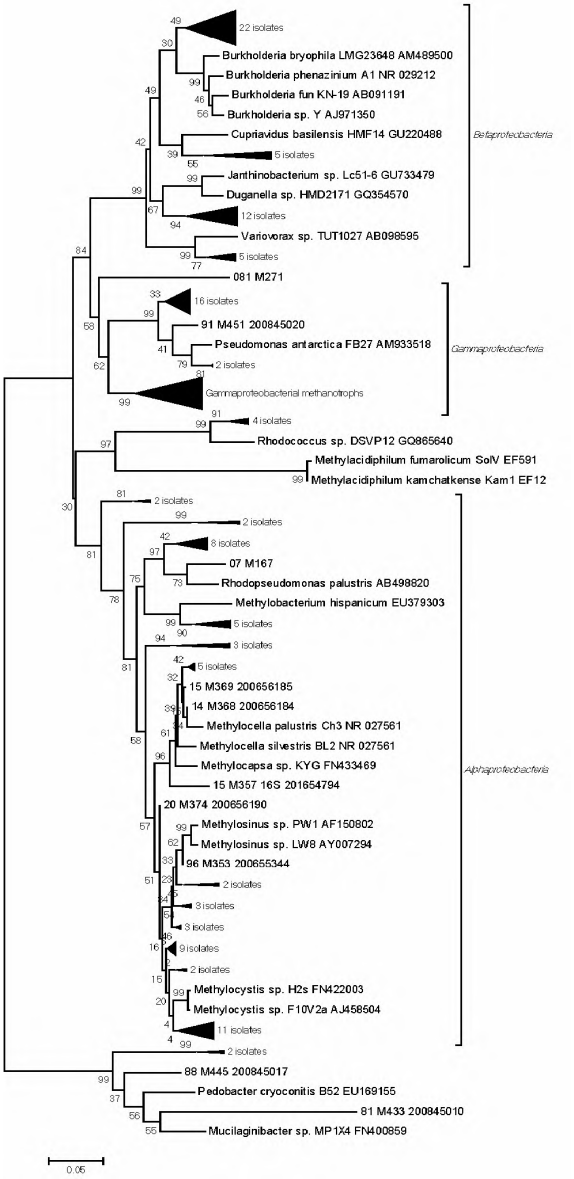

Figure 1 16S rRNA gene phylogenetic tree showing the relationship of sequences obtained from *Sphagnum* enrichments compared to selected cultivated bacteria. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates

To test down to which pH enrichments of endophytic methanotrophs from *Sphagnum* mosses would become active peat water was adjusted to cover a pH range from 2.5 to 5.0. No methane oxidizing activity was measured at 2.5. The enrichment at pH 3.3 was further analyzed and purified on Gelrite plates. The methanotrophic culture obtained in this way showed 99% homology to *Methylocystis* H2s, based on 16S rRNA gene analysis. This microorganism was previously isolated from peat and was reported to have a pH optimum between 6 and 6.5 (Belova *et al.*, 2010).

Upon further testing our methanotrophic culture did result in better growth at higher pH and showed the same optimum, but the culture could also be maintained at pH 3.0.

Phylogenetic analysis

All strains showed to contain a *pmo*A gene and a *mmoX* gene, but no pmoA-like *pxm*A gene (Tavormina *et al.*, 2010). Phylogenetic analysis based on the 16S rRNA genes, *pmo*A genes and *mmoX* genes of all the strains showed that they all belong to the *Alphaproteobacteria*. Among the species isolated strain 29 and M242 were most related to *Methylosinus* spp. and strain M162, M169, M175 and M212 were most related to *Methylocystis* spp. (Figure 2, 3 and 4). The 16S rRNA gene sequences of strains M162, M167, M175, M169 and M212 were 100% identical to *Methylocystis* sp. H2s (FN422003) and *Methylocystis* sp. F10V2a (AJ458504). Compared to each other the 16S rRNA gene of strain 29 and M242 were 98 % identical. Strain 29 showed highest similarity (99%) to *Methylosinus* sp. strain 8 (AJ458486) and *Methylosinus* sp. NCIMB 11126 (Y18946). Strain M242 showed highest similarity to *Methylosinus* sp. SE2 (AJ458478) and *Methylosinus* sp. NCIMB 13214 (AB007840). The *pmo*A gene sequences of strains M162, M167, M175, M169 and M212 were 100% identical to *Methylocystis* sp. H2s (FN422005) and *Methylocystis* sp. F10V2a (AJ459046). The *pmo*A gene of strain 29 and M242 were 97 % identical and both strains showed highest similarity, 96-97 %, to *Methylosinus sporium* 8 (AJ459018).The *mmo*X sequence of M162, M167, M175 and M212 were 100% identical to *Methylocystis* sp. H2s (FN422004), *Methylocystis* sp. F10V2a (AJ458529), and

strain M169 was 98-99 % identical to all those strain. The *mmo*X sequence of strain 29 and M242 are 99.6 % identical and they both showed highest similarity to *Methylosinus* sp. LW3 (AY007287) and *Methylosinus* sp. PW1 (AY007292), both 96 to 97% on DNA level.

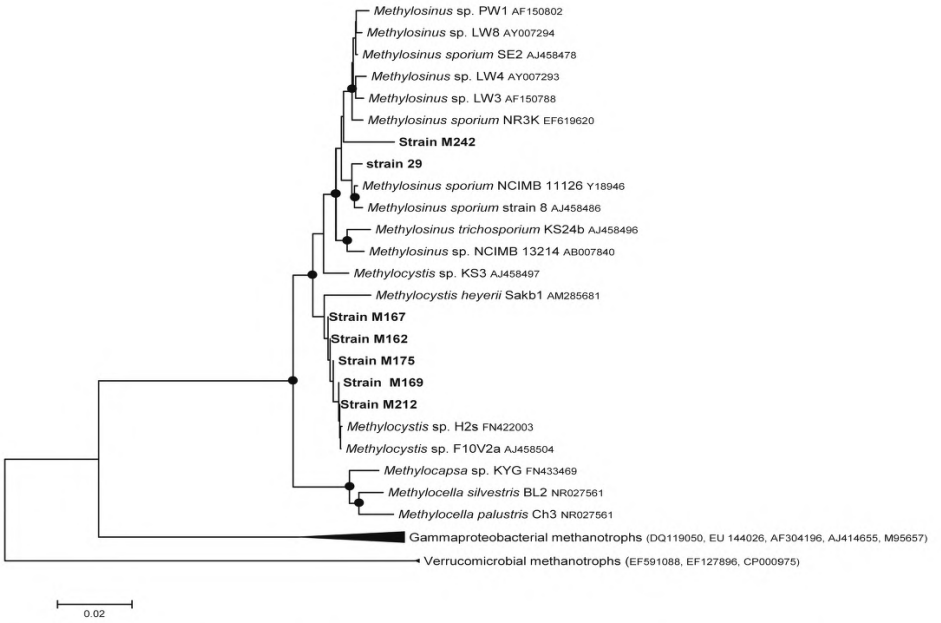

Figure 2 16S rRNA gene phylogenetic tree showing the relationship of the isolated methanotrophic strains to selected methanotrophs. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

Analysis of all 16S rRNA, *pmo*A and *mmo*X genes of strain M162, M167, M175, M169 and M212 indicated that these strains were very similar and genetically identical to *Methylocystis* strain H2s isolated from a peat bog and was shown to be able to use on acetate for survival (Belova *et al.*, 2010).

Strain 29 and M242 were closely related to each other and were shown to belong to the genus *Methylosinus*. Both *Methylocystis* and *Methylosinus* spp. were abundantly found in peat ecosystems ((Dedysh *et al.*, 2001 {Chen, 2008 #445); (Kip *et al.*, 2010). No acidophilic *Methylosinus* sp. has been reported before and

only the moderately acidophilic *Methylocystis* species, isolated from *sphagnum* peat, were characterized in more detail (Dedysh *et al.*, 2007; Belova *et al.*, 2010; Im *et al.*, 2010). Therefore the strains different physiological properties were tested.

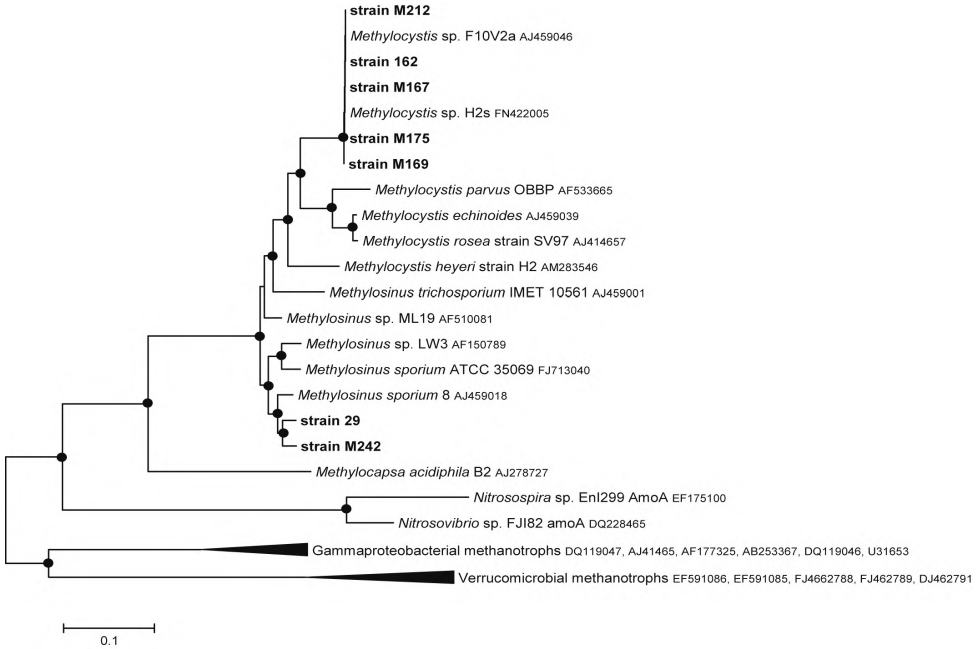

Figure 3 *pmo*A gene phylogenetic tree based on DNA showing the relationship of the isolated strains to selected methanotrophs. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

Physiological properties

The *Methylosinus* related strain 29 showed growth and methane oxidation between pH 3.5 and 7.0, with an optimum at pH 5 to 5.5 and it's temperature range for growth was between 10 and 30 °C with an optimum around 20 to 25 °C. Growth was observed in liquid and solid M2 medium with methanol occurred, but formate, formaldehyde, glucose or acetate did not support growth. This makes strain 29 the first described acidophilic *Methylosinus* strain isolated from *Sphagnum* mosses. Only a neutrophilic *Methylosinus* strain has been previously

isolated from an acidic peat lake (Heyer & Suckow, 1985). However several molecular studies did show the presence of *Methylosinus* spp in peat ecosystems (Dedysh *et al.*, 2003; Chen *et al.*, 2008a).

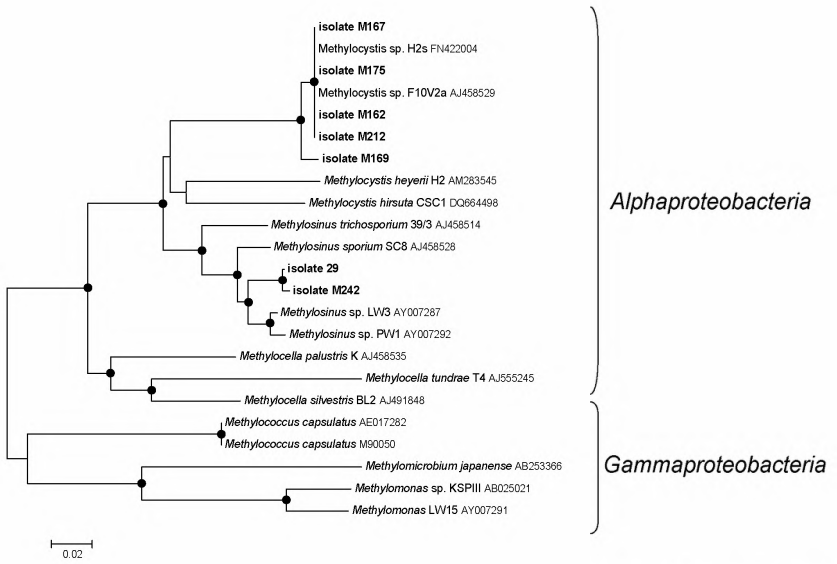

Figure 4 *mmo*X gene phylogenetic tree based on DNA showing the relationship of the isolated strains to selected methanotrophs. The tree was based on Neighbour-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 % are indicated at the node of the branch with a black dot.

The *Methylocystis* related strains M169 and M212 showed growth on liquid and solid M2 medium with methanol, but not with formate, formaldehyde, glucose or acetate. *Methylocystis* strain H2s has been shown to be able to use acetate for survival over long periods of time in the absence of methane and a re-examination of known isolates showed several other *Methylocystis* sp. were also capable of the same survival strategy (Belova *et al.*, 2010). Another new *Methylocystis* strain (SB2) showed slow growth on acetate (Im *et al.*, 2010). Within *Sphagnum* dominated peat bogs the natural pH ranges between 4 and 5, there is a wide temperature-range and a large difference in methane concentrations, which demands flexibility and adaptation of the micro-organisms. Nitrogen fixing capacities were tested by growing the culture on nitrogen-free M2 medium under various oxygen conditions and ethylene formation upon addition

of acetylene. Strain 29 showed growth and ethylene production on nitrogen-free M2 medium at 1, 2, 5 and 10% oxygen. *Sphagnum* dominated peat bogs are limited in all nutrients, also in fixed nitrogen. All strains isolated were able to grow on nitrogen free medium. *Sphagnum* dominated peat bogs are limited in all nutrients, also in fixed nitrogen and the ability to fix nitrogen can be a great advantage in this ecosystem.

Transmission electron microscopy (TEM) showed that both strain 29, M242 and M175 consisted of single coccoid cells that showed a cell wall typical of Gram-negative bacteria (Fig. 5). Cells from both strain 29 and M242 showed the typical sinus shape and were motile. *Methylosinus* related strain M242 showed a small polysaccharide layer or possibly a layer of fimbriae. Cells of all strains showed intracytoplasmic membranes in bundles distributed along the periphery of the cell, which is a typical feature of type-II *Methylocystis/Methylosinus* related methanotrophs (Fig. 5). Both TEM and phase contrast microscopy showed strain 29, M242 and M175 to be pure cultures. In several cases indications of internal cellular storage particles were observed, possibly consisting of polyhydroxy-alkanoates or –butyrates, which are commonly found in methanotrophs.

Strain 29 and M242 both showed the production of spores, visualized with light microscopy and cell cultures remained viable after storage in glycerol stock at -80 ℃, heat-treatment, freeze drying and dessication.

Strain M162, M169 and M175 were not resistant to dessication or freeze drying and no cysts were observed during microscopic examination. However the strains remained viable after heat treatment and storage in glycerol at -80 ℃.

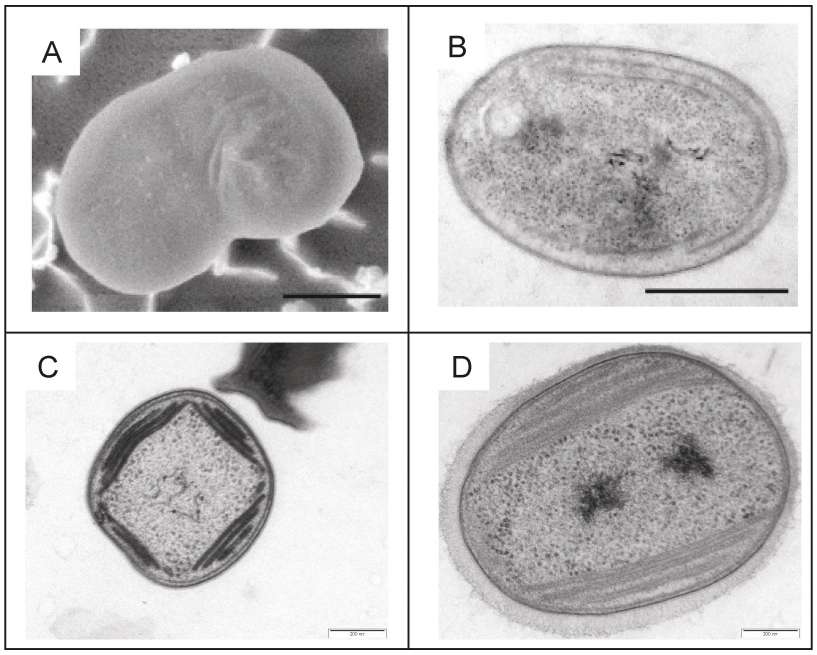

Figure 5. Electron micrographs of high-pressure frozen, freeze-substituted and Epon-embedded cells (A-B) and negatively stained cells (C-D) of strains 29, M242 and M175. **A.** scanning electron micrograph of actively growing cells of strain 29, bar is 5µm. **B.** Transmission electron micropgraph of a cell of strain 29, bar is 5µm. **C.** Transmission electron micrograph of a cell of strain M175. **D.** Transmission electron micrograph of a cell of strain M242

Cellular fatty acids

The proteobacterial methanotrophs possess distinct patterns of phospholipid ester-linked fatty acids (PLFA). Methanotrophic biomarkers are C16:1ω8 and C16:1ω5 for type I and C18:1ω8 for type II methanotrophs (Bodelier *et al.*, 2009).

The major fatty acids of strain 29 and M242 were C16:1ω7, C18:1ω7 and some C16:0 and C18:00 were found (Table 1). Apart from these fatty acids, strain 29 also possessed the hopanoids diplopterol and 2-methyldiplopterol. These were detected as free alcohols. 2-Methyldiplopterol was previously reported to occur in type-II methanotrophs (Summons and Jahnke, 1992). Periodic acid treatment of the total extract resulted in the formation of bishomohopanol and in substantially

smaller amounts of homohopanol, revealing the presence of bacteriohopanetetrol and bacteriohopanepentol derivatives, respectively, which are characteristic for many methylotrophs (Rohmer, 1992).

Table 1 PLFA comparison of strains and other methanotrophic genera. *: combination of c and t measured. R= reference, a: this article, b: Bodelier *et al.*, 2009, c: Dedysh *et al.*,2002, d: Dunfield *et al.*,2003, e: Dedysh *et al.*,2004, f: Dedysh *et al.*,2002, g: Deysh *et al.*,2007.

strain	R	C14:0	C15:0	C16:1 ω8	C16:1 ω7	C16:1 ω6	C16:1 ω5	C16:0	C18:1ω7	C18:0	18: 1 ω8c
Methylosinus strain 242	a	0	0	0	13	0	0	1.5	81.1	4.3	0
Methylosinus strain 29	a	0	0	0	18.8	0	0	1	78.4	1.8	0
Methylosinus sporium sp. KS17	b	0.11			16.1	1.33		1.2	65.67	1.37	
Methylosinus trichosporium sp. SM6	c	0.14			13			1	20.88	0.69	60.33
Methylocella palustris KT	d	0	0.1	0	12.6	0	0.1	5.9	78.6	0.9	0
Methylocella silvestris BL2T	e	0	0	0	8.8		0	3	82.2	1.2	0
Methylocella tundrae (T4T, TCh1 & TY1)	f	2.6-4.1	0	0	7.2-11.3		0	7.2-7.7	59.2-61.7	0.4-0.6	0
Methylocapsa acidiphila B2T	g	0	0	0	4.7	0	0.1	7.3	78.3	7.6	0
Methylocystis heyeri H2T	h	<0.10	0	29	3.39	0.5		1.2	10.86	0.72	31.98

The abundance of C18 PLFA and absence of most of the C16 PLFA in both isolates is in accordance with the type II methanotrophic features. The PFLA distribution of strain 29 and M242 are quite similar to the general PLFA distribution of methanotrophs within the *Alphaproteobacteria* (Table1). Strain 29 and M242 showed highest similarity to the PLFA distribution of *Methylosinus sporium* sp KS17 (Bodelier *et al.*, 2009). All *Methylosinus sporium* isolates, as well as strain 29 and M242 lack 18:1ω8c, while *Methylosinus trichosporium* isolates show an abundance of this lipid.

Detection in environment

Methanotrophic communities of *Sphagnum* mosses and peat soils were previously investigated using a *pmo*A micro-array (Kip *et al.*, 2010) and showed the presence and sometimes abundance of *Methylocystis* and *Methylosinus* spp. Belova *et al* (2010) designed a FISH probe (Mcyst-1273) for the *Methylocystis* strain H2s and showed that this strain represented 17-58% of the microbial population in peat soils of different geographical regions. Although this probe fits (100% match) on strains M162, M167, M169, M175 and M212, FISH on *Sphagnum* plants is rather difficult due to autofluorescent chlorophyll.

In conclusion, this study showed that using M2 medium (Dedysh *et al.*, 1998) only alphaproteobacterial methanotrophs could be isolated. Several isolation techniques resulted in a lot of pure cultures, but unfortunately many were almost identical. Two new *Methylosinus sporium* strains were isolated from *Sphagnum* mosses, strain 29 and M242 which both were able to grow at pH 4.5. All purified isolates showed typical features of type II, alphaproteobacterial methanotrophs. Strain 29 and M242 follow the description of the *Methylsinus* genus as published in Bowman et al. (1993, 2006). They produce spores, are sinus shaped, motile, show inclusions of poly-P-hydroxybutyrate and fix nitrogen. Strains M172, M169 and M175 have similar properties to the genus *Methylocystis* as described by Bowman *et al.* (1993, 2006) and the *Methylocystis* strain H2s isolated from peat (Belova *et al.*, 2010). *Methylosinus sporium* and *Methylocystis* strain H2s were found in other peat ecosystems with molecular surveys and they could play an important role in peat ecosystems. Their isolation and characterization expand our knowledge on methanotrophs and can help us to update the microarray for future molecular surveys.

Materials and methods

Sampling and culture conditions

Sampling sites for *Sphagnum* mosses from the two Dutch acidic *Sphagnum* peat bogs (Mariapeel and Hatertse Vennen) were described in Kip *et al.* (2010). In addition mosses were taken at the nature reserve Parc Naturel Haute Fagne (Belgium; 50°27′0.6"N;

5°55'39"E). *Sphagnum* mosses were thoroughly washed and incubated in 120 ml bottles with 1 ml of methane. After incubation they were either used for dilution series or transferred to a fernbach or a 250 ml bottle with 100 ml filter sterile peat water from the Mariapeel or medium M2. The flasks were closed with a screw cap and a red butyl rubber septum. Upon methane consumption a dilution series was made of the *Sphagnum* moss by crushing the moss with a mortar. Dilutions were made from 10^{-1} to 10^{-9} in medium M2 in 120 ml or 60 ml serum bottle containing 5% liquid medium M2, 5% CO_2 and 1% CH_4 or 5% liquid medium M2 and 1% CH_4. The bottles were sealed with grey butyl rubber septa and methane and carbon dioxide were added before autoclaving.

Bottles were incubated at room temperature on a table shaker at 90 rpm and methane concentrations were measured regularly. Growth and methane consumption was found up to 10^{-7} dilution and aliquots of this dilution were transferred to agarose plates. After colonies appeared, these were restreaked to obtain pure cultures and single colonies, which were used for colony PCR and further culturing.

PCR amplification of the 16S rRNA gene, the pmoA, pxmA and mmoX genes

Single colonies were picked with a sterile toothpick and the same toothpick was transferred to 20 µl sterile MQ. The inoculated MQ was used as a template for PCR. The 16S rRNA gene was amplified with general bacterial primers 616F (Juretschko *et al.*, 1998) and 1492 (Lane, 1991). Both the *pmoA* and the *mmo*X genes were amplified with general primers using the recommended annealing temperatures (Holmes *et al.*, 1995; McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000). *Pxm*A genes were amplified by using the primers *pxm*A230F and *pxm*A732R (Tavormina *et al.*, 2010). The *pxm*A732R cocktail was used to make one degenerate primer *pxm*A732R: 5' TSGCRAACCAYTTRCCGATRTRC 3' and PCR was performed with a gradient from 50-60 °C.

All PCR products were purified using the QIAquick PCR Purifications Kit (Qiagen). DNA sequencing was performed with the primers used in the PCR and for the 16S rRNA gene also the universal bacterial primer 612R (Ehrmann *et al.*, 2003) was used. DNA sequencing was performed by the sequencing facility of the UMC Sint Radboud, Nijmegen. Phylogenetic analyses were conducted with the MEGA4 package (Tamura *et al.*, 2007). Phylogenetic dendrograms were contstructed using the Neighbour-Joining and Maximum Parsony algoritmsas implemented in the MEGA software. Evolutionary distances between sequences were calculated using the DNA and deduced amino acid sequences.

Physiological tests

The pH and temperature optimum tests were performed at least in duplicate with M2 medium. The medium was adjusted to pH 3.5 to 7 by mixing with 0.1 M solutions of H_3PO_4, KH_2PO_4, and K_2HPO_4. Serum bottles (120 ml) containing 12 ml medium were inoculated with an active pre-culture of the isolate. Bottles contained 1-5% (v/v) methane and were sealed with butyl rubber septa and an aluminum cap. After 5-8 days of incubation at room temperature, culture turbidity and methane consumption were analyzed. When no growth occurred within 8 days, the culture was incubated up to 40 days to check for growth. Temperature optimum test was performed at pH 4.7 at 4, 10, 15, 20, 25, 30 and 37℃.

Cultures of different incubation periods were tested for resistance to freeze drying, heat, desiccation and storage in glycerol at -80 ° (Whittenbury *et al.*, 1970a). Viability of the cultures was tested by growth on solid and liquid M2 medium. For the heat treatment the cultures were incubated for 20 min. at 80 degrees. Desiccation resistance was tested by spreading the culture on sterile glass slides and let them dry and cultures were stored in 20% glycerol at -80 °C. All stored cultures were tested for viability of the cells at different time intervals of growth (1, 2, and 4 weeks of growth) and storage (1, 2, 8 and 24 weeks of storage).

Carbon sources

Isolates were tested for growth and methane consumption on M2 medium with methanol (0.5% v/v), acetate (0.1% (w/v) and formate (0.1% w/v) both in liquid medium and on agarose plates. Cultures were incubated for 1 month and monitored daily. Methane consumption and growth by optical density (OD600) were monitored.

Nitrogen fixation

Cultures were tested for growth and methane consumption on nitrogen-free M2 medium with varying oxygen concentrations (20, 10, 5, 2 and 1 %) and methane concentration at 1%. Upon growth on nitrogen-free medium with methane as energy source the cultures were transferred to nitrogen-free medium with 0.1% methanol and the nitrogenase activity assay was performed by adding 2% of acetylene and measuring ethylene production as described before (Khadem *et al.*, 2010).

Analytical techniques

Methane was measured on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh). Turbidity as a measure of growth was analyzed on an Ultraspect K spectrophotometer at 600 nm.

Phospholipid fatty acid (PLFA) determination

Total lipids were obtained from 0.5-5 mg freeze-dried cell material using a modified Bligh and Dyer extraction procedure (Boschker *et al.*, 1998; Bodelier *et al.*, 2009), after which it was separated into three increasingly polar fractions over an activated silicic acid column using chloroform, acetone and methanol respectively. The methanol fraction was subjected to mild-alkaline methanolysis, after which an authentic C19:0 fatty acid standard was added. The fatty acids, released from the phosphate head groups, were methylated using diazomethane. An aliquot of this fraction was used for dimethyl disulfide (DMDS) adduction, as described by Nichols et al. (Nichols *et al.*, 1986) to determine double bond positions of the mono-unsaturated fatty acids. Quantification was performed on a gas chromatograph (HP 6890) equipped with a flame ionization detector (FID) set at constant pressure (100 kPa) using relative retention times. A fused silica column (50 m x 0.32 mm i.d., film thickness 0.1 μm) coated with CP Sil-5CB was used with helium as a carrier gas. Extracts were injected on-column at 70 °C. The temperature increased with 20 °C/min to 130 °C and 4 °C/min to 320 °C followed by an isothermal hold for 20 min. Components were identified using a gas chromatograph–mass spectrometer (Thermo Trace GC Ultra).

Sample preparation for transmission electron microscopy

For strain 29: actively growing cells were collected by centrifugation, embedded in 1% water agar and fixed in 1% OsO_4/2% glutaraldehyde in 50 mM cacodylate buffer (pH 6.5) for 1 h at 4°C. After dehydration in an ethanol series, the samples were embedded in Spurr epoxy resin. Thin sections were cut on a Sorvall MT-5000 Ultra Microtome, stained with 2% (w/v) uranyl acetate in water and then post-stained with lead citrate (Reynolds, 1963). The specimen samples were examined with a JEOL JEM 100 CX-II transmission electron microscope. Cryo-Scanning Electron Microscopy (Cryo-SEM) was performed on active batch cultures to examine cell morphology. A stub with a droplet of culture was frozen in liquid nitrogen. The sample was transferred in a transfer holder under vacuum at liquid-nitrogen temperature to the cold stage at -95°C into a cryo-preparation chamber CT 1500 HF (Oxford Instruments, High Wycomb, UK). The specimen was sputter-coated with 5 nm Pt and conveyed under high vacuum to the cold stage of a scanning electron

microscope equipped with a cold-field emission electron gun (JSM 6300F; JEOL, Tokyo, Japan), analysed and recorded at -180°C using a 5-kV accelerating voltage (Verhoeven *et al.*, 2005).

For strain M242 and M175: actively growing cells were cryofixed by high-pressure freezing, freeze-substituted in anhydrous acetone containing 2% osmium tetroxide, 0.2% uranyl acetate and 1% H_2O or anhydrous acetone containing 2% osmium tetroxide, embedded in Epon resin, sectioned, post-stained and imaged as described previously (van Niftrik *et al.*, 2008). Fixed cells were examined a transmission electron microscope (Tecnai12, FEI Company, Eindhoven, The Netherlands). Images were recorded using a CCD camera (MegaView II, AnalySis).

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Chapter 5

Methane cycle in different *Sphagnum magellanicum* dominated peat bog habitats in Tierra del Fuego.

Nardy Kip, Christian Fritz, Eva Langelaan, Yao Pan, Levente Bodrossy, Alfons J.P. Smolders, Mike S.M. Jetten & Huub J.M. Op den Camp

Submitted for publication

Abstract

Sphagnum peatlands are important ecosystems in the methane cycle. Methanotrophs living in and on the *Sphagnum* mosses are able to act as a methane filter and thereby reducing methane emissions. In contrast to peat ecosystems in the Northern hemisphere peat ecosystems in South America are dominated by one moss species; *Sphagnum magellanicum*, which provides the opportunity to do species-independent comparison studies of different peat bogs. The peat mosses from the different habitats, pool, lawn and hummock, characterized by different *in situ* methane concentrations, were investigated for the activity and diversity of methanotrophs. Potential methane oxidizing activity was found in all *Sphagnum* mosses and a positive correlation was found between activity and *in situ* methane concentration. Although methanotrophic activity of living *Sphagnum* mosses from lawn and hummock was low (<0.5 µmol/gDW/day), the activity of *Sphagnum* litter at the water table, with high *in situ* methane concentrations, was high (23.5 µmol/gDW/day). The methanotrophic diversity was similar in the different habitats and comparable to the methanotrophic diversity found in the Northern Hemisphere. A *pmo*A microarray indicates that both alpha- and gammaproteobacterial methanotrophs was present in all *Sphagnum* mosses, even in the mosses with a low initial methane oxidation activity. Prolonged incubation of *Sphagnum* mosses from lawn and hummock with methane showed the methanotrophic community to be viable and able to respond and adapt within 15 days. This corroborates that in the field the increased methane availability stimulates methanotrophy even in low active mosses. The high abundance of facultative methanotrophic *Methylocystis* species might explain the flexibility and fast adaptation to increased methane concentrations in the system. The insight into these processes is important for our understanding of methane recycling and reduction of emissions towards the atmosphere.

Introduction.

Carbon dioxide and methane are important greenhouse gases and their concentrations are rising rapidly since industrial times (Forster *et al.*, 2007).

Methane and carbon dioxide emissions from peatlands are of importance to the greenhouse effect, but for good prediction models more information is needed about the carbon cycle in peatlands. Micro organisms play an important role in the biogeochemical cycles of these peatlands and the knowledge about the microbial diversity can improve our understanding of the carbon and nutrient turnover of the ecosystem. Within peat ecosystems methane also serves as an important carbon source and methane oxidizing bacteria, methanotrophs have been reported to inhabit these systems. The methanotrophs were shown to be present on and inside *Sphagnum* mosses and can act as a filter for methane, thereby recycling carbon of the system and reducing methane emissions (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010). The methanotrophs live in and on the *Sphagnum* mosses, providing extra carbon dioxide for the moss, which in return provides oxygen to the bacteria. Highest methane oxidation rates have been found in submerged *Sphagnum* mosses from pools, where the mosses are most limited in carbon dioxide (Kip *et al.*, 2010).

Bacterial and methanotrophic communities have been studied in a few *Sphagnum* dominated peat bogs (Opelt and Berg, 2004; Dedysh, 2009), but new discoveries showed that there is still a lot to be discovered (Dedysh, 2009). Microbial communities can be investigated with molecular tools based on the bacterial 16S rRNA genes (Stackebrandt and Goebel, 1994), while methanotrophic communities can be characterized using functional genes like the methane monooxygenase genes, *pmo*A and *mmo*X (Murrell and Jetten, 2009). This enzyme catalyses the first step in the methane oxidation pathway and can only be found in methanotrophs. A *pmo*A based microarray can be used to analyze the methanotrophic community of an ecosystem. Peat bogs are a harsh environment for microbes to live in because of the low pH around 4.5 and the low nutrient content. Methanotrophs occur within the *Proteobacteria* and the *Verrucomicrobia* (Conrad, 2009; Op den Camp *et al.*, 2009). Gammaproteobacterial methane-oxidizing bacteria belong to the type I methanotrophs, which use the ribulose monophosphate pathway for formaldehyde fixation. The type II methanotrophs belong to the *Alphaproteobacteria*, which use the serine pathway for formaldehyde fixation. This genus includes the *Methylocystis-Methylosinus* genera and the acidophilic methanotrophs of the

genera *Methylocella* and *Methylocapsa. Methylocella* species were the first isolated facultative methanotrophs and recently facultative *Methylocystis* and *Methylocapsa* species were isolated as well (Belova *et al.*, 2010; Dunfield *et al.*, 2010; Im *et al.*, 2010). These facultative methanotrophs have been shown to be able to survive a long period without methane in the presence of acetate, an important carbon source in peat ecosystems. This is used as a survival strategy (Belova *et al.*, 2010). The recently described extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum isolated from volcanic areas do not show intracellular membrane structures and they have to be investigated more thoroughly (Op den Camp *et al.*, 2009), however, current molecular techniques used for conventional methanotrophs are not able to detect the verrucomicrobial methanotrophs yet. Most studies have been performed on peatlands in the Northern Hemisphere (Dedysh, 2009) and peatlands in the Southern Hemisphere have so far received less attention. South American peatlands are remote areas without human influence and are showing ecological interesting features (Blanco and de la Balze, 2004; Grootjans *et al.*, 2010). In this study we focused on the methanotrophic activities and microbial communities in *Sphagnum* mosses in peatlands of South America. In the Northern Hemisphere many different *Sphagnum* species occur that cover the different habitats, such as pool, lawn and hummock, but the South American peatlands harbor the same moss species, *Sphagnum magellanicum* that dominates different habitats.

The present study describes methane oxidation activity in *S. magellanicum* of all the various habitats in two different peat bogs in Tierra del Fuego, Argentina. Methanotrophic activity was studied along a gradient of methane availability. The methanotrophic communities of *S. magellanicum* mosses from the different micro habitats in one peat bog were investigated and compared and by the use of several different molecular tools the presence and abundance of gamma- and alphaproteobacterial methanotrophs were shown, in which (facultative) *Methylocystis* species might play an important role.

Results and discussion

Study sites

Samples were taken from two different Patagonian bogs a *Sphagnum* bog, called Andorra and a mixed-cushion bog peatland, called Moat. Average daily air temperatures are 5-6 °C with cold summers around 9°C. Soil temperatures were low and stable at both bogs, throughout the growing season decreasing from 8-12°C at 5 cm below surface to 4-8°C at 100 cm depth. The *Sphagnum* bog was dominated by *Sphagnum magellanicum* (Bridel) with less than 1% cover of vascular plants. *Sphagnum magellanicum* occupies all hydrological niches from pools to hummocks which rise up to 1 meter above the summer water table. In the mixed-cushion bog *Sphagnum magellanicum* mosses occupy margins of pools and also form small lawns (few square meters) embedded in a matrix of evergreen cushion plants (Roig and Collado, 2004). Dominating cushion plants were *Astelia pumila* (G. Forster) R. Br. and *Donatia fasciculares* R.R. et G. Forster covering more than 70%. Pore water analysis reflected acid conditions in both bogs (pH 3.5-4.5). The peatlands studied remained unaffected by anthropogenic alteration such as drainage, agricultural use or elevated atmospheric nutrient deposition. Methane oxidizing activity and emission were investigated in these two contrasting peat bogs. In the Andorra peat bog the *Sphagnum* associated methanotrophic community in and on *Sphagnum* was characterized by molecular techniques.

Methane oxidizing activity tests

Both peatlands showed the typical pool, lawn and hummock micro habitats and were all mainly covered with the same *Sphagnum* species, *Sphagnum magellanicum*, which makes the comparison between different habitats and peat bogs species-independent. Methane oxidizing activities of *Sphagnum* mosses of the two different peat ecosystems were determined (Table 1). Initial methane oxidizing activity of living *Sphagnum* mosses showed to be the highest in pools and very low in hummock and lawns. Highest activity was found in mosses collected from pools in Andorra, which are rich in methane (24 µmol CH_4/day/g DW). Low methane oxidizing activity was found in *Sphagnum* mosses from

hummock and lawns. In Moat the pool is depleted of methane and the methane oxidizing activity was low (0.5 µmol CH_4/day/g DW) and comparable to the drier habitats like lawns and hummocks. The differences in methane oxidation rates between micro habitats and between peatlands show a clear positive correlation between the methane concentration in the pore water and the initial methane oxidation rates in the tested mosses. Water level is an important factor in the methanotrophic activity of *Sphagnum* mosses (Kip *et al.*, 2010), which also influences the *in situ* methane concentrations. In this case the methane concentration was shown to be of even more importance. Pore water methane concentrations in Moat pools were much lower then in Andorra pools, resulting in lower methane oxidation rates at comparable water levels. Highest methane oxidation was found at 20 °C and these rates were double the rates at 10 °C, resulting in a Q10 of around 2. Within the Andorra pools *S. magellanicum* is most abundant, however another *Sphagnum* species was also found, *S. falcatulum*, which also showed methane oxidizing activity, which was lower compared to *S. magellanicum*, again correlated with the pore water methane concentration. Since submerged *Sphagnum* mosses showed highest methane oxidizing activity *Sphagnum* litter samples were taken from the lawn and hummock in Andorra peat at 5 and 55 cm below the water level for lawn and 10 cm below the water level for the hummock sample. These samples showed a high pore water methane concentration and both methane oxidation and methane production were found in these samples (Fig.1).

Methane oxidation of *Sphagnum* litter from the lawn showed methane oxidation rates between 5 to 11 µmol CH_4/day/g DW at 10°C and *Sphagnum* litter from hummock showed rates around 4 µmol CH_4/day/g DW at 10°C.

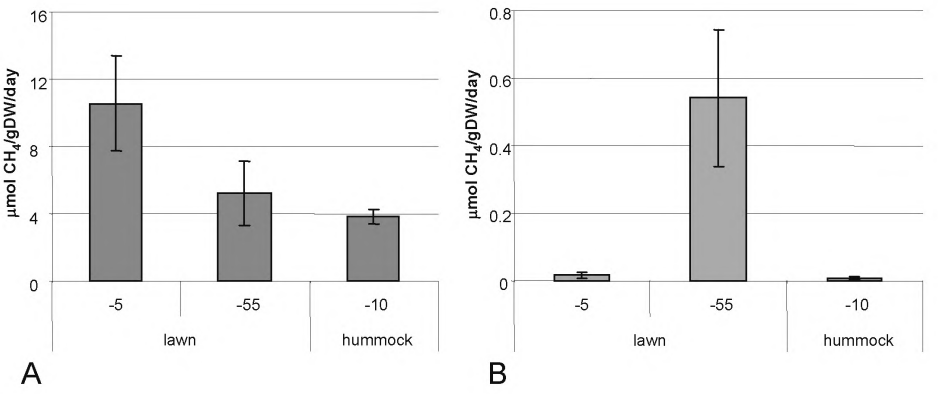

Figure 1. Potential methane oxidation and production of *Sphagnum* litter from Andorra at 10°C. A. Methane oxidizing activity of *Sphagnum* litter in lawn and hummock at different depths, in relations to the water table. B. Methane production rates of *Sphagnum* litter in lawn and hummock at different depths, in relations to the water table. N=4 per depth.

These methane oxidation rates show again a correlation between methane oxidation rates and methane concentrations *in situ*, indicating that the methanotrophic activity in lawn and hummocks occurs at greater depth than in the pools where methane oxidation is found at the surface.

During the year water levels fluctuate in a peat ecosystem and this influences the methanogenic and methanotrophic activities. At these depths the oxic and anoxic conditions constantly fluctuate and therefore also methane production rates were measured. Methane production rates were higher at -55 cm than at -5 cm in the lawn *Sphagnum* litter and were around 0.5 µmol CH_4/day/g DW at 10°C at -55 cm. Submerged *Sphagnum* litter from the hummock showed a methane production rate of 0.07 µmol CH_4/day/g DW at 10°C.

Table 1. Overview of data obtained at the Moat and Andorra peat bogs. Depth indicates the sample depth above or below (-) the water level at the sampling day and mean depth is the sample depth according to the mean water level.

Peat land	**Micro-habitat**	***Sphagnum* species**	**Depth**	**Mean depth**	**[CH_4]**	**Water content**	**Bulk density**	**CH_4 Emission**	**pH**	**CH_4 oxidation rates at 10 °C**
			Cm	**cm**	**mg CH_4/l**	**%**	**g l-1**	**mg $CH_4/m^2/d$**		**µmol CH_4/gDW/da**
Andorra	pool	*S. magellanicum*	-1	-1	35.4 ± 10.9	96.8± 0.2	13.7 ± 1.6	7.6 ± 3.5	4-4.3	23.5 ± 20
Andorra	pool	*S. falcatulum*	-1	-1	9.4± 8.6	97.8± 0.36	5.0 ± 0.3	ND	4-4.3	8.6 ± 3.2
Andorra	lawn	*S. magellanicum*	15	25	1.2± 1.0	94.0± 0.4	24.5 ± 1.6	1.7 ± 1.6	3.5-4.2	0.22 ± 0.25
Andorra	hummock	*S. magellanicum*	50	70	NaN	90.7± 0.15	26.5 ± 3.3	ND	3.5-4.2	0.01 ± 0.005
Moat	pool	*S. magellanicum*	-1	1	1.4 ±1.5	97.7± 0.16	19.4 ± 3.0	5.1 ± 5.9	3.6-4	0.47 ± 0.53
Moat	lawn	*S. magellanicum*	10	8	1.0 ± 0.9	94.8± 0.29	28.3 ± 6.8	5.6 ± 4.7	3.4-4	0.25 ± 0.38
Moat	hummock	*S. magellanicum*	30	25	NaN	91.5± .47	45.5 ± 2.3	ND	3.4-3.6	0.04± 0.0006
Andorra	Lawn	litter *S. magellanicum*	-15	-5	110.6 ± 55.9	92.4	22.3	ND	4-4.5	
Andorra	lawn	litter *S. magellanicum*	-65	-55	657.4 ± 239.8	94.6	24.4	ND	4.3-4.6	
Andorra	hummock	litter *S. magellanicum*	0	-10	23.1 ± 6.3	92.9	28.4	ND	ND	

The differences in methane production rates at these different depts is most probably due to the water level fluctuations that make the 5 cm deep litter sometimes anoxic and sometimes oxic, which greatly influences the oxygen-sensitive methanogens. Methane is both produced and consumed at 55 cm below the water level and remaining methane diffuses upwards where methane production decreases and consumption increases. The production and consumption activities at the same sites show the flexibility of the system to oxic and anoxic conditions, under both conditions microbes take their opportunities and this shows that there is not a real fixed aerobic-anaerobic interface restricting the microbial activities to a certain surface within a peat ecosystem, like e.g. in a rice field (Luke *et al.*, 2010).

Induced methane oxidation in lawn and hummock

Sphagnum mosses from lawn and hummock showed low methane oxidation rates in comparison to pool mosses. Water levels and methane concentrations fluctuate in the lawns and parts can be inundated or dry in different periods of the year. In the Patagonian hummocks mosses grow up to 1 meter above the water level and are very dry. Upon longer incubation of lawn and hummock mosses, methane oxidation increased and methanotrophic growth was observed (Fig. 2 and Table 1). This shows that a viable opportunistic methanotrophic community is present, which is able to quickly respond to changing conditions. Lawn mosses were able to respond within 10 to 15 days while hummock mosses showed a slower response (i.e. at least 3 weeks). Figure 2B shows an exponential increase in methane consumption, indicating methanotrophic growth. These data show the flexibility of these methanotrophic species is large with a relative fast response to more opportunistic conditions. Methanotrophs present in lawn reacted faster to the methane provided than those in hummocks, most probably due to the *in situ* fluctuations that are bigger in lawns than in hummocks.

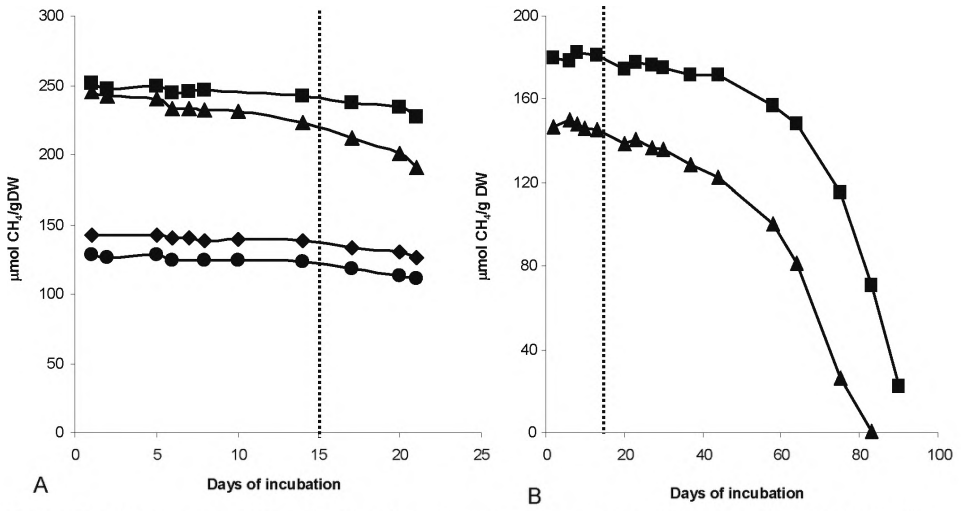

Figure 2. Methane consumption by *Sphagnum* mosses from different microhabitats. A *Sphagnum* mosses from Andorra. Closed square and closed triangle: two different moss samples from the lawn micro habitat, closed diamant and closed circle: two different moss samples from the hummock micro habitat. B. *Sphagnum* mosses from Moat. Filled square and filled triangle: two different moss samples from the lawn micro habitat.

Methane emission

Methane emission rates of both peatlands were generally low, reflecting low summer temperatures, low carbon turnover caused by the nutrient poor *Sphagnum* peat and high methane oxidation rates. Emission rates were found to be the highest in pools (>300 µmol $CH_4/m^2/d$) in the *Sphagnum* bog (Andorra) coinciding with highest methane pore water concentrations (Table 1). Emissions were substantially lower when surface peat was depleted in methane and water levels well below the surface allowed oxygenation of surface peat. Lowest emissions were found in the lawn in the *Sphagnum* bog (Andorra). At this site average water table was 5.5 cm deeper and vascular plant cover was extremely low (50-100 shoots/m^2) compared to the lawn in Moat (500-800 shoots/m^2). Two measurements even revealed a net consumption of 5.1 µmol CH_4 m^{-2} d^{-1} and 252 µmol CH_4 m^{-2} d^{-1}, respectively. In the Andorra pool the methane concentration is approximately 30 times higher then in the lawn, but emissions are only 4.5 times higher. This difference can be explained by methanotrophic consumption, thereby significantly reducing methane emissions to the atmosphere. At lawn sites the

layer of potential methane oxidation is much thicker, starting at the highly active methanotrophic *Sphagnum* litter to a low activity by methanotrophs in the *Sphagnum* mosses in the aerated part. Therefore lawns of *Sphagnum* mosses act as a methane consuming sponge (Daulat and Clymo, 1998). Methane emission is dependent on production, consumption and transport types. Methane and oxygen availability determine presence and activity of methanotrophs and water level is an important regulator of both methane and oxygen and can partly explain the methanotrophic activities found in *Sphagnum* mosses and litter. (Daulat and Clymo, 1998; Kip *et al.*, 2010; Larmola *et al.*, 2010). However, if methane concentrations are low in the ecosystem, e.g. Moat peatland, overall methanotrophic activity is low and independent of the water level. Availability might also be spatially more variable than in many other wetlands. Wet *Sphagnum* bogs decompose slowly, resulting in wide pores for infiltrating rain in Patagonia (Kleinebecker 2008, Price & Whittington 2010). This slow decomposition, even above the water table, causes a low oxygen demand (<100 µmol/l soil/d, Fritz, personal communication). Therefore, oxygen can episodically be present at depts below the water table providing growing conditions for methanotrophs in water saturated habitats (e.g. litter).

Methanotrophic community analysis

Methanotrophic bacteria inside and on *Sphagnum* mosses play an important role in the carbon recycling in peat bogs by reducing methane emissions to the atmosphere (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010). The methanotrophs in and on *Sphagnum* mosses benefit from a stable environment along the methane gradient and are provided with oxygen and the *Sphagnum* mosses benefit from extra carbon dioxide for photosynthesis and therefore the methanotrophic community inside and strongly attached on the mosses were investigated. Since so far studies have only looked at peatlands in the Northern Hemisphere, it would be interesting to get an insight into the diversity of the methanotrophs in the *Sphagnum* mosses and peat litter from the different habitats in the Southern Hemisphere. This was investigated using a functional gene marker encoding for methane mono oxygenase, called *pmo*A. A clone library was made from the *Sphagnum* pool mosses of Andorra: 14 clones belonged to the *Alphaproteobacteria* and 2 clones belonged to the *Gammaproteobacteria.*

Thirteen clones showed highest homology to *Methylocystis* spp. (>92%, DNA homology), one clone showed highest homology to *Methylocapsa acidiphila* and two clones showed 80% homology to isolated *Methylomonas* spp. based on DNA sequences. The clones phylogenetically cluster with *Methylomonas* strain M5 (see chapter 3) and they showed 87 and 88% homology to an mRNA based environmental clone (EF644619) from *Sphagnum* peat soil from the United Kingdom (Chen *et al.*, 2008a) (Fig. 3).

The *Methylocystis* related clones phylogenetically cluster together with the facultative *Methylocystis* species. All isolated methanotrophs from peat soil belong to the *Alphaproteobacteria*, among which the *Methylocapsa* spp. and *Methylocystis* spp., of which the later is commonly found in all peat ecosystems (Dedysh, 2009).

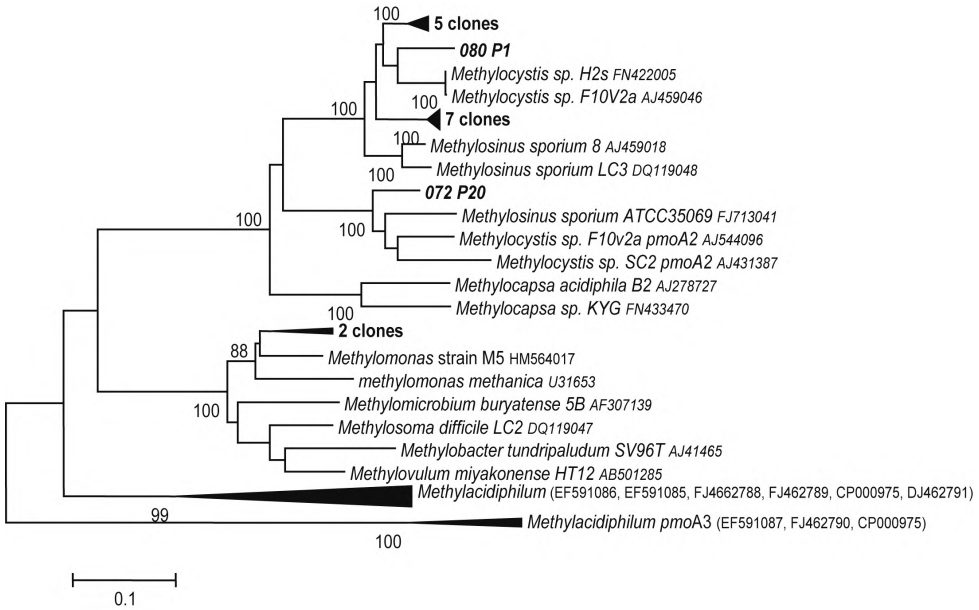

Figure 3. *pmo*A gene phylogenetic tree based on DNA showing the relationship of the clones to selected methanotrophs. The tree was based on Neighbor-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >80 are indicated at the node of the branch.

The *Methylocystis* spp. are abundantly present in this *pmo*A clone library, which correlates to other studies from peatlands (Dedysh, 2002; Chen *et al.*, 2008b). The two gammaproteobacterial clones might indicate the presence of a new species in this ecosystem. None of the isolated gammaproteobacterial methanotrophs are able to grow below pH 5.5 (Bowman, 2006) and using molecular studies they have not been found in other acidic ecosystems, except the mentioned peat ecosystem harboring a new type I methanotrophs (Chen *et al.*, 2008a). So far only a few acidophilic methanotrophs have been isolated from peat and this has been a great effort. However isolations would give us a better insight into new methanotrophic species and these results show possibly undiscovered methanotrophs in this peat ecosystem.

In order to screen the methanotrophic communities of more samples in a faster way a microarray method was used.

pmoA microarray analysis

The method to investigate methanotrophic communities is based on a *pmo*A microarray (Bodrossy *et al.*, 2003), which represents *pmo*A gene sequences of all cultured and non-cultured methanotrophs known. The *pmo*A PCR products from the two pool and hummock samples and the litter samples of lawn (-55 cm below the water level) and hummock (-10 cm below the water level). As exemplified by Figure 4 all the mosses showed a high rather similar methanotrophic community, as revealed by a similar hybridization patterns on the microarray of the different samples. The total *pmo*A diversity of the *Sphagnum* mosses was very high (Fig.4) compared to other studies using the same microarray on peat soils (Chen *et al.* 2008a), peat based upland soils (Cebron *et al.* 2007) and rice fields (Vishwakarma *et al.* 2009), but quite similar to the methanotrophic communities in *Sphagnum* mosses from other peat bogs around the world (Kip *et al.*, 2010). The microarray results showed abundance of both type I (*Gammaproteobacteria*) and type II (*Alphaproteobacteria*) methanotrophs and the presence of many different species, which were not found before in other studies. The submerged S*phagnum* mosses, *S. magellanicum* and *S. falcatulum*, showed a higher hybridization with the type II probes over type I. The two different *Sphagnum* species showed some differences in hybridization, where *Sphagnum falcatulum* seems to have less or no

hybridization with probes related to *Methylomonas* sp. which are showing hybridization in *Sphagnum magellanicum*.

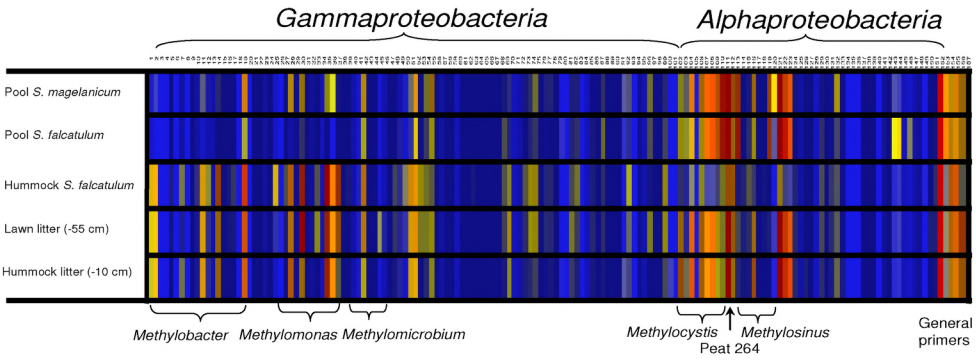

Figure 4. Representation of the results for the *pmo*A based microbial methanotrophic community analysis microarray. Colour coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). A larger version of figure 6 can be found in the appendix and a list of the probes in the respective order is given in Appendix Table S1.

Activity of methanotrophs in *Sphagnum* was shown to be species-dependent based on functional moss characteristics related to water-holding capacity, water content and growth density (Larmola *et al.*, 2010). Variable configuration of hyaline cells, water storage ability and connectivity to methane containing pore water may play an important role in the diversity of methanotrophs in different *Sphagnum* species.

The pool mosses and litter samples showed a similar hybridization pattern indicating a similar methanotrophic community at the surface as below the water table, the type II methanotrophs seem to be more abundant in the litter of lawn and hummock. The type II probes showed that both *Methylocystis* sp. and *Methylosinus* sp. were present in all the *Sphagnum* mosses, although the hummock *Sphagnum* mosses showed a much lower abundance compared to all other samples. The microarray confirms the presence of methanotrophs in the hummock which showed very low initial methane oxidation, but upon longer incubation both lawn and hummock mosses showed an increase in

methanotrophic activity. Unfortunately there was not enough PCR product from the lawn mosses to perform the microarray. The methanotrophs detected in the hummock mosses could be present in a dormant state, waiting for the right growth conditions to come along. The hummock sample is the only sample that showed low methane oxidizing activity and it could be a strong indication the *Methylocystis-Methylosinus* species are responsible for methane oxidation. Other studies using the same microarray (Chen *et al.*, 2008a; Chen *et al.*, 2008c) showed that *Methylocystis* sp. are abundantly present in peat soils. Also, the presence of *Methylocystis* sp. was demonstrated in the *pmo*A clone library of the pool samples. Furthermore, a probe targeting a group of uncultivated type II methanotrophs from a peatland, Peat264 hybridized with the PCR product. Type II methanotrophs are commonly found in peat- and other ecosystems and several acidophilic methanotrophs have been isolated (Dedysh, 2009). The microarray also showed a strong signal with type Ia probes, which target the gamma-proteobacterial genera *Methylomonas*, *Methylobacter* and *Methylomicrobium*. A broad diversity of *Methylomonas* and *Methylobacter* was detected, except in the *S. falcatulum*. Two clones of the *pmo*A microarray were distantly related to *Methylomonas* species, which might still be able to hybridize with the *Methylomonas* related probes. The type Ib specific probes, which target the thermotolerant and thermophilic gammaproteobacterial genera *Methylococcus*, *Methylothermus* and *Methylocaldum* showed a weaker signal, but still indicated a diverse type Ib community across the analyzed samples. As mentioned before no gammaproteobacterial methanotrophs have been detected in acidic ecosystems before which indicated that these are most probably new methanotrophic species and thus far no gammaproteobacterial methanotroph has been isolated from peat.

In general methanotrophs are believed to be obligate methanotrophs, however several recent isolates have been shown to be facultative (Dedysh *et al.*, 2005; Belova *et al.*, 2010). Using two different molecular tools the presence of *Methylocystis* species has been shown in all the *Sphagnum* mosses and litter from the peat ecosystem. Several *Methylocystis* species are facultative methanotrophs and have been recently shown to be able to grow or survive on acetate (Belova *et al.*, 2010; Im *et al.*, 2010), which would be a great advantage in a peat ecosystem since methane and acetate are generally both present, but their concentrations can fluctuate, especially with the water level. *Methylocystis* species can live on acetate

in times of low to zero methane concentrations, while other obligate methanotrophs have to survive using different strategies like forming spores or capsules (Whittenbury *et al.*, 1970; Bowman *et al.*, 1993; Rothfuss *et al.*, 1997). Another advantage of *Methylocystis* species is the presence of two different *pmo*A genes which have different affinities for methane (Baani and Liesack, 2008). Most methanotrophs harbor only one single type of pMMO, defining whether it is able to grow under high or low methane concentrations and this seemed to be species specific, until the *pmo*A2 gene was found in *Methylocystis* species. The presence of *Methylocystis* species makes the ecosystem flexible and is able to work under high, low and zero methane conditions and able to react fast to new conditions.

Methylocella sp., which are commonly found in Northern peatlands (Dedysh *et al.*, 2000; Dedysh *et al.*, 2004) are the only known methanotrophs that do not have a *pmo*A gene and are therefore not detected in this microarray. Detection of *Methylocella* spp. by screening with *mmo*X based PCR on the two pool samples did not result in any PCR product, indicating *mmo*X-possessing methanotrophs are not abundantly present (Kip *et al.*, 2010) or that the PCR primers are not suitable. The PCR primers used are not able to amplify Verrucomicrobial *pmo*A for which no general primers have been developed yet. To look at the general bacterial community and the presence of methanotrophs that could be missed with the *pmo*A screening a 16S rRNA clone library was performed.

Bacterial communities in Sphagnum mosses

To investigate the total bacterial community a clone library based on the 16S rRNA gene was performed of the two pool *Sphagnum* mosses from Andorra peat, *S. magellanicum* and *S. falcatulum* respectively. Both 16S clone libraries showed the presence of *Alphaproteobacteria* (17%), *Verrucomicrobia* (13%) and *Gammaproteobacteria* (2%) (Fig. 5) and since the distribution of bacterial genera among the two species was comparable an average was made.

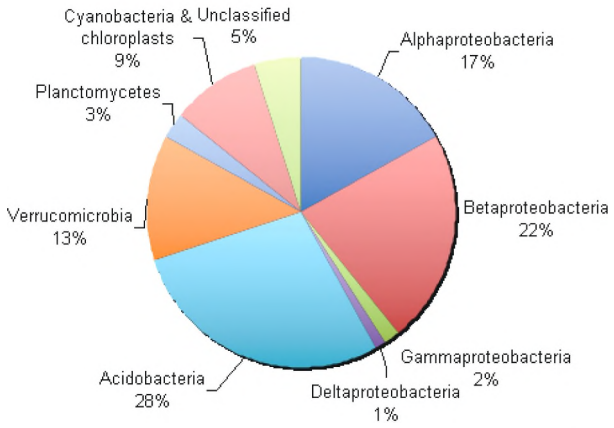

Figure 5. Phylogenetic classification from two 16S rRNA clonelibraries from Patagonian *Sphagnum*.

The 16S clone libraries showed a very diverse set of bacteria to be present inside or on *Sphagnum* mosses. Compared to other studies the microbial community in *Sphagnum* peat soils (Opelt and Berg, 2004; Dedysh *et al.*, 2006; Kulichevskaya *et al.*, 2007a) is comparable to the microbial community found here, inside and attached on the *Sphagnum* mosses of the Patagonian peatlands. Most of the clones showed sequence similarity to isolates or environmental samples originating from peat ecosystems, of which most of them originate from Siberian acidic peat bogs. This indicated that similar bacterial communities can be found in peatlands in the Northern and Southern hemisphere implying there is no big geographical difference in microbial diversity in peat bogs. Four out of five classes of *Proteobacteria* were present in the 16S rRNA clone library; *Alfa-*, *Beta-*, *Gamma* and *Deltaproteobacteria.* 42 % of the clones belonging to the *Alphaproteobacteria* showed a 96-97% to *Acidophaera rubrifaciens*, a member of the *Rhodospirullales* an acidophilic bacteriochlorophyll-producing bacterium isolated from acidic hotsprings and mine drainage (Hiraishi *et al.*, 2000). This bacterium is closely related to *Acidosoma sibirica*, an acidophilic bacterium isolated from a *Sphagnum* peat bog (Belova *et al.*, 2009). Other clones also belonged to the *Rhodospirullales* family, of which several showed high homology to isolates from peat bogs, like *Telmatospirillum siberiense* (Sizova *et al.*, 2007). Another 42% of the clones of the *Alfaproteobacteria* belonged to the order of

Rhizobiales, to which also all isolated methanotrophs belong of which four clones show high homology (98-99%) to the methanotrophic symbiont of *Sphagnum* found in the Mariapeel (Raghoebarsing *et al.*, 2005). Unfortunately this methanotrophic symbiont has not been cultivated yet. Only a few methanotrophs have been isolated from peat ecosystems, all belonging to the *Alphaproteobacteria* but no close relatives to those were found in the clone library. There is a possibility more unknown methanotrophs belong to this order, but so far these 16S rRNA sequences cannot reveal a metabolic importance of the obtained clones. From other environments methanotrophs have been isolated belonging to the *Gammaproteobacteria* and *Verrucomicrobia*, but so far none from peat ecosystems and none of the clones showed high homology to other isolates methanotrophs of these classes. The clones belonging to the *Verrucomicrobia* were almost all highest related to *Opititus* spp. of which some were isolated from rice fields, they have never been demonstrated to oxidize methane (Janssen *et al.*, 1997). Only three verrucomicrobial methanotrophs are known and all are thermophilic and originating from volcanic regions, none of the clones were closely related to these methanotrophs. As shown with the microarray more often relatives to thermophilic methanotrophs can be detected in peat ecosystems, which might indicate the presence of new methanotrophic species. Verrucomicrobial methanotrophs are recently discovered and opened new possibilities to find new and unknown methanotrophs in all methane rich ecosystems. Two clones belonged to the *Deltaproteobacteria* being closest relative to *Bdelvibrio bacteriovorus* and *Spirobacillus cienkowskii*, both pathogens. Pathogens have been shown to occur abundantly in *Sphagnum* (Opelt *et al.*, 2007), but so far their presence in these ecosystems has not been understood. Besides these classes an abundance of *Acidobacteria* (28%) and *Betaproteobacteria* (22%) was found, both classes have been found to occur in peat ecosystems, but their role has not been discovered yet. Belova and coworkers (Belova *et al.*, 2006) described the presence of *Burkholderia* species, belonging to the *Betaproteobacteria* in Western Siberian, Canadian and Estonian peat bogs and Opelt and coworkers showed the abundance of the genus *Burkholderia* of endo and ectophytic bacterial populations in bryophytes (Opelt and Berg, 2004; Opelt *et al.*, 2007). These *Burkholderia* species are antagonistic bacteria with antimicrobial activity, which is thought to be used by the *Sphagnum* mosses as a

defense strategy against fungi (Opelt *et al.*, 2007). *Acidobacteria* have been detected and isolated from peat (Pankratov *et al.*, 2008; Kulichevskaya *et al.*, 2010), where they most likely grow on various heteropolysaccharides and galacturonic acid, which are released during decomposition of *Sphagnum* moss and vascular plant debris. Three percent of the clones belonged to the *Planctomycetes,* which are often detected in *Sphagnum* dominated peatlands (Dedysh *et al.*, 2006; Kulichevskaya *et al.*, 2006) and several have been isolated (Kulichevskaya *et al.*, 2007b; Kulichevskaya *et al.*, 2008; Kulichevskaya *et al.*, 2009) and several clones show homology to these isolates. A few clones belonged to the *Bacteriodetes*, showing sequence similarity to the *Sphingobacteria* genus containing *Mucilaginibacter* spp., which were isolated from a *Sphagnum* peat bog. This species was shown to degrade pectin, xylan and laminarin in peat. The 16S rRNA clone library was able to detect a broad variety of bacteria, which is comparable to other studies. However, no clones were found to be related to isolated methanotrophs and only a few clones were related to the symbiotic methanotroph from *Sphagnum* moss. The used primer set is a general primer set that, however, does not always anneal to DNA isolated from methanotrophs (Kip, personal communication), which could explain why methanotrophs were not represented in this clone library. It could be that the used primer set does not detect methanotrophs in environmental samples or is biased against methanotrophs. Described 16S rRNA clone libraries performed on peat also showed only a few clones with homology to known methanotrophs (Dedysh *et al.*, 2006; Raghoebarsing, 2006). Another explanation for the lack of methanotrophic sequences in this clone library could be that there are more unknown methanotrophs present that are not detected by the primer set or the DNA isolation technique is not able to extract methanotrophic DNA as easily as DNA of other bacteria. The primer set does anneal to Verrucomicrobial methanotrophs, which were neither found within these clone libraries.

Final conclusion and discussion

The Patagonian peat bogs were ideal for this study since the peat bogs are pristine and the different micro habitats are harbouring the same *Sphagnum* species: *S. magellanicum.* This study revealed a high activity and diversity of

methanotrophic bacteria in Patagonian *Sphagnum* bogs. High activity is correlated with high methane concentrations and the water level and methanotrophic activity reduced methane emissions to the atmosphere. Methane oxidation in pools takes place in the floating rafts of *Sphagnum* mosses, where they are provided with oxygen and the mosses get carbon dioxide of the methanotrophs in return. In the lawn and hummock the oxygen-methane levels are different and we showed that methane oxidation takes place deeper in the *Sphagnum* layer around and below the water table, where methane concentrations are higher and even methane oxidation and production were found at the same place. Under normal conditions oxygen penetrates to about 10 cm below the water table. Input of oxygen into the system can come from heavy rainfall events or buoyancy flow (Rappoldt *et al.*, 2003). Wet *Sphagnum* bogs decompose slowly resulting in wide pores for infiltrating rain in Patagonia. This slow decomposition causes a low oxygen demand (below 100 µmol l^{-1} soil d^{-1}, Fritz, personal communication). Therefore oxygen can be present at depts below the water table enabling methanotrophs to function.

Low methane oxidation was measured in the upper layers of the lawn and hummock, but after a longer incubation period methane oxidation was detected. A snow and ice cover during winter can lead to a substantial increase in methane availability allowing for episodic methane oxidation (Tokida *et al.*, 2007; Gazovic *et al.*, 2010). The microarray showed the presence of methanotrophs in the hummock mosses and the diversity of methanotrophs was between almost all the samples was surprisingly comparable considering the origin of all the different micro habitats where methane and oxygen concentrations are very different.
Bacterial and methanotrophic communities in peatlands from the Southern hemisphere are comparable to those on the Northern hemisphere. The same families and classes were found which does not exclude the presence of new strains or species. The *pmo*A clone library showed the presence of new unknown methanotrophs.

The high abundance of *Methylocystis* spp. indicates they are very probably the key players in the ecosystem. The capacity to use acetate as a carbon source and to consume methane at both high and low concentrations might

represent an important part of the survival strategy of these kind of methanotrophs in peatlands and might explain why they are found in abundance in all *Sphagnum* dominated peatlands and other ecosystems (Belova *et al.*, 2010). *Sphagnum* mosses have been shown to release substantial amounts of compounds such as phenolic compounds, amino acids, carbohydrates, hydrocarbons and fatty acids (Fenner *et al.*, 2004) which could stabilize (facultative) methanotrophic communities during low methane conditions. The flexibility and fast adaptation of the methanotrophic symbionts and mosses may differ substantially between *Sphagnum* species. The cooperation between methanotrophs and *Sphagnum* species make the ecosystem flexible since they are able to adjust to any circumstances and conditions, like shifts in methane or oxygen concentrations. More research is needed to understand this plant-microbe interaction and their role within the ecosystem. Many methanotrophs are able to fix nitrogen (Bowman *et al.*, 1993) and this could be another important survival strategy which is important in these pristine peat ecosystems with very low nitrogen deposition (Aldous, 2002).

Materials and methods

Study sites description

The two used sites: the pure *Sphagnum* bog, called Andorra (54° 45' S; 68° 20' W, 200 m a.s.l.) and the mixed-cushion bog peatland, called Moat (54° 58'; 66° 44' W, 40 m a.s.l.). Average daily air temperatures are 5-6°C with cold summers around 9°C. July is usually the coldest month with 2°C. Soil temperature was low and stable at both bogs, through out the growing season decreasing from 8-12°C at 5 cm below surface to 4-8°C at 100 cm depth. The *Sphagnum* bog was dominated by *Sphagnum magellanicum* (Bridel) with less than 1% cover of vascular plants like *Empetrum ssp.*, *Nothofagus ssp.*, *Rostkovia ssp*, *Carex ssp.*, *Marsipospermum ssp* and *Tetroncium ssp. Sphagnum magellanicum* occupies all hydrological niches from pool to hummocks rising up to 1 meter above the summer water table (Baumann 2006). Other *Sphagna* like *Sphagnum falcatulum* (*S. cuspidatum* coll.) and *Sphagnum cuspidatum* co-exist only in pools (cf. Kleinebecker 2007). In poor fens *Sphagnum fimbriatum* can be found next to *S. magellanicum* (Köpke 2005, Grootjans 2010). In contrast, the mixed-cushion bog consists of little *Sphagnum magellanicum*. The *Sphagnum* mosses occupy margins of pools and form also small lawns (few square

meters) embedded in a matrix of evergreen cushion plants (Roig & Collado 2004, Gebser 2008). Dominating cushion plants were *Astelia pumila* (G. Forster) R. Br. and *Donatia fasciculares* R.R. et G. Forster covering more than 70%. The soil below cushion plants is densely packed with tap roots (1-2 mm diameter) and fine roots exceeding depths of 120 cm causing thorough methane oxidation (Grootjans *et al.* 2010, Fritz unpublished data). Lawns of *Sphagnum magellanicum* hosted abundantly vascular plants compared to the *Sphagnum* bog. Pore water reflected acid conditions in both bogs (pH 3.5-4.5). Peatlands studied remained unaffected by anthropogenic alteration such as drainage, agricultural use or elevated atmospheric nutrient deposition.

Pore water methane concentration and pH measurements

Pore water was sampled at depths of 5-10 cm from pools and lawns prior collecting moss and litter samples for incubation. For deeper samples we estimated methane concentrations by adding/substracting the linear increase (some 11 µmol CH_4/ l/cm depth) to methane concentrations determined 30 cm below the water level. Anaerobic peat water samples were taken using 5 cm ceramic cups (Eijkelkamp Agrisearch Equipment ®, Giesbeek, the Netherlands), connected to vacuum infusion flasks (40 ml) after sampling 150 ml to exclude internal stagnant sampler water. The 40 ml glass infusion flasks had a sample-head space ratio of usually 1:2. As internal standard 1 ml of ultra pure ethane gas (Airliquide ®) was added after sampling and flasks were stored at 4°C during less than 2 weeks until analysis. Microbial modification of samples was hampered by adding 0.1 mg $HgCl_2$ (0.1 ml of 0.1 %). Methane and ethane concentrations were measured in the head space after vigorous shaking releasing >96% of methane to the headspace. Values of pH were determined one day after collection using a handheld (Consort ® C933) and a standard pH electrode (SP10T). Additional water samples were drawn with 60 ml-syringes.

Methane emissions

Methane emissions were assessed using dark static PVC chambers (3700 cm^3, 15 cm high) with bleeds of 4 mm PVC hose. PVC frames were installed 2 months before measuring and removable chamber tops were sealed to the frame by the water filled rim. At lawns in the Sphagnum bog lawns two collars were installed (different site than earlier reported, Fritz unpublished) and one collar at pool sites. Gas samples were taken in the morning and in the afternoon at the same day at all sites per peatland. Sampling took place in December 2008 (spring), February 2009 (summer) and late March 2009 (autumn) allowing for some variability in water level. Temperature differed less than 3 K during

sampling. Gas samples where taken with a double sided needle for 60 minutes in 20 minute intervals in pre-vacuumed 12 ml glass vial with butyl stopper (Exetainer ®). At sites with very low emissions an additional sample was taken after 360 min. Samples were stored cool and analyzed within one week. Emission data are presented for 32 of 40 measurements where the linear slope fitted $r^2 > 0.75$. Rejected time series were probably subjected to ebullition as observed by unexpectedly high methane concentration at t=20 min depleting afterwards.

Bulk density

Water content and bulk density at cushion plants site was determined by sampling carefully the upper 5 cm of mosses in a 10 cm × 10 cm box. Remnants of vascular plant were excluded. Deeper samples were taken using a D-Section corer (ID 4.7 cm; Eijkelkamp Agrisearch Equipment ®, Giesbeek, the Netherlands). The samples were dried at 70°C for 2 days.

Methane oxidation tests

Whole *Sphagnum* mosses were thoroughly washed and incubated in 120 ml bottles with 1 ml of methane. Methane was measured on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh). Methane production tests were performed in 60 ml bottles containing only dinitrogen gas. Methane oxidation tests were performed at 10, 15 and 20 °C, in the dark. *Sphagnum* Samples were taken in December 2007, March 2008 and April2008. Litter samples were taken in April and December 2008.

gDNA isolation

Sphagnum mosses were washed with sterile demineralized water after sampling and kept frozen at -20°C. Genomica DNA was isolated as described before (Kip *et al.*, 2010) and was stored at 4 °C.

16S rRNA gene and pmoA gene clonelibraries

PCR amplification of the 16S rRNA gene was performed with two general bacterial 16S rRNA gene primers: 616F and 630R (Juretschko *et al.*, 1998). PCR amplification of the *pmoA* gene was performed with two general *pmoA* gene primers: A682R and A189F (Holmes *et al.*, 1995). All PCRs were performed in a gradient from 50 to 60 degrees and PCR products were combined. All PCR products were purified using the QIAquick PCR Purifications Kit (Qiagen). DNA sequencing was performed with the primers used in the

PCR and for the 16S rRNA gene also the universal bacterial primer 612R (Ehrmann *et al.*, 2003) was used. Used *pmoA* gene primers were specific only for known methanotrophic A*lpha-* and *Gammaproteobacteria*, primers were not expected to hybridize with the *pmoA* gene of methane-oxidizing *Verrucomicrobia.* The pGEM-T Easy Vector System Kit (Promega) was used for ligation of the *pmoA* and 16S rRNA gene amplifications. Ligation was performed as prescribed by the manufacturer. 16S rRNA gene ligation mix was transformed to Top10 competent *E. coli* cells (Invitrogen™) by heat shock exposure (30-45 seconds at 42°C). *pmoA* gene ligation mix was transformed by heat shock exposure to XL-1 Blue competent *E. coli* cells, constructed as described by Inoue *et al.* (1990). Plasmid DNA with ligated 16S rRNA gene was isolated with the FastPlasmid Mini Kit (Eppendorf) following the instructions of the manufacturer. pDNA with ligated *pmoA* gene was isolated with the E.Z.N.A.™ Plasmid Miniprep Kit (EZNA™). Partial 16S rRNA gene fragments and complete *pmoA* gene sequences were sequenced with M13 forward and reverse primers (Invitrogen™), targeting vector sequences adjacent to the multiple cloning site. pDNA sequencing was performed by the sequencing facility of the UMC Sint Radboud, Nijmegen. Clone library sequences and their closes relatives were analyzed using MEGA version 4 (Tamura *et al.*, 2007). All sequences were aligned automatically using the alignment tool of MEGA4 (ClustalW). Phylogenetic analyses of the gene sequences were performed by applying neighbor-joining analyses (Saitou & Mei, 1987).

Microarray

The *pmo*A based microarray is performed as described in Kip *et al.* (2010).

Chapter 6

*pmo*A microarray and deep sequencing reveal two dominant methanotrophic genera in *Sphagnum* mosses from a peat bog

Nardy Kip, Bas E. Dutilh, Yao Pan, Levente Bodrossy, Alex Hoischen, Mike S.M. Jetten & Huub J.M. Op den Camp

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Abstract

Sphagnum peatlands are important ecosystems in the methane cycle. Methanotrophs in these ecosystems have been shown to reduce methane emissions and provide extra carbon to *Sphagnum* mosses. However, little is known about the diversity and identity of the methanotrophs present in and on *Sphagnum* of peatlands. In this study, we applied a *pmo*A microarray and deep 454 pyrosequencing to a *pmo*A PCR product from *Sphagnum* mosses to investigate the presence of known and unknown methanotrophs and to compare the two methods. Both techniques showed comparable results and revealed an abundance of *Methylomonas* and *Methylocystis* species in *Sphagnum* mosses from a Dutch peat bog. The advantage of the microarray analysis was that it was fast and cost effective, especially when many samples had to be screened, while pyrosequencing was advantageous in providing *pmo*A sequences of many unknown or uncultured methanotrophs present in the *Sphagnum* mosses and, thus, provided more detailed insight into the microbial diversity.

Introduction

Methane is an important greenhouse gas and, as for all other greenhouse gases, its concentration is rising rapidly since pre-industrial times (Forster *et al.*, 2007). Methanotrophs can act as a sink for methane. These microbes occur in many different ecosystems like rice paddies, soils, volcanic areas and peat bogs (Hanson and Hanson, 1996; Conrad, 2009; Op den Camp *et al.*, 2009). In 2005, our group showed that methanotrophs also occur in the hyaline cells of *Sphagnum* mosses (Raghoebarsing *et al.*, 2005). Moreover, using a *pmo*A microarray, we recently detected a wide variety of methanotrophic symbionts in and on the mosses, showing the global prevalence of this symbiosis (Kip *et al.*, 2010). Acidic peat bogs are the most extensive type of wetlands, occupying about 3% of the total land area and storing about 30% of carbon in soils globally. Methanotrophs present in these peatlands can act as a filter for methane, thereby reducing methane emissions (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010) and recycling carbon within the ecosystem. Besides the environmental importance of methanotrophs for the reduction of methane emissions into the atmosphere, they are also applied in environmental bioengineering (Jiang *et al.*, 2010),

biotechnological applications (Trotsenko *et al.*, 2005) and waste water treatment (Chang and Alvarez-Cohen, 1997).

Methanotrophs occur in three monophyletic lineages within the *Proteobacteria* and one lineage within the *Verrucomicrobia* (Conrad, 2009; Op den Camp *et al.*, 2009). Gammaproteobacterial methane-oxidizing bacteria belong to the type I methanotrophs that use the ribulose monophosphate pathway for formaldehyde fixation and have disc-shaped intracellular membranes (ICMs) that occur throughout the cell. The type II methanotrophs belong to the *Alphaproteobacteria* and comprise two lineages which use the serine pathway for formaldehyde fixation and have ICMs along the periphery of the cell. This genus includes the *Methylocystis-Methylosinus* genera and the acidophilic methanotrophs of the genera *Methylocella* and *Methylocapsa*. The recently described extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum isolated from volcanic areas do not show intracellular membrane structures and still need to be characterized further (Op den Camp *et al.*, 2009).

Peatbogs are a harsh environment for microbes to live in because of the low pH, between 3.8 and 4.8, and a low nutrient content. The isolation of methanotrophs from these ecosystems is a challenge. After using very diluted oligotrophic media, several methanotrophs could be isolated from peatlands (Dedysh *et al.*, 1998; Dedysh *et al.*, 2000; Dedysh *et al.*, 2002; Dedysh *et al.*, 2004) all belonging to the *Alphaproteobacteria*, but molecular tools have shown that many remain uncultured (Chen *et al.*, 2008b; Dedysh, 2009; Kip *et al.*, 2010). Such molecular tools are necessary to gain insight into these microbial communities and to identify new species. Methanotrophic communities can be studied with general taxonomic markers like 16S rRNA, or using functional markers of methanotrophs like the genes encoding the methane monooxygenase enzymes, *pmo*A and *mmo*X. The *pmo*A gene is the most commonly used marker. It encodes a subunit of the particulate (membrane bound) methane monooxygenase, and is present in all aerobic methanotrophs except *Methylocella* spp. The *mmo*X gene can only be found in some methanotrophs and encodes a subunit of the soluble methane monooxygenase. A *pmo*A based microarray (Bodrossy *et al.*, 2003) was developed to rapidly screen the methanotrophic

community. The microarray probes are based on *pmo*A genes from known methanotrophic isolates and from environmental samples. This molecular screening tool quickly provides information about the methanotrophic communities and indicates the sites that hold a potential to discover unknown species. Conversely, clone libraries are labor intensive and not quantitative. Finally next-generation sequencing is an attractive alternative owing to the growing read length of pyrosequencing (currently 450 nucleotides) and the large number of reads (500.000 per plate), although it is more expensive.

Here, we compare 454 pyrosequencing of a *pmo*A PCR product with a *pmo*A microarray to investigate a methanotrophic community in *Sphagnum* mosses. Since microarray analysis showed the presence of both type I and type II methanotrophs, the more quantitative pyrosequencing approach was used to measure their abundances and phylogenetic analysis of the *pmo*A sequences might reveal unknown methanotrophs.

Results and discussion

In this study a methanotrophic community living in and on *Sphagnum* mosses from a Dutch peat bog, Hatertse Vennen, was investigated using both a *pmoA* microarray and by 454 pyrosequencing of the *pmo*A PCR product. *Sphagnum* mosses from the studied peat ecosystem were tested for methane oxidation activity by incubating the thoroughly washed *Sphagnum* mosses with methane. All investigated samples showed methane oxidation rates, between 20 and 40 µmol/g DW/day at 20 °C, comparable to previous studies (Raghoebarsing *et al.*, 2005; Kip *et al.*, 2010).

Methanotrophic community in Sphagnum investigated using 454 pyrosequencing

The *pmo*A PCR product, obtained using genomic DNA from *Sphagnum* mosses as a template, was sequenced using titanium 454 pyrosequencing. To reduce problems during the sequencing run we mixed the *pmo*A PCR products with 16S rRNA PCR products from a non-related experiment. The *pmo*A and 16S rRNA

genes were separated based on Blast searches. In total, we obtained 134,330 *pmo*A reads. The *Gammaproteobacteria* represented a total of 78,488 reads (58%) of which 80% where highly related to *Methylomonas* spp. The *Alphaproteobacteria* represented 53,378 reads or 40% of the reads and almost all of them (95%) were highly related to *Methylocystis* spp. These results show the clear abundance of *Methylomonas* and *Methylocystis* species in the tested *Sphagnum* mosses of the Hatertse Vennen.

Methanotrophic community in Sphagnum investigated using a pmo*A microarray*

DNA was isolated from non-incubated *Sphagnum* mosses (two independent samples) and a PCR based on general *pmo*A primers, was performed. The PCR products were hybridized to the microarray (Fig. 1A). Other studies using the same microarray in peat soils (Chen *et al.* 2008a), rice fields (Vishwakarma *et al.* 2009) and upland soils (Cebron *et al.* 2007) revealed a much less diverse methanotrophic community in comparison to our microarray results. Nevertheless, the diversity was similar to previously analyzed *Sphagnum* mosses from Siberia and Patagonia (Kip *et al.*, 2010).

The microarray showed an abundance of *Methylobacter, Methylomonas, Methylomicrobium, Methylocystis* and *Methylosinus* genera. Other studies using the same microarray (Chen *et al.*, 2008a; Chen *et al.*, 2008b) also showed the abundance of *Methylocystis* sp. in peat soils. Type II alphaproteobacterial methanotrophs were commonly found in peat and other ecosystems and several acidophilic methanotrophs have been isolated (Dedysh, 2009). Type I gammaproteobacterial methanotrophs have not often been detected in peat ecosystems (Dedysh *et al.*, 2002). Because *Methylocella* sp., a common methanotroph in Northern peatlands (Dedysh *et al.*, 2000; Dedysh *et al.*, 2004), does not possess a *pmo*A gene, it can not be detected on our microarray. We assessed the abundance of *Methylocella* sp. separately using a *mmo*X PCR clone library (see below).

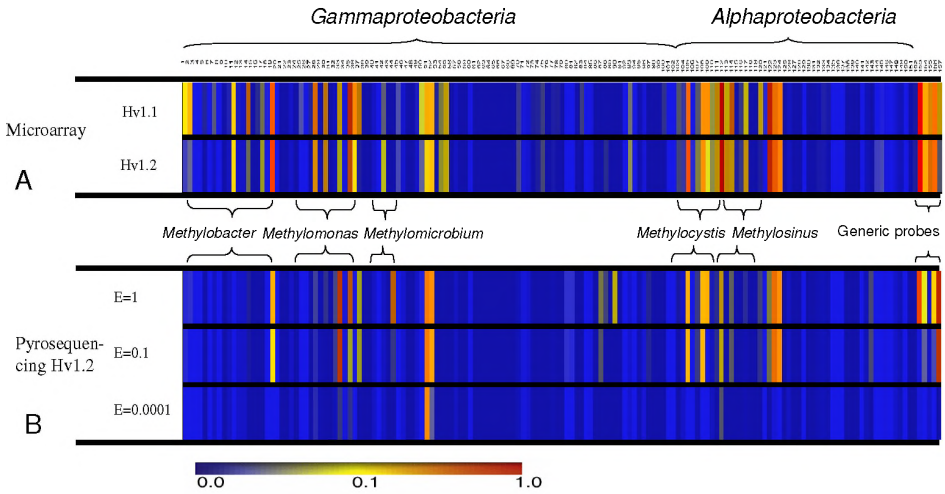

Figure 1. A *pmo*A based microbial methanotrophic community analysis microarray of two different mosses, Hv1.1 and Hv1.2. B results of the BlastN mapping microarray probes on 454 titanium sequencing reads of Hv1.2 using different E-values. Colour coding bar represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates that 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). Hv1.1 and Hv1.2 represent two independent samples. A larger version of figure 6 can be found in the appendix and a list of the probes in the respective order is given in Appendix Table S1.

Microarray data compared to deep sequencing data

The probes present on the microarray are ca. 24 nucleotides long. These sequences were queried by BlastN against all 454 *pmo*A reads. The number of reads that mapped to a probe was recorded at different E-value cutoffs and compared to the hybridization intensity of the microarray (Fig. 1B). We required high E-value cutoffs due to the short length of the reads. Figure 1B shows that a high signal intensity on the microarray correlates well with the number of 454 sequencing reads identified as hits in the BlastN search. The presence and abundance type I methanotrophs, especially *Methylomonas, Methylomicrobium* and *Methylobacter* species and the type II methanotrophs *Methylocystis* and *Methylosinus* species were revealed using both methods. Probes that showed abundance among the sequencing reads but not on the microarray were those designed based on marine and freshwater sediment clones related to

Methylococcus and *Methylocaldum* spp., some probes targeting *Methylomicrobium* spp., *Methylomonas* spp., *Methylobacter* spp., some environmental type I related probes and the general type Ib probes Ib453 and Ib559 (see Appendix Table 1). The sequencing data confirms the high abundance of type I methanotrophs which were previously considered to represent only a very low percentage of the total methanotrophic community (Dedysh, 2009). However, the studies summarized by Dedysh (2009) were performed on peat soils and not on living *Sphagnum* mosses as we did. Probes that showed hybridization on the microarray but did not yield BlastN hits among the sequencing reads were: marine sediment clones (probe SWI1-375 and SWI1-377), the *Methylomicrobium album* probe Mmb304 and the *Methylococcus* sp. probe Mc396.

Phylogenetic analysis of the methanotrophic community in Sphagnum

Since the *pmo*A microarray showed in such a high diversity of methanotrophs in the *Sphagnum* microbiota we also determined the phylogenetic distribution of the reads by mapping them to a *pmo*A database, containing *pmo*A and *amo*A sequences of cultivated and environmental origin. In addition, we constructed a maximum likelihood phylogenetic tree from the database sequences that mapped at least ten reads (98% of all reads are represented), plus several reference sequences (Fig. 2).

The family of *Gammaproteobacteria* represents a total of 78,488, which is 58% of the total amount of reads of which 62,445 reads where highly related to *Methylomonas* spp., this represents 47% of the total and 80% of Gammaproteobacterial reads. The *Alphaproteobacteria* represented 53,378 reads or 40% of the total amount of reads, almost all (94%) were highly related to *Methylocystis* spp.. This shows the clear abundance of most probably one or just a few species of *Methylomonas* and *Methylocystis* species to be present on and in the tested *Sphagnum* mosses of the Hatertse Vennen.

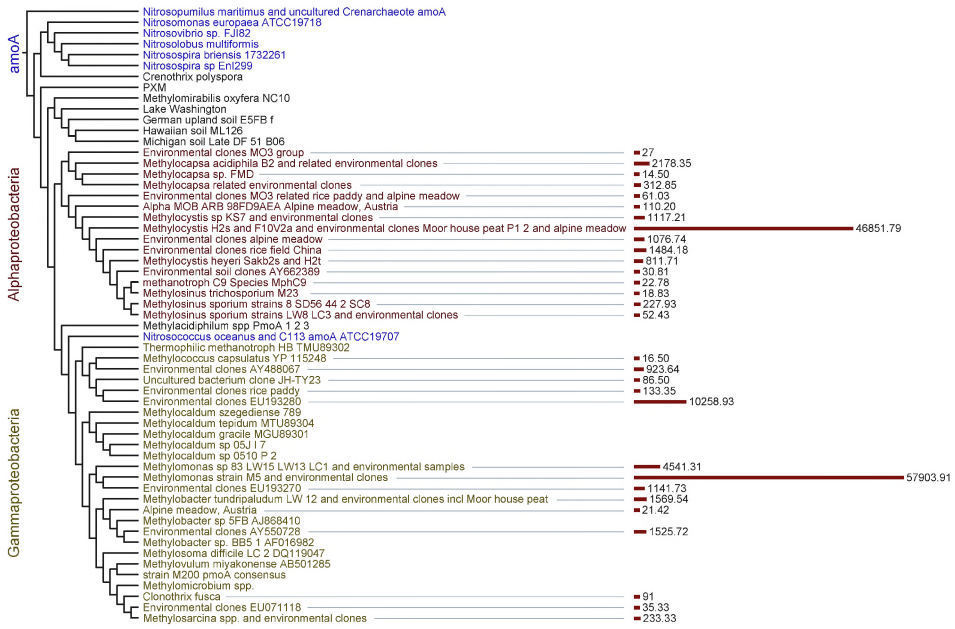

Figure 2. Representation of the results for the *pmo*A reads mapped on a phylogenetic tree representing cultured and uncultured *pmo*A and *amo*A sequences. Red bars indicate the total amount of reads found for that branch. Accession codes and sequences per branch can be found in Appendix Table 3 (www.ru.nl/microbiology/dissertations/dissertations).

Methylocystis sp. are commonly found in any ecosystem where methane is present (Dedysh *et al.*, 2007; Chen *et al.*, 2008b) and active *Methylomonas* species have been detected in peat ecosystems (Chen *et al.*, 2008a), however, not in the high numbers we report here. In general, microbial community analysis performed on peat soils showed a large abundance of *Alphaproteobacteria,* often more than three times the amount of *Gammaproteobacteria* (reviewed in Dedysh, 2009). However these studies were performed on peat and no studies have been performed on living *Sphagnum* mosses before. Verrucomicrobial *pmo*A reads were not found.

mmo*X clone library*

Because the molecular tools outlined above are based on the *pmo*A gene, they cannot detect methanotrophs of the *Methylocella* genus, which does not possess

this gene (Dedysh *et al.*, 2000). Still, *Methylocella* sp. is commonly found in peat ecosystems, so we document their presence in the *Sphagnum* mosses using a *mmo*X-based PCR with 7 primer sets. Only the primer set mmoX1-mmoX2 resulted in a small amount of PCR product. Only using a nested PCR approach more primer combinations resulted in PCR product. Different combinations of the primers F882, 1403R, mmoXA and mmoXB were needed to obtain a small amount of PCR product, indicating *Methylocella* sp. not to be abundant. These PCR products were not sufficient for titanium 454 sequencing, so we prepared and sequenced clone libraries, which resulted in 21 sequences identical to the known *mmo*X gene of *Methylocella* sp. (Fig. 3), and two sequences related to *Methylobacter* sp. (data not shown). Although this analysis shows the presence of small amounts of *Methylocella* sp. in the *Sphagnum* mosses, not all methanotrophs contain the *mmo*X gene. Therefore, this gene is not a good functional marker to test the abundance of other methanotrophs.

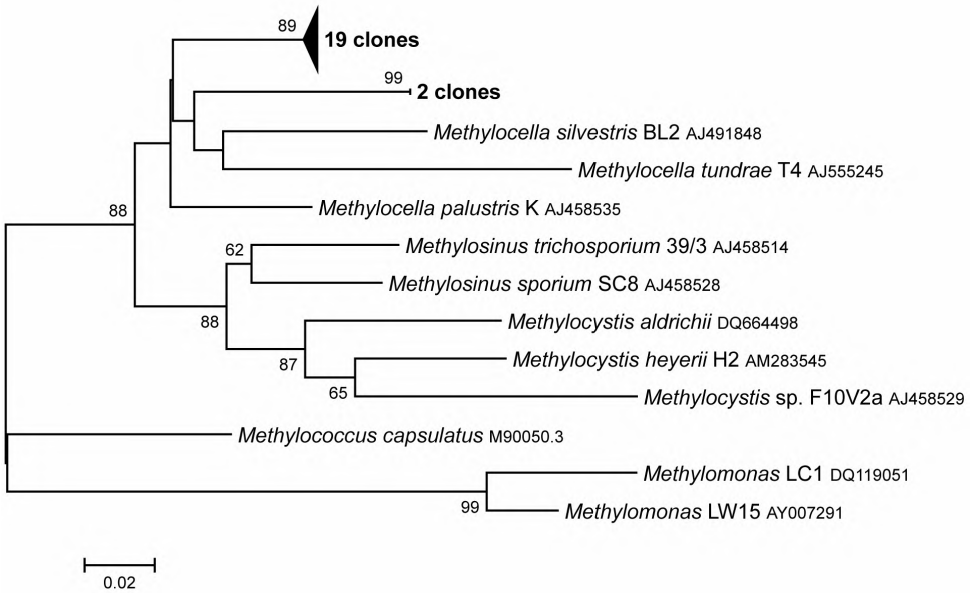

Fig 3. *mmo*X gene phylogenetic tree, based on DNA, showing the relationship of the clones to selected methanotrophs. The tree was based on Neighbour-Joining analysis using a bootstrap test of 500 replicates. Bootstrap values of >60 are indicated at the node of the branch.

Detection of Verrucomicrobial methanotrophs

The general primers used do not amplify Verrucomicrobial *pmo*A genes and therefore a new set of primers was designed to detect these new species.in the *Sphagnum* mosses. The primers are based on the *pmo*A1 and 2 of the three methanotrophic strains of the *Methylacidiphilaceae* family (Op den Camp *et al.*, 2009). A PCR product was obtained using DNA from *Sphagnum* mosses from the Hatertse Vennen as a template and this product was used to hybridize onto the microarray. Hybridization was found with the probes specific for *Verrucomicrobia*, however the hybridization intensity could not be calculated since, the calculation is based on the presence of the general A189 forward primer and the validation of the probes is still ongoing.

Final conclusions and discussion

Overall, the results of the pyrosequencing and the microarray are comparable and show the abundance of both *Gammaproteobacterial* as well as *Alphaproteobacterial* genera. The high abundance of two genera, *Methylomonas* and *Methylocystis*, became more obvious after deep PCR sequencing. By comparing the microarray with the probes mapped to the deep sequencing reads, we obtained a rather similar pattern. The differences might be due to differential hybridization or they could be the result of the different PCR techniques used to amplify the *pmo*A sequences for the microarray and for pyrosequencing (see Methods). Another influencing factor is the T7-tag needed for the microarray procedure. It has been shown that even a GC clamp can influence PCR results (Bourne *et al.*, 2001) and as a T7-tag consists of seven nucleotides, its effect on hybridization might be considerable. Furthermore, although the primers we employ have been extensively used to characterize methane oxidizing communities (Henckel *et al.*, 2000), PCR inherently biases the results in any study. A primer combination with A682r is also able to amplify the homologous *amo*A gene of ammonia oxidizers, and was shown to recover mainly *amo*A genes (Bourne *et al.*, 2001). However, a nested PCR of 189f/mb661 on the 189f/682r PCR product can retrieve the *pmo*A sequences. Primers Mb661r and A650r are more specific in amplifying *pmo*A genes, but Mb661r misses the clusters USC-

alfa and RA21 (Bourne *et al.*, 2001) and certainly the rice clusters taxonomically located between *pmo*A and *amo*A (Luke *et al.*, 2010). Moreover, A650r showed biases in recovering mainly *Methylococcus capsulatus pmo*A sequences and none related to *Methylomonas* or *Methylomicrobium* (Bourne *et al.*, 2001). As all recovered sequences showed the abundance of *Methylomonas* and *Methylocystis*, we conclude that this general result cannot be attributed to a bias in any of the primers.

Materials and methods

Sampling and methane oxidation test

Samples were collected from an acidic *Sphagnum* peat bog at Hatertse Vennen (the Netherlands). Whole *Sphagnum* mosses were thoroughly washed and incubated in 120 ml bottles with 1 ml of methane. Methane was measured on a Hewlett-Packard model 5890 gas chromatograph equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh).
gDNA isolation and microarray analysis were performed as described in Kip *et al.* (2010).

pmo*A PCR sequencing*

The *pmo*A PCRs were performed as described above for the microarray. No nested PCR was used. We could only use the primer sets A189-Mb661 and A189-A650 (Holmes *et al.*, 1995; Bourne *et al.*, 2001) because the sequencing size limit at that moment was around 500 nt. Verrucomicrobial primers VpmoA216- VpmoA622 showed several PCR products of different size and the expected size of around 444 bp was excised from an agarose gel and purified with a QIAEX II Gel Extraction Kit (Qiagen). After purification a low quality and low amount of PCR product was left, which in the end was 1% of the final concentration. The other PCR products were purified using the QIAquick PCR Purifaction Kit (Qiagen). Titanium 454 sequencing of a mixture of all PCR products was performed by the Department of Anthropogenetics (UMC St. Radboud, Nijmegen, NL).

Phylogenetic analysis of pmo*A pyrosequencing reads*

*pmo*A PCR products were mixed in a ~1:1 ratio with 16S rRNA PCR products from an non-related experiment. After sequencing, we extracted a total of 134,330 *pmo*A reads by Blast searches against a large *pmo*A and *amo*A database containing 3,849 unique sequences. These included the *pmo*A sequences from isolates and environmental

sequences that were used to design the probes for the *pmo*A microarray (Bodrossy *et al.*, 2003), supplemented with novel sequences, e.g. from more recent literature. Using an E-value cutoff of 0.1 ensured the exclusion of 16S rRNA reads. Each pyrosequencing read was then assigned to its top *pmo*A gene in this database, and conversely, for each database sequence, we counted the number of reads that mapped to it with a top score. In case of multiple equally-scoring top hits we divided the read equally. The 270 sequences with at least ten mapped reads represented 132,865 (98.9%) of all *pmo*A reads. Together with a selection of characterised *pmo*A sequences, we constructed a phylogenetic tree as follows. First, we translated the DNA sequences into protein, and determined the correct frame of translation, as the database contained the reverse complement of the *pmo*A gene in some cases. Then, these protein sequences were aligned using ClustalW 2.0.12 with default parameters (Larkin *et al.*, 2007). The amino acids in the alignment were then translated back into the original codons of DNA, which was used as input for PhyML with the following parameters: approximate likelihood ratio test (SH-like branch supports), HKY85 model, estimated Ts/tv ratio, estimated proportion of invariable sites (5.38%), 4 substitution rate categories, estimated gamma distribution parameter, mean of rate class, maximum likelihood nucleotide equilibrium frequencies, optimised tree topology, NNIs tree topology search, BioNJ starting tree optimised branch lengths and substitution model parameters (Guindon *et al.*, 2009). For visualization, we collapsed several of the branches (see Fig. 3) and the details are presented in Supplemental Table 3.

mmo*X clonelibrary*

The *mmo*X genes were amplified with general primers using the recommended annealing temperatures (McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000). The PCR program was as described above for the first PCR of the microarray. For the primerset mmoX1-X2 a touchdown 73-63 °C was performed. For the primer sets f882-r1403, f882-mmoXB, mmoXA-mmoXB, mmoXA-r1403, f882-9r and mmoXA-r1403 a touchdown from 63-53 and gradients between 65-50 were performed. Nested PCRs were performed by using a 100times diluted PCR product as template. All PCR products were purified using the QIAquick PCR Purifaction Kit (Qiagen). DNA sequencing was performed with the primers used in the PCR. DNA sequencing was performed by the sequencing facility of the Radboud University Nijmegen Medical Centre. Phylogenetic analyses were conducted with the MEGA4 package (Tamura *et al.*, 2007).

Verrucomicrobial pmoA primer design

The primers based on the *pmo*A1 and *pmo*A2 of all three verrucomicrobial methanotrophs were designed: VpmoA216:GGAAAGAymGrATGTGGTGGCC (forward) and VpmoA622: GTTTCnACCATnCGnATrTAyTCAGG (reverse). The PCR performed with the VpmoA primer set was performed as described before for the touchdown PCR with the A189/T7-mb661. Initial validation was performed using a pure culture of *Methyloacidiphilum fumarolicum* SolV and shown to amplify its pmoA genes. Positive PCR samples hybridized on the updated *pmoA* microarray and the PCR products showed positive signal with all the Verrucomicrobial *pmoA1* and *pmoA2* probes and no signal with the Verrucomicrobial *pmoA3* probes. Unfortunately it was not possible to calculate hybridization intensity since calculations are based on the general pmoA forward primer hybridization intensity and this primer was not used in this PCR.

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Chapter 7

Summary, integration and perspectives

Methane is a greenhouse gas which contributes to global warming and its related environmental changes. In the last decades much research has been focused on methane sources and sinks in the global methane cycle. Important ecosystems in the carbon and methane cycle are wetlands. Peat wetlands store about one third of the worldwide carbon in soil and produce and consume vast amounts of methane (Gorham, 1991). Northern peatlands emit about 12 % of the global total methane (Wuebbles and Hayhoe, 2002). This has significant implications for global environmental changes since methane is 25 times more effective as a greenhouse gas compared to carbon dioxide and on a 200 year time scale it contributes to 22% of the total climate forcing of the long lived greenhouse gases (Lelieveld *et al.*, 1998). Methane cycling in peatlands involves a number of biogeochemical processes, which are governed by various physical, chemical and biological factors (Segers, 1998). Knowledge of sources and sinks and the effects of environmental changes to these ecosystems are essential in order to predict future climate changes better and to design mitigation strategies. Within peatlands, methane is not only produced but also consumed and therefore recycled within the system. In the last fifteen years a lot of research has been conducted on methanotrophs in ecosystems like rice fields, soils and peatlands and it has expanded the understanding of the ecosystems and the functioning of methanotrophs. The discovery of an extreme acidophilic methanotrophs outside the known proteobacterial families of methanotrophs demonstrated that there is still a lot to be discovered (Dunfield *et al.*, 2007; Pol *et al.*, 2007). In 2005, methanotrophs that live in symbiosis with *Sphagnum* mosses were discovered in a Dutch peat bog. This finding implies methane emissions could be reduced and methanotrophs provide extra carbon to the mosses for photosynthesis. This discovery was the basis of this research in which the geographical and environmental distribution of these symbiotic methanotrophs was studied. This study is a cooperation of scientist from different fields, like ecology, geochemistry, hydrology and microbiology. This joint expertise is necessary to get a better understanding of the biogeochemical processes in peat bogs from all over the world. This PhD study was focused on the methanotrophic community composition within and on *Sphagnum* mosses and the isolation of these symbiotic methanotrophs.

This thesis shows that indeed methanotrophic symbionts occur in peatlands all over the world (chapter 2). Further, new acidophilic methanotrophs from the *Sphagnum* mosses were isolated (chapter 3 and 4). Finally the diversity of the methanotrophic community in peat mosses was detected by new molecular methods (chapter 5 and 6).

Global prevalence of methanotrophic symbionts in *Sphagnum* mosses (chapter 2)

By testing methane oxidation rates of *Sphagnum* mosses from different peat ecosystems it was shown that methanotrophic symbionts are present in peat bogs from all over the world. Labeling experiments with $^{13}CH_4$ showed incorporation into *Sphagnum* lipids upon submerged incubation, indicating carbon dioxide produced by the methanotrophs is used by the plant. *Sphagnum* mosses live under carbon limited conditions which makes any extra carbon very useful. Methanotrophic communities appeared to consist of both *Alpha-* and *Gammaproteobacteria*, while in peat soils *Alphaproteobacteria* usually seemed to be most abundant. Mosses from North Siberia showed methane oxidation even below 4 °C and were the only ones that showed a different hybridization pattern on the *pmoA* microarray compared to all the other mosses from around the world. These North Siberian *Sphagnum* mosses even consumed methane, at high rates, when incubated on ice.

Sphagnum removal studies showed that *Sphagnum* mosses can reduce emissions up to 30%. *In situ* measurements in North Siberia revealed reductions up to 15% (Frans-Jan Parmentier, personal communication). Global environmental changes will influence the peat ecosystem and the emissions of these systems. Many factors have to be taken into account like temperature, precipitation, droughts, anthropogenic activities, the effect of elevated levels of green house gases and also, in permafrost regions, methane hydrates/clathrates. All these factors not only affect the ecosystem, but also enhance or decrease other factors of which many are currently unknown. In recent years global warming was most prominent in the Northern regions and rising temperatures will result in longer growing seasons, thus increased production and consumption and

Sphagnum species might change or disappear due to temperature increase. Another result in several areas is the melting of the permafrost, which might also be a risk for melting of methane hydrates that are present. Globally the influence of humans plays a big role in ecosystem changes. Many peatlands are converted into agricultural lands, leading to enhanced oxidation of the carbon in the peat, releasing stored carbon in a much faster way. Another important anthropogenic change is the elevated nitrogen deposition into ecosystems, this can change an ecosystem dramatically and might cause increased growth of vascular plants and disappearance of *Sphagnum* mosses (Tomassen *et al.*, 2003).

We demonstrated highest methane oxidation to occur in submerged mosses, originating from pools. Non submerged mosses from lawn and hummock showed very low methane oxidation at the surface, however, submerged *Sphagnum* litter from lawn and hummock vegetation sites showed similar oxidation rates to pool mosses, when sampled just below the water table (chapter 5). Methane production rates are dependent on the water level (Moore and Dalva, 1993; Kettunen *et al.*, 1999) and the results obtained in this study show the same effect for methane oxidation rates. The influence of methane oxidation by symbiotic methanotrophs on the global methane and carbon cycle cannot be calculated with the obtained methane oxidation rates measured in the laboratory. In addition to controlled laboratory experiments, it is important to quantify microbial processes *in situ* at field scale to obtain rates that may be more representative for the environment studied. However, the results obtained do indicate that they should be implemented in global models calculating and predicting methane and carbon dioxide levels in the atmosphere. *In situ* studies in a Canadian peat bog were used to make in a climate change model in which it was shown that an increase of temperature resulted in a decrease in methane emissions, but on the other hand they found a 26 times increase of methane emissions with a 15% increase of precipitation (Lai, 2009), which demonstrates the influence of many factors in the ecosystem. In connection with the need to improve the predictions of changes in atmospheric greenhouse gas concentrations and hence global climate, considerable research efforts have been directed towards biogeochemical cycling of methane in peat ecosystems over the past two decades. In order to get a good overview of fluxes and processes in the ecosystem

the integration of many more experiments and techniques are necessary. This also requests scientist of different expertise to work together as is stimulated by the Darwin centre for Biogeosciences. The ecological, microbiological, hydrological, meteorological and biogeochemical processes all need to be simulated in a complicated global model in order to understand the ecosystem and to make good predictions for the future. The magnitude of methane fluxes varies considerably among peatlands types and vegetation. Methane gas is liberated from peat via three main pathways, namely diffusion, ebullition and plant mediated transport. The vegetation types within the peatlands are determining the amount of plant mediated transport to the atmosphere and play a more important role in nutrient rich fens compared to ombrotrophic peat bogs, where hardly any vascular plants grow. The magnitude of methane flux from peatlands is a function of substrate availability and quality, water table dept, temperature, pH, vegetation type and productivity and field measurements. Due to varying integrative effects of these factors, methane emissions from peatlands exhibit high spatial variation among peatlands types and microtopographic locations and more research is needed to come to a consensus (Lai, 2009). It is hard to deal with the 'scaling' problem to extrapolate local field measurements into a global or even only an ecosystem model, but hopefully more research and cooperation between scientists will improve this.

Discovery and isolation of symbiotic methanotrophs

In chapter two the methanotrophic bacterial communities of *Sphagnum* mosses from different peatlands were analyzed using a *pmo*A microarray. This showed the presence of both *Alphaproteobacteria*, including *Methylocystis* spp. and *Methylosinus* spp. and *Gammaproteobacteria*, among which representatives of the genera *Methylobacter*, *Methylomonas* and *Methylomicrobium* were detected. Studies performed on peat soils already showed the abundance of *Methylocystis* spp. and many times a low abundance of *Gammaproteobacteria* was found in peat soils (reviewed in Dedysh, 2009). However, this review paper did also mention the awaiting of isolation of the peat inhabiting type I methanotrophs. Isolation of methanotrophs from peat ecosystems using the widely used Whittenbury medium was not successful and by using a diluted medium, designated medium M2,

Dedysh and coworkers were able to isolate new acidophilic methanotrophs from peat ecosystems (Dedysh *et al.*, 1998). Using this M2 medium for enrichments and isolations described in this thesis only alphaproteobacterial methanotrophs were isolated (Chapter 4). Several new strains were obtained in a pure culture, belonging to the *Methylosinus* family. Besides those new strains very often the recently described *Methylocystis* strain H2s was isolated from different peat ecosystems. In order to enrich and isolate gammaproteobacterial methanotrophs, which were detected with the *pmo*A microarray in all *Sphagnum* mosses, a new medium was designed based on the peat water composition of the Dutch Mariapeel peat bog. This resulted in the isolation of two new gammaproteobacterial methanotrophs, strain M5 belonging to the genus *Methylomonas* and strain M200 most closely related to *Methylovulum* sp. (Chapter 3).

It is generally accepted that the majority of the bacteria present in an ecosystem are so far non-culturable and that the medium used determines the success rate. However, the recent discoveries of new methanotrophic species distantly related to known isolated strains, e.g. verrucomicrobial methanotrophs and within the Gammaproteobacteria the new *Methylovulum* species and strain M200 indicate that cultivation of bacteria is necessary to update and extend our knowledge on bacterial diversity and our molecular tools. To show their presence in and on the *Sphagnum* plants fluorescence in situ hybridization (FISH) can be used. FISH on *Sphagnum* peat mosses is difficult since the chlorophyll in the living *Sphagnum* causes too much autofluorescence to be able to detect bacteria. FISH is only possible on fresh samples that are examined within 24 hours after sampling. This made FISH on the samples from other countries impossible. Methane oxidizing activity has only been shown as a total rate of the methanotrophic community. The contribution to this activity by isolated methanotrophs can be proven by several experiments. For example a microarray based on mRNA could be performed to investigate the active methanotrophic community. This does require fast freezing of the sample in order to get a good overview of the *in situ* situation since mRNA can be rather unstable and be degraded in a short period of time. Another option would be to perform a stable isotope probing (SIP) experiment in combination with a clone library analysis,

e.g. 16S rRNA based (Morris *et al.*, 2002). Also a combination can be made like SIP-PLFA or SIP-DNA in combination with multiple displacement amplification to make a fosmid metagenomic library (Chen *et al.*, 2008a; Chen *et al.*, 2008b). Within a SIP experiment one has to incubate the samples using conditions that mimic the *in situ* conditions and concentrations to get a good representation of what happens in the ecosystem. In environmental samples many other microorganisms are present that can cross-feed on methanotrophs which makes the interpretation of the data difficult (Morris *et al.*, 2002; Radajewski *et al.*, 2002). Unfortunately using the SIP method with only labeled methane might be less suitable detect methanotrophs like *Methylocella* and *Methylocystis* spp. since they are facultative methanotrophs and when for example acetate is present in the environmental samples, these methanotrophs might have a preference to consume this substrate.

Study of two different Patagonian peatlands

Within the Patagonian peatland one *Sphagnum* species dominated all the different micro habitats of pool, lawn and hummock which provided a good ecosystem to test species independent methane oxidation and methanotrophic composition. Methane oxidation was shown to be related to the water levels (Chapter 2), but in chapter five it was demonstrated that methane concentration in the peatland is another important factor. Submerged pool mosses from an ecosystem with a low methane content showed much lower methane oxidation rates compared to submerged pool samples from a pool with a high methane concentration. Here *Sphagnum* litter, from just below the water table, showed both methane oxidation and production. This shows that most of the methane oxidation in lawn and hummock micro habitats is taking place around the water table, where methane concentrations are highest and that there is a correlation between methane oxidation activity and methane concentration.

A 16S ribosomal RNA clone library revealed a highly diverse bacterial community and the presence of clones related to the methanotrophic symbiont identified by Raghoebarsing *et al.*, (2005). General PCR primers were used to include any new methanotroph which would not be detected with methanotroph

specific primers. However, using general primers no close relatives of known methanotrophs were found at all. This is a common problem in environmental microbiology, since often the bacteria of interest are not abundant in the total bacterial community or there is a PCR bias. It would be best to test other combinations of both general and specific PCR primers. However, nowadays metagenomic analysis would be the preferred option, which enables detection less abundant species (see chapter 6).

Methanotrophic communities were analyzed with a *pmo*A clone library and *pmo*A microarray and showed a rather comparable community between pool mosses and lawn and hummock litter. Hummock mosses, with low methane oxidation rates, lacked the abundance of *Methylocystis-Methylosinus* species which indicates that these could be the most active members of the methanotrophic community within the Patagonian peat bogs. Recently it has been shown that besides *Methylocella* species also the *Methylocystis* genus contains facultative methanotrophic strains (Belova *et al.*, 2010; Im *et al.*, 2010). The ability to consume acetate is a good survival strategy and might explain the overall abundance of *Methylocystis* species within peat bogs. Another important factor within peat bogs, which was not addressed here, is the low nitrogen concentration within a peat bog ecosystem. The Patagonian peat bogs are so remote that there is no human influence, which results in a very low nitrogen deposition. Several methanotrophs are able to fix nitrogen and this ability could give them an advantage above others which cannot do this.

Detection of methanotrophs

Methanotrophic communities present in and on *Sphagnum* mosses were investigated using a *pmo*A microarray, 454 titanium sequencing of *pmo*A PCR product and 16S rRNA, *pmo*A and *mmo*X clone libraries. Almost all methanotrophs contain the *pmo*A gene, encoding for the particulate membrane bound methane monooxygenase, except *Methylocella* spp., which only contain the soluble methane monooxygenase, *mmo*X. In order to get a good overview of the methanotrophic community both genes should be assessed using molecular tools. Other tools to detect methanotrophs are fluorescence in situ hybridization

(FISH) and phospholipid fatty acid (PLFA) analysis (Murrell *et al.*, 1998), as discussed above.

Using molecular tools methanotrophic communities in peat have revealed the abundance of alphaproteobacterial methanotrophs, in particular *Methylocystis* and *Methylocella* species (reviewed in Dedysh, 2009). The presence of gammaproteobacterial methanotrophs was shown before in peat ecosystems, but they were never found to be abundant. In this thesis the presence and abundance of Gammaproteobacteria in and attached on *Sphagnum* mosses has been shown using different techniques. The *Methylomonas* species seem to be an important part of the methanotrophic community. Chapter 3 describes the isolation of strain M5, which shows highest homology to *Methylomonas* and phylogenetically clusters together with the clones obtained in the study of Chen and coworkers (2008b). The *pmo*A clone library of the Patagonian peat moss also revealed clones belonging to the same cluster and the pyrosequencing of *pmo*A PCR products from the Hatertse Vennen peat ecosystem in the Netherlands also showed the abundance of *Methylomonas* related reads. Most of those reads phylogenetically cluster together with strain M5 described in chapter 3. In SIP study on peat soils Chen and coworkers (2008b) discovered type I clones related to *Methylomonas* and *Methylobacter* species to be active within the peat soils. *Sphagnum* moss and peat soil consist of the same plant material, but are two different niches, which might cause the differences found by different research groups investigating methanotrophs in peat ecosystems. *Sphagnum* mosses have been shown to leak sugars and amino acids (Fenner *et al.*, 2004), which can be used by the bacteria present in the hyaline cells. This supply of nutrients can influence the microbial composition. Also in rice fields a difference has been found in methanotrophic composition between soil and rhizosphere (Luke *et al.*, 2010). The differences between gamma (type I) and alphaproteobacterial (type II) methanotrophs have been shown in several studies. Gammaproteobacterial methanotrophs were shown to thrive in environments with high methane and oxygen levels (Hanson and Hanson, 1996), higher nutrient contents and higher pH (Dedysh, 2009). In rice fields it was shown that type II methanotrophs are present in unplanted and unfertilized soil and upon fertilization and planting type I methanotrophs are activated and both type I and II can be found. Activation of

type I methanotrophs could be an issue in peat ecosystems closer to rural areas with anthropogenic influences, such as nitrogen deposition. However, more remote areas like Patagonia and North Siberia have little anthropogenic influence and still show the presence of type I methanotrophs. The plants in an ecosystem can influence the methanotrophic composition, e.g. in ricefields type I methanotrophs were shown to thrive only in the presence of rice plants, which could indicate that plant exudates stimulating type I methanotrophs, which might also be the case in *Sphagnum* mosses.

A recent study on methanotrophs in floodplains (Steenbergh *et al.*, 2009) showed that different types of proteobacteria to have different life strategies; type Ia invests more in reproduction upon favorable conditions explaining the presence of these methanotrophs in rizospheres and surface layers of lake sediments. In contrast, type II methanotrophs were shown to invest in survival and longevity, which can be advantageous in environments where nutrients are limiting. The presence of both types of methanotrophs can be expected in many environments where methane is present. The abundance of the different types depends on the conditions and fluctuations in the ecosystem. Besides the different life strategies other strategies should be taken into account, e.g. the survival and performance under nitrogen or copper limiting conditions and the ability to form resting structures. However, these survival abilities are scattered throughout the known methanotrophic genera and not restricted to a certain type of methanotrophs (Hanson and Hanson, 1996).

Using FISH Dedysh and co-workers detected gammaproteobacterial methanotrophs to be less then 1% of the community (Dedysh *et al.*, 2001). However, FISH on strain M5 showed to be rather difficult, probably because of the polysaccharide layer around the cells. This layer is commonly found in methanotrophs of both gamma- and alphaproteobacteria and possibly could make FISH detection difficult. Another difficulty is the isolation of DNA, which in molecular ecology has always been a point of discussion (Tebbe and Vahjen, 1993; Fitzpatrick *et al.*, 2010; Pan *et al.*, 2010). DNA recovery of all the resident microorganisms can be difficult and the different DNA isolation techniques can end up in different results (Pan *et al.*, 2010). One way to address this problem is

to use direct PCR on the environmental samples, however knowledge on the optimal PCR conditions are required and it has been shown that this technique also has biases due to PCR conditions and lysing efficiency of the micro organisms in the sample. All isolated methanotrophs in this thesis have been used in colony PCR and only on the gammaproteobacterial strain M200 the colony PCR never worked. This again might be due to the polysaccharide layer around the cells which might inhibit cell lysis or the PCR. Environmental samples need to be diluted to reduce the PCR inhibitors present in the environment and this dilution will result in detection of only abundant species (Fode Vaughan, 2001). Besides possible differences in DNA isolation the primer choice is of even more influence. In the microarray and pyrosequencing experimental set ups several different primers and touchdown PCR were used to minimize the primers biases. The most commonly used primer sets for studying *pmo*A diversity are A189f/A682r (Holmes *et al.*, 1995), A189f/A650 (Bourne *et al.*, 2001) and A189f/mb661r (Costello and Lidstrom, 1999). A combination with A682r is able to amplify the homologous *amo*A gene of ammonia oxidizers and was shown to recover mainly *amo*A genes. Using a combination with the reverse primers mb661r and A650 only *pmo*A genes are amplified, but Mb661r misses the clusters USC-α and RA21 (Bourne *et al.*, 2001) and certain rice clusters located in between *pmo*A and *amo*A (Luke *et al.*, 2010). On the other hand A650r showed biases in recovering mainly *Methylococcus capsulatus pmo*A sequences and no *Methylomonas* or *Methylomicrobium*- associated *pmo*A sequences (Bourne *et al.*, 2001). All sequences recovered using the different primer sets showed the abundance of *Methylomonas* and *Methylocystis*-associated *pmo*A sequences which cannot be contributed to the bias of any of the primers as found in the study of Bourne *et al.* (2001). Molecular techniques always are based on current knowledge and need to be updated and re-evaluated upon new discoveries. The former general *pmo*A PCR primers are not general anymore after the discovery of the verrucomicrobial methanotrophs (Op den Camp *et al.*, 2009), which contain three different *pmo*A genes. None are detectable with the currently used general *pmo*A PCR primer sets.

When using PCR based sequences for microbial diversity studies one has to take into account the errors produced by the polymerase used in the PCR. Taq

polymerases lack the proofreading activity and the use of a high fidelity PCR polymerases with proofreading activity are recommended when focusing upon a highly conserved gene. For the *pmo*A based PCRs performed in this study the use of the high fidelity polymerase, Phusion (Finnzymes), was not possible since many unspecific PCR products were formed and the PFU enzyme (Stratagene and Fermentas) did not result in enough PCR product, while conventional Taq polymerase resulted in the specific PCR products of the expected size. An alternate method to investigate the methanotrophic community would be a PCR-independent method using multiple displacement amplification used for whole community genome amplification (WCGA) (Erwin *et al.*, 2005; Chen *et al.*, 2008b; Neufeld *et al.*, 2008). This technique requires less template DNA then any other molecular technique and is therefore very useful for the analysis of environmental samples in which DNA quantities can often be a problem. However, Bodelier and collegues (2009) showed that caution should be taken using this method since type I methanotroph *pmo*A sequences were preferentially amplified over those from type II methanotrophs, which was suggested to be due variation in GC content (Bodelier *et al.*, 2009a). Nevertheless the technique is useful in case the data would not be accessible with other techniques, e.g. low DNA content or when quantification of community composition is not important. In general the bias is most probably caused by the low amounts of template and can be overcome using higher concentrations.

Another method to detect methanotrophic diversity is the analysis of specific lipids, PLFA. The proteobacterial methanotrophs possess distinct lipid profiles, which enables the differentiation between them. The type I methanotrophs contain mainly PLFA with 14 (14C) or 16 carbon atoms (16C) and type II mainly lipids with 18 carbon atoms (C18) and the different genera can be distinguished by the presence and abundance of specific lipids. In environmental research certain PLFAs are used as biomarkers for the different methanotrophs, type I specific PLFAs are C16:1ω8c and C16:1 ω5t while C18:1ω8c is specific for type II (Bodelier *et al.*, 2009b). They are commonly used in combination with stable isotope probing (SIP) experiments (Chen *et al.*, 2008a; Shrestha *et al.*, 2008) which enables the identification of active methanotrophs. To be able to identify the methanotrophs, PLFA profiles of isolates methanotrophs need to be

known and also here pure cultures of new methanotrophic bacteria expand the knowledge and the phylogenetic resolution of PFLA analysis. Unfortunately many PLFA analyses on environmental samples did not reveal more then the presence of type I and type II methanotrophs, due to the limitation of available profiles of isolated methanotrophs. This was improved by the analysis of many type II isolates in 2009 (Bodelier *et al.*, 2009b). However, in view of recent description and isolations of new gamma and alphaproteobacterial methanotrophs (chapter 3; chapter 4; Belova *et al.*; 2010, Iguchi *et al.*, 2010; Im *et al.*, 2010) a new update or re-evaluation might be necessary. Additionaly, so far no specific lipids have been detected in the new verrucomicrobial methanotrophs (Dunfield *et al.*, 2007; Pol *et al.*, 2007). Therefore the PLFA analysis should always be accompanied by other molecular studies in order to draw conclusions on the methanotrophic diversity.

All the pitfalls in the different approaches make clear that integration of different approaches is necessary to get a good overview of the microbial diversity in environmental samples. Therefore different approaches were integrated to get insight into the diversity of methanotrophic symbionts in *Sphagnum* mosses in this study.

Biogeography of methanotrophs

Methanotrophs occur in all kinds of environments, but three major ecosystems are investigated on the presence and activity of aerobic methanotrophs: peatlands, rice paddies and soils. However, most studies were mainly confined to local scales. In this study we have tried to get a better understanding of the methanotrophic communities in *Sphagnum* mosses in peat ecosystems all around the world. So far all studies were focused on the peatlands in the Northern Hemisphere, especially Siberian peatlands. Within the recent years several studies have been performed on the diversity of methanotrophs, showing the abundance of *Alphaproteobacteria*, mostly *Methylocystis* species. This species appears not only in peat ecosystems but in many other ecosystems like soils, rice paddies and landfill soils. As mentioned above these species are able to survive without methane, using acetate, which could be the reason they are so widespread.

The hypothesis that methanotrophic diversity would show a unique pattern as found in the other kingdoms around the different niches and thus show biogeographical patters could not be confirmed by the data described this thesis. Here we showed a similar phylogenetic biogeographical pattern; the presence of *Methylocystis –Methylosinus*, *Methylomonas* and *Methylobacter* was shown in all the *Sphagnum* mosses tested from all over the world. This shows environmental factors are more determining methanotrophic communities rather than geographical differences.

Verrucomicrobial methanotrophs in peat bogs?

Since the discovery of the methanotrophic *Verrucomicrobia* in volcanic regions the quest has started to find them in other ecosystems and to find new unknown methanotrophs outside the proteobacterial family. The verrucomicrobial methanotrophs are acidophilic (pH <3.0) and thermophilic (55 °C, Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). Since these conditions are not commonly found, the question to be answered will be if verrucomicrobial methanotrophs are restricted to geothermal habitats. Conventional *pmo*A primers are not able to anneal to the *pmo*A sequences of these verrucomicrobial methanotrophs, which means that they always escaped detection. 16S rRNA clone libraries showed the presence of *Verrucomicrobia* in peat ecosystems (Raghoebarsing, 2006; Dedysh, 2009), but they are never closely related to the methanotrophic *Verrucomicrobia*. A new set of primers was designed based on the *pmo*A 1 and 2 gene sequences of the verrucomicrobial methanotrophs in order to detect them specifically in environmental samples. The microarray was updated with new probes based on the *pmo*A genes of the methanotrophs belonging to the *Verrucomicrobia*. The primer set was tested on all genomic DNA tested from all the *Sphagnum* samples, but only two samples showed a positive result: Hatertse Vennen and Western Siberia (pool). Hybridization on the microarray showed positive results with only the verrucomicrobial *pmo*A probes. This is a hint to the presence of *Verrucomicrobial* methanotrophs in peatlands, but much more research is necessary to find more neutrophilic and mesophilic verrucomicrobial methanotrophs.

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Samenvatting en uitleg

Methaan is een belangrijk broeikas gas wat bijdraagt aan het broeikaseffect en andere daaraan gerelateerde effecten. Methaan is een deel van de koolstofcyclus. Koolstof is aanwezig in bijna alles op deze wereld, in de levende wezens, in de grond, in de lucht en in het water. De koolstof cyclus is van groot belang voor het broeikaseffect. Koolstofdioxide en methaan zijn de twee belangrijkste gassen in de atmosfeer, die zorgen voor opwarming van de aarde. Het is dus belangrijk om te weten waar er veel koolstofdioxide en methaan wordt geproduceerd, maar ook waar het wordt geconsumeerd. Hierin spelen veengebieden een belangrijke rol. Veengebieden zijn in Nederland algemeen bekend. Vroeger werd er veel veen afgegraven en gebruikt als brandstof (turf). Dit veen is ontstaan door ophoping van organisch materiaal, voornamelijk veenmossen die door de lage zuurgraad van het veenwater en de lage concentratie aan nutriënten niet goed konden worden afgebroken. Hierdoor hoopt zich een grote laag aan deels verteerd plantenmateriaal op. Dit is samengedrukt tot veen, wat uiteindelijk na vele duizenden jaren bruinkool wordt en uiteindelijk steenkool. Deze ophoping van plantenmateriaal zorgt voor een grote opslag van koolstof, waardoor de veengebieden van het Noordelijk halfrond een derde deel van het in de wereld in bodems opgeslagen koolstof bevatten. Naast deze opslag van koolstof wordt nog steeds veel koolstofdioxide en methaan geproduceerd. Dit wordt geproduceerd door de micro-organismen in het veengebied.

De veengebieden die in dit proefschrift onderzocht, zijn hoogvenen die vooral begroeid zijn door veenmossen, ook wel *Sphagnum* mossen genoemd. Deze worden in Nederland nog veel in de bloemsierkunst gebruikt. In deze venen word koolstof vastgelegd in plantenmateriaal, ook wel biomassa genoemd, door fotosynthese. Bij de fotosynthese wordt ook zuurstof geproduceerd door de mos. Als deze mossen afsterven en komen bovenop de veenbodem waar ze (deels) worden omgezet door micro-organismen naar uiteindelijk methaan en koolstofdioxide. De gassen welke naar boven diffunderen. In het water drijven en groeien de veenmossen, waarin bacteriën zitten die het methaan met behulp van zuurstof kunnen omzetten. Deze bacteriën leven in symbiose met de veenmossen. Het mos zorgt voor een stabiele leefomgeving en toevoer van zuurstof, en mogelijk ook andere nutriënten, en de bacterie zet methaan en zuurstof om in koolstof dioxide, dat weer als koolstofbron dient voor de plant. Deze symbiose is

in 2005 gevonden in een veengebied in Nederland en in hoofdstuk twee dit proefschrift is aangetoond dat de symbiose voorkomt in alle (geteste) veengebieden over de hele wereld. Met behulp van koolstofisotopen werd bepaald dat de koolstof die van de methanotrofe bacteriën komt voor een derde bijdraagt aan de biomassa van de plant. Het veenwater is over het algemeen erg nutriënt – arm en bevat niet veel opgeloste koolstofdioxide die beschikbaar is voor de plant. Een bacterie die je extra levert wil je dan wel dichtbij hebben. In de veenpoelen, waarin de mossen helemaal onder water staan, is deze symbiose het meest van belang omdat de mossen geen koolstofdioxide uit de lucht kunnen opnemen. Deze poelen stoten ook het meeste methaan uit (emissie). Deze symbiose is dus wel degelijk van belang voor de hoeveelheid methaan die word uitgestoten en de hoeveelheid methaan die in het systeem word gerecycled. De modellen die voorspellingen doen over de koolstofcyclus over de hele wereld in relatie tot het broeikaseffect zullen rekening moeten houden met deze symbiose en de aan of afwezigheid van *Sphagnum* mossen in een veengebied. Andere wetenschappers hebben met behulp van modellen laten zien dat ongeveer 15% van de methaan word omgezet en recyclet in het ecosysteem. Met het opwarmen van de aarde worden allerlei processen versneld of vertraagd. Methaanproductie in de veengebieden word verwacht te versnellen en in dit proefschrift hebben we laten zien dat ook methaanconsumptie versneld bij een hogere temperatuur. Maar temperatuur is niet het enige broeikaseffect, ook effecten als extreme weersomstandigheden zoals droogte of overstroming, meer of minder neerslag en verhoogde atmosferische concentraties van koolstofdioxide en het verdwijnen van de permafrost kunnen een grote invloed uitoefenen op alle processen in veengebieden. De meest uitgestrekte veengebieden komen voor in het Noordelijk halfrond met een koud klimaat. Het opwarmen van de aarde is het grootst naarmate je dichter bij de polen komt en een te snelle opwarming zou bijvoorbeeld kunnen leiden tot het verdwijnen van de *Sphagnum* mossen waardoor er meer methaan vrij komt en waardoor de aarde nog meer opwarmt. Aan de andere kant zorgt de opwarming van de aarde ervoor dat de permafrost ontdooit en er kan meer regen vallen, waardoor er meer veengebieden onder water komen te staan en er meer methaan wordt geconsumeerd. Al deze processen zijn zeer complex en de effecten zijn niet alleen direct maar ook indirect en het is

daarom van belang om alle processen goed te bestuderen om goede modellen te maken, die nauwkeurige voorspellingen kunnen doen.

Een belangrijk onderdeel van dit proefschrift is de het vaststellen van de bacteriële samenstelling die voorkomt in de mossen, waarbij de meeste aandacht gericht is op de bacteriën die methaan kunnen consumeren, de methantrofe bacteriën, ook wel methanotrofen genoemd (alle hoofdstukken). Met behulp van verschillende moleculaire technieken is gekeken naar de verschillende soorten die voorkomen in en op de mossen. De variëteit aan methanotrofe bacteriën die is gevonden in de mossen is groot maar ook gelijk tussen alle mossen van verschillende landen en werelddelen. Dit laat zien dat bepaalde soorten het goed doen in veenmossen en anderen niet. Het ecosysteem is zuur en heeft weinig nutriënten en is daarom een moeilijke omgeving om te leven en te overleven. Er zijn verschillende soorten methanotrofen geïsoleerd en opgekweekt uit veen, maar nog nooit uit een mos zelf. Hoofdstukken drie en vier van dit proefschrift beschrijven de isolatie en beste kweekmethoden voor een aantal methanotrofe bacteriën uit veenmossen, waarvan een heel aantal nog nooit eerder gevonden zijn. De isolaties van nieuwe bacteriën is van belang om meer te weten te komen van het ecosysteem en om moleculaire technieken, die gebaseerd zijn op kennis verkregen door isolaties, te vernieuwen. In hoofdstukken vijf en zes wordt meer specifiek gekeken naar bepaalde veengebieden. In hoofdstuk vijf wordt er gefocust op veengebieden in Vuurland. Hier zijn veengebieden die heel ver van de bewoonde wereld afliggen en dus ongerept zijn en ze worden gedomineerd door één soort mos, waardoor het een goed systeem is om te bestuderen. Hier zijn de verschillende microhabitatten van de mossen,in het Engels: pool (poel), lawn (slenk) en hummock (bult) beter bestudeerd en werd er bewezen dat de methaanconcentratie van groot belang is op de activiteit van de methanotrofe bacteriën en dat de bacteriële soorten die gevonden worden in de verschillende microhabitatten nagenoeg hetzelfde zijn. Hier werd een bepaald genus, *Methylocystis* spp, gevonden die van belang kan zijn voor de activiteit en flexibiliteit binnen dit veen-ecosysteem. Verschillende soorten van dit genus kunnen naast methaan ook op acetaat leven, deze stoffen zijn beide aanwezig in het ecosysteem maar de concentraties ervan fluctueren nogal en daarom is het voordelig om een alternatieve koolstofbron te hebben. Hierdoor kunnen deze

bacteriën beter overleven in het systeem en zijn ze waarschijnlijk meer aanwezig. Ook is aangetoond dat er in hummocksoorten die een lage activiteit hebben wel degelijk methanotrofen zitten die binnen korte tijd kunnen reageren op veranderde condities zoals hogere methaan concentraties. Het feit dat methanotrofen overal aanwezig zijn, maakt het systeem flexibel en geeft het de mogelijkheid zich aan te passen aan de fluctuerende condities van het ecosysteem. De aanwezigheid van de verschillende soorten methanotrofe bacteriën is in hoofdstuk zes beschreven voor mossen uit het veengebied 'de Hatertse Vennen'. Hier zijn twee moleculaire technieken vergeleken en de data twee dominante methanotrofe genera. Deze zijn waarschijnlijk het meest van belang binnen dit ecosysteem en zijn ook veelvuldig gevonden in de mossen van andere veengebieden. Een aantal nieuwe methanotrofen die geïsoleerd zijn (hoofdstuk 3 en 4) behoren ook tot deze genera en onderzoek naar de groei omstandigheden van deze bacteriën kan ons meer leren over hun rol in het ecosysteem.

De resultaten van dit proefschrift geven ons meer inzicht in de processen die zich afspelen binnen veengebieden. De recycling van koolstof binnen het systeem is van belang voor de koolstof cyclus van de wereld. De recycling is overal op aarde in veengebieden met veenmos gevonden en de variëteit van methanotrofe bacteriën bleek groot maar ook erg vergelijkbaar over de hele wereld. Hierdoor is het ook makkelijker om resultaten van verschillende veenmosecosystemen met elkaar te vergelijken. Het waterniveau en de methaanconcentraties zijn van groot belang op de activiteit van de methanotrofen en de symbiose zorgt voor extra koolstof toevoer naar de plant. Dit alles laat zien dat we zuinig moeten zijn op onze veengebieden en de gebieden die we nog hebben moeten behouden en beschermen. In Nederland zijn een heel aantal projecten waarbij oude afgegraven veengebieden worden hersteld, maar hier is veel geduld bij nodig.

Dankwoord

Aknowledgements

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En natuurlijk alle Darwin collega's voor de gezellige Darwin dagen en workshops.

Bedankt iedereen!
Thanks you all!

Curriculum Vitae

Nardy Kip werd geboren op 9 december 1980 te Haulerwijk, gemeente Oostellingwerf. Daar begon ze haar basisschool 'de Schalmei', in 1991 verhuisde de familie Kip naar Vorden en daar heeft ze de basisschool afgemaakt op basisschool 'het Hoge'. Hierna behaalde ze haar VWO diploma op het 'Baudartius College' te Zutphen. In 1999 begon ze haar Studie Bioprocestechnologie aan de Wageningen University and Research centre, waarvoor ze afstudeervakken heeft gedaan bij de vakgroepen Fytopathologie (WUR) en Microbiologie (WUR) en een stage by Fitopathologia (Naples, Italy). Na wat andere projecten aan de WUR koos ze voor een promotieonderzoek bij de vakgroep Microbiologie aan de Radboud Universiteit te Nijmegen, waar ze tegenwoordig als post-doc werkt.

Appendix

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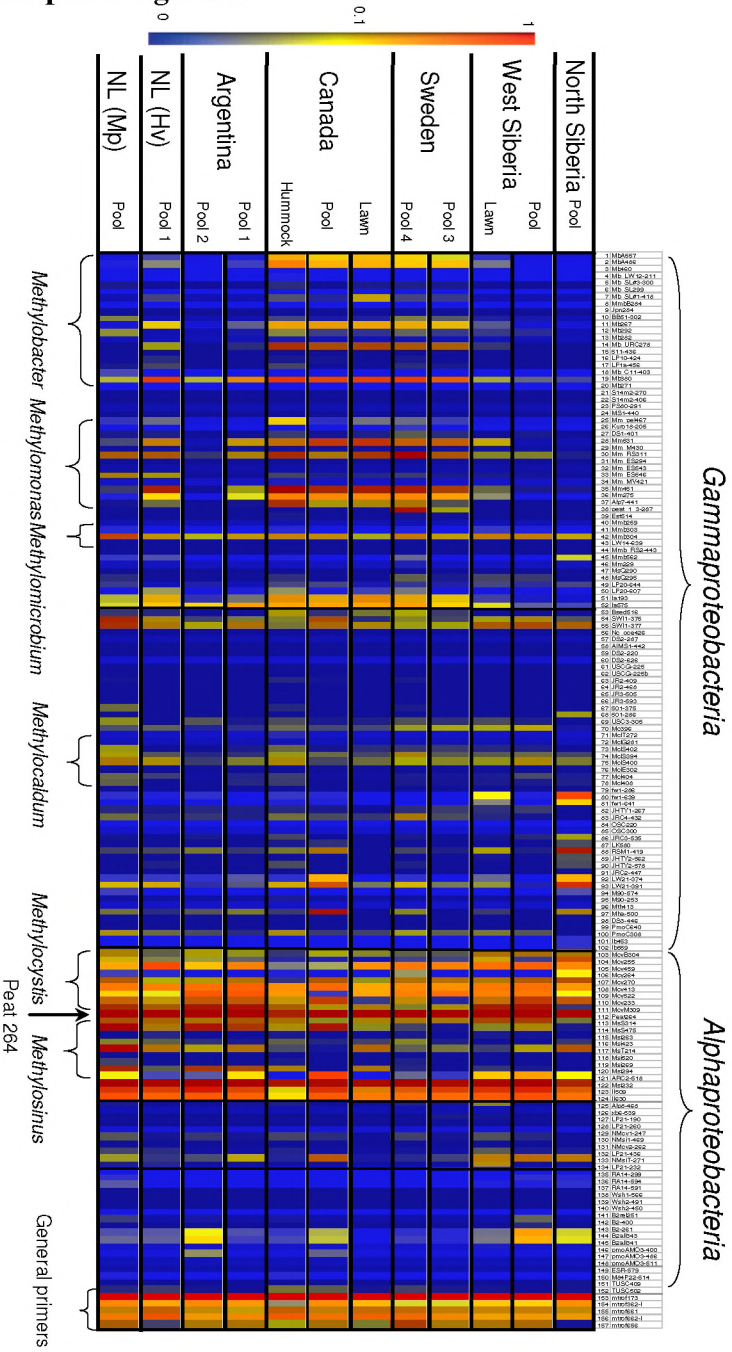
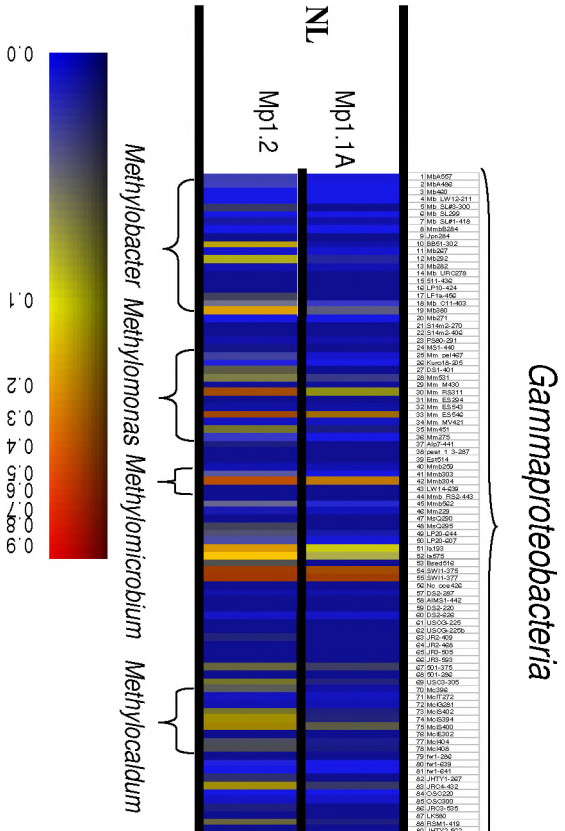
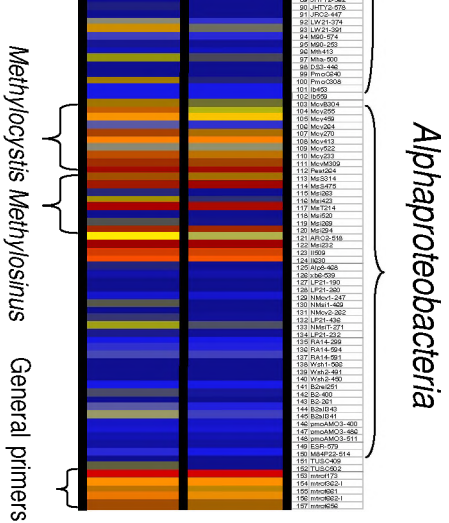
Chapter 2 figure 6

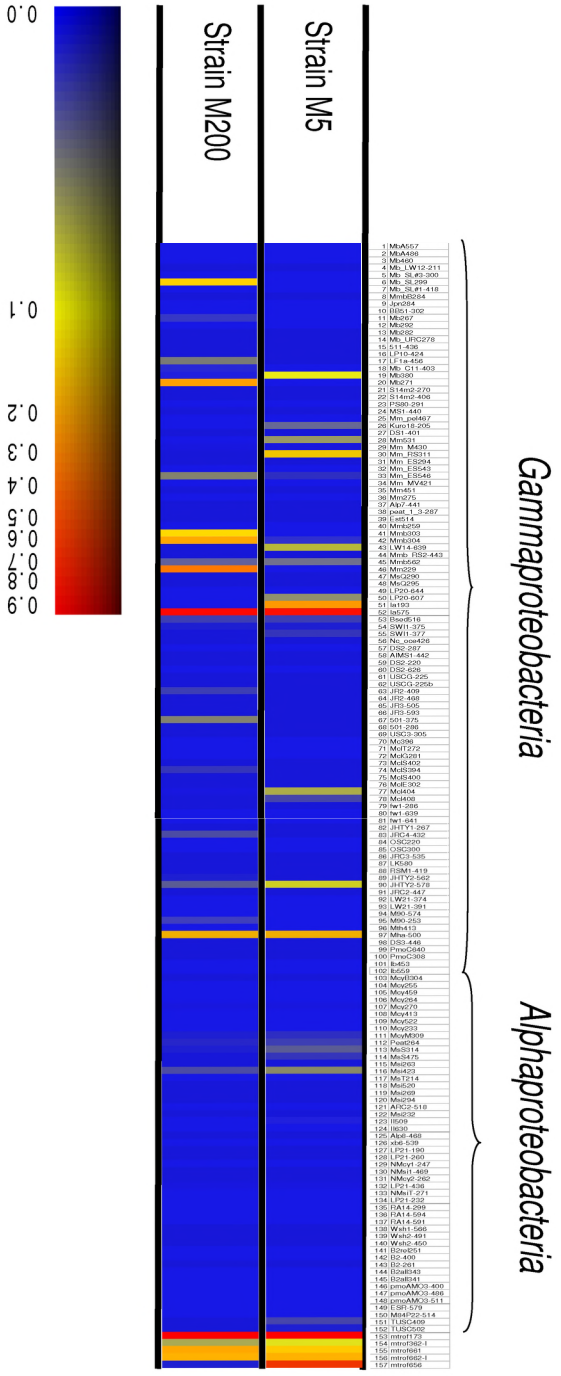


Chapter 3 figure 1

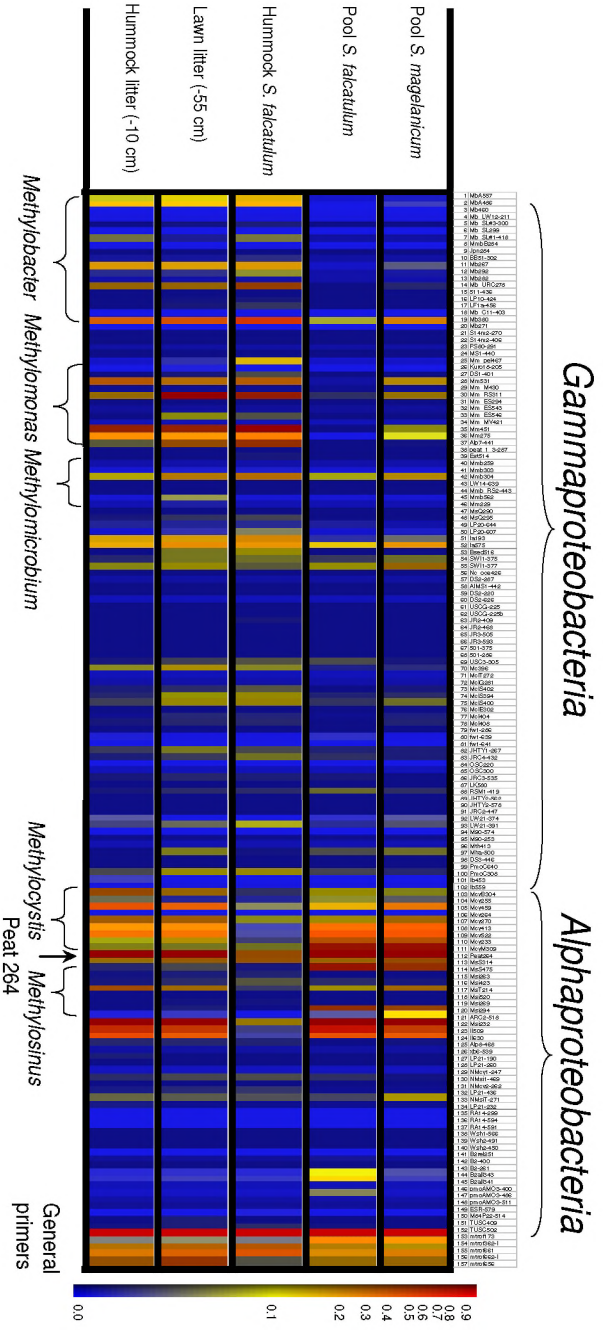



Alphaproteobacteria
Methylocystis Methylosinus
General primers

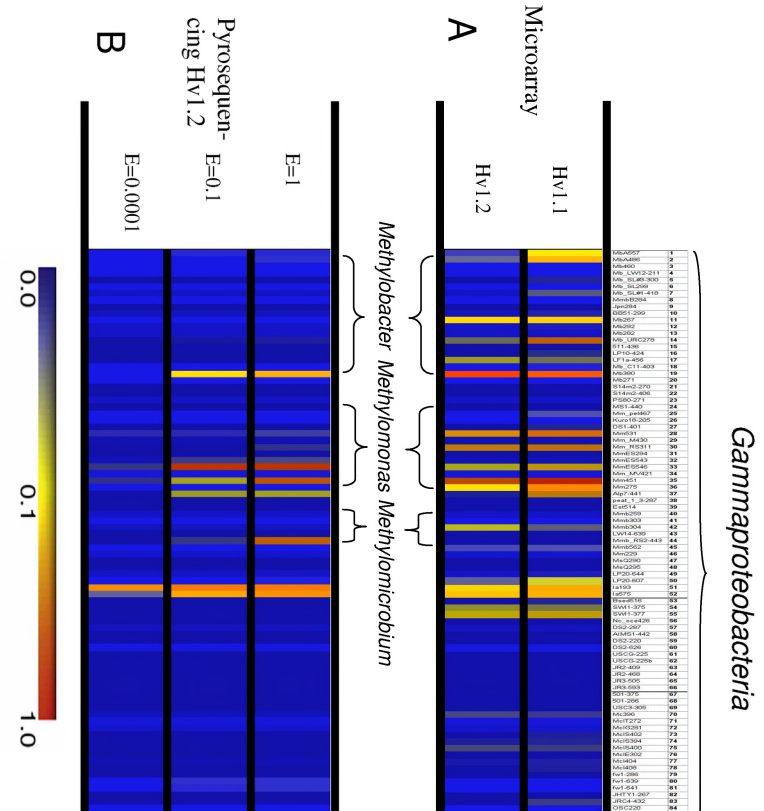
Chapter 3 figure 7



Chapter 5 figure 4



Chapter 6 figure1



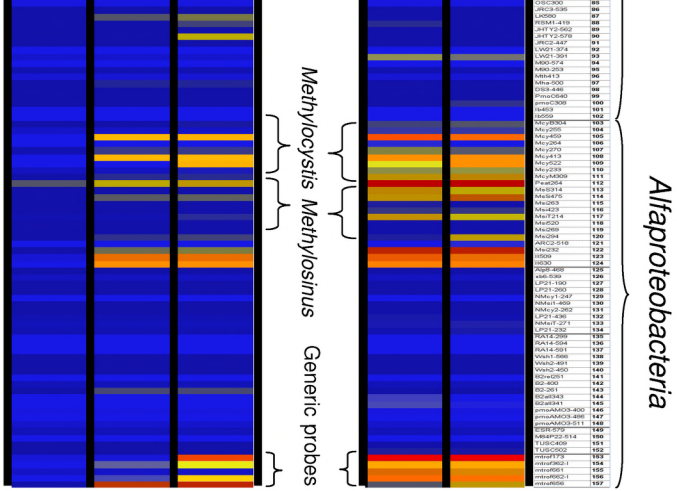

Methylocystis
Methylosinus
Generic probes
Alfaproteobacteria

Table 1. Probes used for microarray analysis. Column numbers correspond to the order in which the probes are arranged on the microarray analysis.

Number	Name	Intended specificity
1	MbA557	*Methylobacter*
2	MbA486	*Methylobacter*
3	Mb460	*Methylobacter*
4	Mb_LW12-211	*Methylobacter*
5	Mb_SL#3-300	*Methylobacter*
6	Mb_SL299	soda lake *Methylobacter* isolates and clones
7	Mb_SL#1-418	soda lake *Methylobacter* isolates and clones
8	MmbB284	*Mmb. Buryatense - same region as Jpn284, but 3 MM vs. that one Methylobacter and Japanese strain related*
9	Jpn284	clone Jpn 07061
10	BB51-302	*Methylobacter*
11	Mb267	*Methylobacter*
12	Mb292	*Methylobacter*
13	Mb282	*Methylobacter*
14	Mb_URC278	*Methylobacter*
15	511-436	*Methylobacter*
16	LP10-424	*Methylobacter* LP 10 group
17	LF1a-456	*Methylobacter* LF 1a group
18	Mb_C11-403	*Methylobacter*
19	Mb380	*M.bacter broad group A universal?*
20	Mb271	*Methylobacter*
21	S14m2-270	*Marine type Ia cluster, S14m#2*
22	S14m2-406	*Marine type Ia cluster, S14m#2*
23	PS80-291	clone PS-80
24	MS1-440	*Marine type Ia cluster, Marine sediment #1*
25	Mm_pel467	*Methylomicrobium pelagicum*
26	Kuro18-205	*Marine type Ia cluster, Kuro18*
27	DS1-401	Deep sea cluster #1
28	Mm531	*Methylomonas*
29	Mm_M430	*Methylomonas*
30	Mm_RS311	*Mm.methanica, RS clade(10-286)*
31	Mm_ES294	*Methylomonas*
32	Mm_ES543	*Methylomonas*
33	Mm_ES546	*Methylomonas*
34	Mm_MV421	*Methylomonas*
35	Mm451	*Methylomonas*
36	Mm275	*Methylomonas*
37	Alp7-441	*Alpine soil Methylomonas, Alp#7 (10-282)*
38	peat_1_3-287	*Mehtylomonas*-related peat clones
39	Est514	*Methylomicrobium*-related clones
40	Mmb259	*Methylomicrobium album* + Landfill *M.microbia*
41	Mmb303	*Methylomicrobium album*
42	Mmb304	*Methylomicrobium album* + Landfill *M.microbia* and related
43	LW14-639	*Methylomicrobium LW14 group*
44	Mmb_RS2-443	*Methylomicrobium, Mmb_RS2*
45	Mmb562	*Mmb. album* and *Methylosarcina*
46	Mm229	*Deep-branching M.monas (?) group (WHmb3 related group)*
47	MsQ290	*M.sarcina quisquilliarum related*
48	MsQ295	*M.sarcina quisquilliarum*
49	LP20-644	*Methylomicrobium*-related clones
50	LP20-607	*LP20 group (Type Ia, deep branching-Mmb?)*
51	Ia193	Type I a (*M.bacter-M.monas-M.microbium)*
52	Ia575	Type I a (*M.bacter-M.monas-M.microbium-M.sarcina)*
53	Bsed516	*Marine sediment #2, Bsed*
54	SWI1-375	*Marine sediment #2, SW#1*
55	SWI1-377	*Marine sediment #2, SW#1*
56	Nc_oce426	*Nitrosococcus oceani*
57	DS2-287	*Deep sea #2, subgroup (N.coccus and Deep sea Type Ia 10-298)*

58	AIMS1-442	*Deep sea #2, AIMS#1*
59	DS2-220	*Deep sea #2, subgroup*
60	DS2-626	*Deep sea #2, subgroup*
61	USCG-225	Upland soil cluster Gamma
62	USCG-225b	Upland soil cluster Gamma
63	JR2-409	JR cluster #2 (California upland grassland soil)
64	JR2-468	JR cluster #2 (California upland grassland soil)
65	JR3-505	JR cluster #3 (California upland grassland soil)
66	JR3-593	JR cluster #3 (California upland grassland soil)
67	501-375	*Methylococcus*- related marine and freshwater sediment clones
68	501-286	*Methylococcus*- related marine and freshwater sediment clones
69	USC3-305	Upland soil cluster #3
70	Mc396	*Methylococcus*
71	MclT272	*Methylocaldum tepidum*
72	MclG281	*Methylocaldum gracile*
73	MclS402	*Methylocaldum szegediense*
74	MclS394	*Methylocaldum szegediense* and related
75	MclS400	*Methylocaldum szegediense* and related
76	MclE302	*Methylocaldum* E10
77	Mcl404	*Mc.capsulatus-Mcl.tepidum-Mcl. Gracile-Mcl.Szeg* and related
78	Mcl408	*Methylocaldum*
79	fw1-286	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones
80	fw1-639	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones
81	fw1-641	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones
82	JHTY1-267	*JH-TY#1*
83	JRC4-432	Japanese rice cluster #4
84	OSC220	Finnish organic soil clones and related
85	OSC300	Finnish organic soil clones and related
86	JRC3-535	Japanese Rice Cluster #3
87	LK580	fw-1 group + Lake Konstanz sediment cluster
88	RSM1-419	*RSM#1*
89	JHTY2-562	*JH-TY#2*
90	JHTY2-578	*JH-TY#2*
91	JRC2-447	Japanese Rice Cluster #2
92	LW21-374	LW21 group
93	LW21-391	LW21 group
94	M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones
95	M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones
96	Mth413	*Methylothermus*
97	Mha-500	*Methylohalobius - M.thermus and related ?*
98	DS3-446	Deep sea cluster #3
99	PmoC640	*PmoC*
100	PmoC308	*PmoC*
101	Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)
102	Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)
103	McyB304	*M.cystis B (parvus/echinoides/*strain M)
104	Mcy255	*M.cystis B (parvus/echinoides/*strain M)
105	Mcy459	*Methylocystis*
106	Mcy264	*Methylocystis*
107	Mcy270	*Methylocystis*
108	Mcy413	*Methylocystis*
109	Mcy522	*Methlocystis* A + peat clones
110	Mcy233	*Methylocystis*
111	McyM309	*M.cystis strain M and related*
112	Peat264	peat clones
113	MsS314	*Methylosinus sporium*
114	MsS475	*Methylosinus sporium*
115	Msi263	*Methylosinus sporium* + 1 *Msi.trichosporium subclaster*
116	Msi423	*Methylosinus*
117	MsT214	*Methylosinus trichosporium* OB3b and rel.
118	Msi520	*Methylosinus trichosporium*
119	Msi269	*Methylosinus trichosporium*
120	Msi294	*Methylosinus*

121	ARC2-518	*Deep branching type II clade ARC2 - Methylosinus trichosporium 15-084 group?*
122	Msi232	*M.sinus*+ most *M.cystis* -considered as additional Utype II Uprobe
123	II509	Type II
124	II630	Type II
125	Alp8-468	*Type II novel pmoA, Alpine cluster Alp#8*
126	xb6-539	Novel *pmoA* copy of type II and related environmental clones
127	LP21-190	Novel *pmoA* copy of type II and related environmental clones
128	LP21-260	Novel *pmoA* copy of type II and related environmental clones
129	NMcy1-247	Novel *pmoA* copy of *M.cystis #1* (?)
130	NMsi1-469	Novel *pmoA* copy of *M.sinus*
131	NMcy2-262	Novel *pmoA* copy of *M.cystis #2* (?)
132	LP21-436	*Mcy + Msi novel pmoA #1 groups*
133	NMsiT-271	Novel *pmoA* copy of *M.sinus trichpsporium* (?)
134	LP21-232	Novel *pmoA* copy of type II and related environmental clones
135	RA14-299	RA14 related clones
136	RA14-594	RA14 related clones
137	RA14-591	RA14 related clones
138	Wsh1-566	Watershed + flodded upland cluster 1
139	Wsh2-491	Watershed + flodded upland cluster 2
140	Wsh2-450	Watershed + flodded upland cluster 2
141	B2rel251	*Methylocapsa*-related clones
142	B2-400	*Methylocapsa*
143	B2-261	*Methylocapsa*
144	B2all343	*Methylocapsa* and related clones
145	B2all341	*Methylocapsa* and related clones
146	pmoAMO3-400	clone pmoA-MO3
147	pmoAMO3-486	*MO3 group*
148	pmoAMO3-511	*MO3 group*
149	Ver330	*Verrucomicrobia, all pmoA1+pmoA2*
150	Ver307	*Verrucomicrobia, all pmoA2*
151	Ver285	*Verrucomicrobia, Ma.fum pmoA2+Ma.kam. pmoA2*
152	Ma_F1-594	*Ma.fum. pmoA1*
153	Ma_I1-312	*Ma.inf. pmoA1*
154	Ma_I1-401	*Ma.inf. pmoA1*
155	Ma_F3-638	*Ma.fum. pmoA3*
156	Ma_F3-542	*Ma.fum. pmoA3*
157	ESR-579	ESR (Eastern Snake River) cluster
158	M84P22-514	environmental clones of uncertain identity
159	TUSC409	Tropical Upland Soil Cluster #2
160	TUSC502	Tropical Upland Soil Cluster #2
161	mtrof173	Universal
162	mtrof362-I	Methanotrophs
163	mtrof661	Methanotrophs
164	mtrof662-I	Methanotrophs
165	mtrof656	Methanotrophs

Table 2. Mapping results of the deep PCR 454 sequencing reads with the microarray probes. Deep PCR sequencing reads were mapped against the microarray probes. Amount of reads mapping with the probes were counted at a cut off of different E-values (E=0.0001, 0.1 and 1). The percentage was based on the counted reads relatively to the total amount of reads (134.330 reads). The lowest E-value was obtained with the highest homology between the probe sequence and the reads.

pmoA template sequencing of Hv 1.2									
				total amounts			%		
nr	Name	Intended specificity	lowest Evalue	E=0.0001	E=0.1	E=1	E=0.0001	E=0.1	E=1
1	MbA557	*Methylobacter*	5.36E-07	7	2634	2700	0	2	2
2	MbA486	*Methylobacter*	1.91E-06	1337	1826	5874	1	1	4
3	Mb460	*Methylobacter*	1.91E-06	370	412	416	0	0	0
4	Mb_LW12-211	*Methylobacter*		0	0	0	0	0	0
5	Mb_SL#3-300	*Methylobacter*		0	0	0	0	0	0
6	Mb_SL299	soda lake *Methylobacter* isolates and clones	5.36E-07	1	2	17	0	0	0
7	Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	4.12E-08	2	2	56	0	0	0
8	MmbB284	*Mmb. Buryatense - same region as Jpn284, but 3 MM vs. that one Methylobacter and Japanese strain related*	9.17E-05	2	2	2	0	0	0
9	Jpn284	clone Jpn 07061		0	0	0	0	0	0
10	BB51-302	*Methylobacter*	0.4	0	0	339	0	0	0
11	Mb267	*Methylobacter*	9.17E-05	1067	1170	1187	1	1	1
12	Mb292	*Methylobacter*	1.23E-03	0	1	3	0	0	0
13	Mb282	*Methylobacter*	4.04E-03	0	1	14	0	0	0
14	Mb_URC278	*Methylobacter*	1.91E-06	4	980	1180	0	1	1
15	511-436	*Methylobacter*	0.08	0	2	8	0	0	0
16	511-436L	*Methylobacter 511 group*	0.03	0	4	11	0	0	0
17	LP10-424	*Methylobacter* LP 10 group	0.04	0	1	3	0	0	0
	LF1a-456	*Methylobacter* LF 1a group	8.87E-03	0	1	27	0	0	0
19	Mb_C11-403	*Methylobacter*	0.03	0	3	950	0	0	1
20	Mb380	*M.bacter broad group A universal?*	6.97E-07	5	16726	26315	0	12	20
21	Mb271	*Methylobacter*	3.23E-03	0	323	369	0	0	0
22	S14m2-270	*Marine type Ia cluster, S14m#2*	0.04	0	0	9	0	0	0
23	S14m2-406	*Marine type Ia cluster, S14m#2*	1.00E-05	9	0	302	0	0	0

24	PS80-291	clone PS-80	1.64E-03	0	1	2	0	0	0
25	MS1-440	*Marine type Ia cluster, Marine sediment #1*		0	0	0	0	0	0
26	Mm_pel467	*Methylomicrobium pelagicum*	1.63E-03	0	1	4	0	0	0
27	Kuro18-205	*Marine type Ia cluster, Kuro18*		0	0	0	0	0	0
28	DS1-401	Deep sea cluster #1	0.1	0	0	1	0	0	0
29	Mm531	*Methylomonas*	2.65E-05	1990	2212	2966	1	2	2
30	Mm_M430	*Methylomonas*	0.5	0	0	11	0	0	0
31	Mm_RS311	*Mm.methanica, RS clade(10-286)*	0.03	0	1	1995	0	0	1
32	Mm_ES294	*Methylomonas*	1.49E-07	2	2	6	0	0	0
33	Mm_ES543	*Methylomonas*	5.36E-07	298	2715	3159	0	2	2
34	Mm_ES546	*Methylomonas*	1.49E-07	2300	44233	45874	2	33	34
35	Mm_MV421	*Methylomonas*		0	0	0	0	0	0
36	Mm451	*Methylomonas*	1.91E-06	2065	6942	30834	2	5	23
37	Mm275	*Methylomonas*	9.17E-05	1548	1778	1873	1	1	1
38	Alp7-441	*Alpine soil Methylomonas, Alp#7 (10-282)*	4.12E-08	47	6551	6983	0	5	5
39	peat_1_3-287	*Mehtylomonas*-related peat clones	3.11E-04	0	720	732	0	1	1
40	Est514	*Methylomicrobium*-related clones		0	0	0	0	0	0
41	Mmb259	*Methylomicrobium album* + Landfill *M.microbia*	0.5	0	0	1	0	0	0
42	Mmb303	*Methylomicrobium album*		0	0	0	0	0	0
43	Mmb304	*Methylomicrobium album* + Landfill *M.microbia* and related		0	0	0	0	0	0
44	LW14-639	*Methylomicrobium LW14 group*	0.03	0	7	394	0	0	0
45	Mmb_RS2-443	*Methylomicrobium, Mmb_RS2*	7.05E-03	0	2276	24845	0	2	18
46	Mmb562	*Mmb. album* and *Methylosarcina*	4.65E-04	0	269	811	0	0	1
47	Mm229	*Deep-branching M.monas (?) group (WHmb3 related group)*	8.87E-03	0	267	318	0	0	0
48	MsQ290	*M.sarcina quisquilliarum related*		0	0	0	0	0	0
49	MsQ295	*M.sarcina quisquilliarum*		0	0	0	0	0	0
50	LP20-644	*Methylomicrobium*-related clones	0.03	0	12	349	0	0	0
51	LP20-607	*LP20 group (Type Ia, deep branching-Mmb?)*	8.06E-03	0	313	2082	0	0	2
52	Ia193	Type I a (*M.bacter-M.monas-M.microbium)*	5.36E-07	38762	46045	46720	29	34	35
53	Ia575	Type I a (*M.bacter-M.monas-*	6.69E-06	6964	36459	48622	5	27	36

		M.microbium-M.sarcina)							
54	Bsed516	*Marine sediment #2, Bsed*	0.02	0	36	36	0	0	0
55	SWI1-375	*Marine sediment #2, SW#1*		0	0	0	0	0	0
56	SWI1-377	*Marine sediment #2, SW#1*		0	0	0	0	0	0
57	Nc_oce426	*Nitrosococcus oceani*		0	0	0	0	0	0
58	DS2-287	*Deep sea #2, subgroup (N.coccus and Deep sea Type Ia 10-298)*		0	0	0	0	0	0
59	AIMS1-442	*Deep sea #2, AIMS#1*		0	0	0	0	0	0
60	DS2-220	*Deep sea #2, subgroup*		0	0	0	0	0	0
61	DS2-626	*Deep sea #2, subgroup*		0	0	0	0	0	0
62	USCG-225	Upland soil cluster Gamma		0	0	0	0	0	0
63	USCG-225b	Upland soil cluster Gamma		0	0	0	0	0	0
64	JR2-409	JR cluster #2 (California upland grassland soil)		0	0	0	0	0	0
65	JR2-468	JR cluster #2 (California upland grassland soil)		0	0	0	0	0	0
66	JR3-505	JR cluster #3 (California upland grassland soil)	0.55	0	0	1	0	0	0
67	JR3-593	JR cluster #3 (California upland grassland soil)		0	0	0	0	0	0
68	501-375	*Methylococcus*- related marine and freshwater sediment clones		0	0	0	0	0	0
69	501-286	*Methylococcus*- related marine and freshwater sediment clones	0.79	0	0	302	0	0	0
70	USC3-305	Upland soil cluster #3		0	0	0	0	0	0
71	Mc396	*Methylococcus*		0	0	0	0	0	0
72	MclT272	*Methylocaldum tepidum*	3.23E-03	0	3	3	0	0	0
73	MclG281	*Methylocaldum gracile*		0	0	0	0	0	0
74	MclS402	*Methylocaldum szegediense*		0	0	0	0	0	0
75	MclS394	*Methylocaldum szegediense* and related		0	0	0	0	0	0
76	MclS400	*Methylocaldum szegediense* and related		0	0	0	0	0	0
77	MclE302	*Methylocaldum* E10		0	0	0	0	0	0
78	Mcl404	*Mc.capsulatus-Mcl.tepidum-Mcl. Gracile-Mcl.Szeg* and related	5.66E-03	0	3	3	0	0	0
79	Mcl408	*Methylocaldum*	1.02E-03	0	3	3	0	0	0
80	fw1-286	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones		0	0	0	0	0	0

81	fw1-639	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	1.02E-03	0	3695	3974	0	3	3
82	fw1-641	fw-1 group: *M.coccus-M.caldum* related marine and freshwater sediment clones	1.02E-03	0	3712	4349	0	3	3
83	JHTY1-267	*JH-TY#1*		0	0	0	0	0	0
84	JRC4-432	Japanese rice cluster #4		0	0	0	0	0	0
85	OSC220	Finnish organic soil clones and related	1.91E-06	5	6	6	0	0	0
86	OSC300	Finnish organic soil clones and related	5.66E-03	0	13	13	0	0	0
87	JRC3-535	Japanese Rice Cluster #3	0.08	0	27	29	0	0	0
88	LK580	fw-1 group + Lake Konstanz sediment cluster	1.31E-04	0	3698	5101	0	3	4
89	RSM1-419	*RSM#1*	1.64E-03	0	29	2906	0	0	2
90	JHTY2-562	*JH-TY#2*	5.16E-04	0	64	66	0	0	0
91	JHTY2-578	*JH-TY#2*	5.68E-04	0	62	8680	0	0	6
92	JRC2-447	Japanese Rice Cluster #2		0	0	0	0	0	0
93	LW21-374	LW21 group	6.69E-06	559	1275	1361	0	1	1
94	LW21-391	LW21 group	3.11E-04	0	138	138	0	0	0
95	M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones	3.11E-04	0	11	12	0	0	0
96	M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones	9.17E-05	2	2	2	0	0	0
97	Mth413	*Methylothermus*	5.36E-07	1	2	2	0	0	0
98	Mha-500	*Methylohalobius - M.thermus and related ?*	0.02	0	1567	1568	0	1	1
99	DS3-446	Deep sea cluster #3	0.45	0	0	1	0	0	0
100	PmoC640	*PmoC*	0.4	0	0	17	0	0	0
101	PmoC308	*PmoC*		0	0	0	0	0	0
102	Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)	3.11E-04	0	441	444	0	0	0
103	Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)	2.65E-05	7	35	1056	0	0	1
104	McyB304	*M.cystis B (parvus/echinoides/*strain M)	1.02E-03	0	32	992	0	0	1
105	Mcy255	*M.cystis B (parvus/echinoides/*strain M)	1.02E-03	0	238	396	0	0	0
106	Mcy459	*Methylocystis*	6.69E-06	52	29245	34505	0	22	26
107	Mcy264	*Methylocystis*	3.11E-04	0	431	449	0	0	0
108	Mcy270	*Methylocystis*	6.69E-06	420	2408	2661	0	2	2

109	Mcy413	*Methylocystis*	9.17E-05	2430	42483	43133	2	32	32
110	Mcy522	*Methlocystis* A + peat clones	3.23E-03	0	1350	35423	0	1	26
111	Mcy233	*Methylocystis*	9.17E-05	87	928	1256	0	1	1
112	McyM309	*M.cystis strain M and related*	0.02	0	1730	1740	0	1	1
113	Peat264	peat clones	6.69E-06	3501	8813	10819	3	7	8
114	MsS314	*Methylosinus sporium*	0.02	0	3	3	0	0	0
115	MsS475	*Methylosinus sporium*	3.23E-03	0	3883	3967	0	3	3
116	Msi263	*Methylosinus sporium* + 1 *Msi.trichosporium subclaster*	2.65E-05	1	1	3	0	0	0
117	Msi423	*Methylosinus*	2.65E-05	13	13	13	0	0	0
118	MsT214	*Methylosinus trichosporium* OB3b and rel.	3.23E-03	0	12	2109	0	0	2
119	MsT343	*Methylosinus trichosporium* OB3b and rel.	6.69E-06	1	8257	16894	0	6	13
120	MM_MsT343	*Methylosinus trichosporium* OB3b and rel. - MM control probe!	6.69E-06	8195	17371	28776	6	13	21
121	Msi520	*Methylosinus trichosporium*		0	0	0	0	0	0
122	Msi269	*Methylosinus trichosporium*	0.03	0	13	17	0	0	0
123	Msi294	*Methylosinus*	3.23E-03	0	2065	2709	0	2	2
124	ARC2-518	*Deep branching type II clade ARC2 - Methylosinus trichosporium 15-084 group?*	2.65E-05	382	568	574	0	0	0
125	Msi232	*M.sinus*+ most *M.cystis* -considered as additional type II probe	3.11E-04	0	4513	5316	0	3	4
126	II509	Type II	1.02E-03	0	45111	46942	0	34	35
127	II630	Type II	3.23E-03	0	46290	48407	0	34	36
128	Alp8-468	*Type II novel pmoA, Alpine cluster Alp#8*		0	0	0	0	0	0
129	xb6-539	Novel *pmoA* copy of type II and related environmental clones	9.58E-03	0	17	17	0	0	0
130	LP21-190	Novel *pmoA* copy of type II and related environmental clones	3.11E-04	0	3	3	0	0	0
131	LP21-260	Novel *pmoA* copy of type II and related environmental clones	0.08	0	4	5	0	0	0
132	NMcy1-247	Novel *pmoA* copy of *M.cystis #1* (?)	0.11	0	0	1	0	0	0
133	NMsi1-469	Novel *pmoA* copy of *M.sinus*	0.99	0	0	3	0	0	0
134	NMcy2-262	Novel *pmoA* copy of *M.cystis #2* (?)	5.36E-07	1	1	1	0	0	0
135	LP21-436	*Mcy + Msi novel pmoA #1 groups*	0.5	0	0	2	0	0	0

136	NMsiT-271	Novel *pmoA* copy of *M.sinus trichpsporium* (?)	0.25	0	0	23	0	0	0
137	LP21-232	Novel *pmoA* copy of type II and related environmental clones	1.02E-03	0	3	10	0	0	0
138	RA14-299	RA14 related clones	0.08	0	2	2	0	0	0
139	RA14-594	RA14 related clones	3.11E-04	0	3	3	0	0	0
140	RA14-591	RA14 related clones	2.65E-05	3	3	3	0	0	0
141	Wsh1-566	Watershed + flodded upland cluster 1	1.05E-04	0	5	7	0	0	0
142	Wsh2-491	Watershed + flodded upland cluster 2		0	0	0	0	0	0
143	Wsh2-450	Watershed + flodded upland cluster 2	6.47E-03	0	18	19	0	0	0
144	B2rel251	*Methylocapsa*-related clones		0	0	0	0	0	0
145	B2-400	*Methylocapsa*		0	0	0	0	0	0
146	B2-261	*Methylocapsa*	2.65E-05	1	2956	3150	0	2	2
147	B2all343	*Methylocapsa* and related clones	4.13E-04	0	7	33	0	0	0
148	B2all341	*Methylocapsa* and related clones	4.65E-04	0	7	169	0	0	0
149	pmoAMO3-400	clone pmoA-MO3		0	0	0	0	0	0
150	pmoAMO3-486	*MO3 group*		0	0	0	0	0	0
151	pmoAMO3-511	*MO3 group*	1.02E-03	0	1	1	0	0	0
152	Ver330	*Verrucomicrobia, all pmoA1+pmoA2*		0	0	0	0	0	0
153	Ver307	*Verrucomicrobia, all pmoA2*		0	0	0	0	0	0
154	Ver285	*Verrucomicrobia, Ma.fum pmoA2+Ma.kam. pmoA2*		0	0	0	0	0	0
155	Ma_F1-355	*Ma.fum. pmoA1*	0.1	0	0	58329	0	0	43
156	Ma_F1-594	*Ma.fum. pmoA1*	0.45	0	0	9	0	0	0
157	Ma_I1-312	*Ma.inf. pmoA1*		0	0	0	0	0	0
158	Ma_I1-401	*Ma.inf. pmoA1*		0	0	0	0	0	0
159	Ma_F3-638	*Ma.fum. pmoA3*		0	0	0	0	0	0
160	Ma_F3-542	*Ma.fum. pmoA3*		0	0	0	0	0	0
161	ESR-579	ESR (Eastern Snake River) cluster	7.25E-03	0	1	3	0	0	0
162	M84P22-514	environmental clones of uncertain identity		0	0		0	0	0
163	TUSC409	Tropical Upland Soil Cluster #2	0.79	0	0	2	0	0	0
164	TUSC502	Tropical Upland Soil Cluster #2	0.02	0	4	4	0	0	0
165	mtrof173	Universal	0.11	0	0	77749	0	0	58
166	mtrof362-I	Methanotrophs	1.02E-03	0	16724	39763	0	12	30
167	mtrof661	Methanotrophs	0.07	0	786	883	0	1	1
168	mtrof662-I	Methanotrophs	1.02E-03	0	4475	20778	0	3	15
169	mtrof656	Methanotrophs	9.58E-03	0	44814	53849	0	33	40
170	NmNc533	*Nitrosomonas-*	0	0	0	0	0	0	0

		Nitrosococcus							
171	Nsm_eut381	*Nitrosomonas eutropha*	0	0	0	0	0	0	0
172	PS5-226	*Nitrosomonas-Nitrosococcus* related clones	0	0	0	0	0	0	0
173	Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	0	0	0	0	0	0	0
174	NsNv207	*Nitrosospira-Nitrosovibrio*	0	0	0	0	0	0	0
175	NsNv363	*Nitrosospira-Nitrosovibrio*	0	0	0	0	0	0	0
176	SV308	*Svalbard clade*	0	0	0	0	0	0	0
177	SVrel583	*Svalbard clade and related*	0	0	0	0	0	0	0
178	Nit_rel471	AOB related clones/probably methanotrophs	0	0	0	0	0	0	0
179	Sed585	*Ssedi#1*	0	0	0	0	0	0	0
180	Sed422	*Ssedi#1 and related*	0	0	0	0	0	0	0
181	Nit_rel223	AOB related clones/probably methanotrophs	0	0	0	0	0	0	0
182	Nit_rel417	*Arctic soil related #1, subgroup*	0	0	0	0	0	0	0
183	Nit_rel419	*Arctic soil related #1, subgroup*	0	0	0	0	0	0	0
184	Nit_rel526	*JRC#1+CCd#1 groups*	0	0	0	0	0	0	0
185	Nit_rel652	*Arctic soil MOB*	0	0	0	0	0	0	0
186	ARC529	AOB related clones/probably methanotrophs	0	0	0	0	0	0	0
187	Nit_rel470	AOB related clones/probably methanotrophs	0	0	0	0	0	0	0
188	Nit_rel351	AOB related clones/probably methanotrophs	0.3	0	0	1	0	0	0
189	gp17-438	*environmental clones of uncertain identity - gp17*	0.1	0	0	6	0	0	0
190	Nit_rel304	AOB related clones/probably methanotrophs - *Crenothrix* and related	0	0	0	0	0	0	0
191	NLw303	*environmental clones of uncertain identity - NL wetland*	0	0	0	0	0	0	0
192	M84P105-451	environmental clones of uncertain identity	0	0	0	0	0	0	0
193	WC306_54-385	environmental clones of uncertain identity	0	0	0	0	0	0	0
194	WC306-54-516	environmental clones of uncertain identity	0	0	0	0	0	0	0
195	gp23-454	environmental clones of uncertain identity	0	0	0	0	0	0	0
196	MR1-348	environmental clones	0	0	0	0	0	0	0

		of uncertain identity							
197	gp619	environmental clones of uncertain identity	0	0	0	0	0	0	0
198	gp391	environmental clones of uncertain identity	0	0	0	0	0	0	0
199	gp2-581	environmental clones of uncertain identity	0	0	0	0	0	0	0
200	RA21-466	clone RA21 - environmental clone of uncertain identity	0.79	0	0	24	0	0	0
201	hyaBp	spiking control (*hyaB* gene of E.coli)	0	0	0	0	0	0	0

Table 3. Sequences of the phylogenetic tree (fig.3, Chapter 6). Names used in the phylogenetic tree are indicated in the column 'clade'. ID 1 gives number from the *pmo*A-ARB database and ID2 shows the accession codes (if available). ARB numbers in column "ID2" refer to unpublished *pmo*A sequences provided by Levente Bodrossy.

Clade	Amount of species_in_clade	Amount of mapped reads	ID1	ID2	Environment
Nitrosopumilus_maritimus_and_uncultured_Crenarchaeote_amoA_DQ148610_	3	0.00	Nitrosopumilus_maritimus_SCM1	EU239959	
		0.00	Uncultured_crenarchaeote_MX_4_10	EU23995	
		0.00	uncultured_crenarchaeote_MX_6_9	DQ148610	
ORI_DQ228465_Nitrosovibrio_sp_FJI82_amoA	1	0.00	Nitrosovibrio_sp_FJI82_amoA	DQ228465	
ORI_EF175100_Nitrosospira_sp_Enl299_AmoA	1	0.00	Nitrosospira_sp_Enl299_AmoA	EF175100_	
ORI_Nitrosomonas_europaea_ATCC19718	1	0.00	Nitrosomonas_europaea_ATCC19718		
ORI_Nitrosospira_briensis_1732261	1	0.00	Nitrosospira_briensis_1732261		
ORI_gi3282756_Nitrosolobus_multiformis	1	0.00	Nitrosolobus_multiformis	gi3282756	
ORI_gi4105846_Thermophilic_methan	1	0.00	Thermophilic_methan	gi4105846	
ORI_gi84627398_Crenothrix_polyspora_11	1	0.00	Crenothrix_polyspora_11	gi84627398	
pxm	4	0.00	Methylomicrobium_album_pxm	EU722431	
Methylomirabilis	2	0.00	Methylomonas_sp_LW13_pxm	EU722432	

_oxyfera_NC101.000000		0.00	Methylomonas_methanica_S1_pxm	EU722433	
		0.00	Methylobacter_marinus_A45_pxm	EU722430	
		0.00	NC10_like bacteria		
ORI_EU723731_Hawaiian_soil_ML126 ML0Yyyy9	1	0.00	Methylomirabilis_oxyfera_Ooij		
		0.00	Hawaiian_soil_ML126 ML0Yyyy9	EU723731	Acacia koa forest soil
Environmental_clones_MO3_group	2	16.00	B36	EU647289	abandoned methane-collected landfill cover soil
Methylocapsa_acidiphila_B2_and_related_environmental_clones_	3	11.00	Der16	EU071102	landfill cover site
		801.95	uncultured_bacterium_Bakchar31	AJ278729	
Methylocapsa sp. FMD	1	688.20	methanotroph_B2	AJ278727	
		688.20	Methylocapsa_acidiphila_B2_pmoA	CT005238	
		14.50	Methylocapsa_sp._FMD	ARB_2FD201F	
Methylocapsa_related_environmental_clones_	9	78.61	LOPB13.4	AF358054	Peat soil microcosm pH-6.8
		69.81	RB-60	ARB_ABDFF6B	Roman Bath hot freshwater fw
		57.87	peat11-4	ARB_72674CB	Moorhouse Peat
		41.09	peat1-7	ARB_BB5EBC9F	Moorhouse Peat
		16.88	japan_yumoto_6-48	AY236518	Japan Yumoto hot spring freshw
		13.95	soda_lake_4-51	ARB_684FE46A	Transbaikal soda lake sediment

		12.22	SL_4.117	AY550709	Transbaikal soda lake sediment
		11.22	LOPA12.6	ARB_9BF268F0	Peat soil microcosm pH-6.8
		11.22	uncultured_bacterium	AF358043	Peat soil microcosm SIP
Environmental_clones_MO3_related_rice_paddy_and_alpine_meadow	3	36.03	20DN-G7	ARB_711FF1E0	Alpine meadow
Alpha MOB ARB 98FD9AEA No_Na251	1	13.00	No_Nam52	ARB_4831E003	Alpine meadow
		12.00	uncultured_bacterium_Rr90a-43	AJ543418	flooded rice paddy, rice roots
		110.20	No_Na251	ARB_98FD9AEA	Alpine meadow
Methylocystis_sp_KS7_and_environmental_clones_AB280424	27	153.35	RSM74	AM910135	rhizosphere soil from rice field
Methylocystis_H2s_and_F10V2a_and_environmental_clones_Moor_house_peat_P1_2_and_alpine_meadow_EF644604	103	126.88	Methylocystis_sp._KS7	AJ459034	Lake Kinneret (Israel) oxic su
		116.75	soda_lake_5-70	AY236087	Transbaikal soda lake sediment
		87.59	GP16	EU244577	tropical agricultural soil
		82.93	CM2	EU131054	coal mine soil
		73.50	GuyF4.M13rev_K07.ab1	ARB_A44271E1	
		60.89	RSM55	AM910125	rhizosphere soil from rice field
		47.82	methanotroph H8	EU359003	soil-water interface from temperate rice fields

		42.67	pmoM-14	AB113639	Subsurface geothermal water in
		32.08	R4.13.PmoA-6	AB280424	Methane-consuming sludge
		31.52	18DN-B7	ARB_E79D1505	Alpine meadow
		23.36	18DN-H6	ARB_D4170268	Alpine meadow
		22.10	1		
		21.13	18DN-F7	ARB_13D93B72	Alpine meadow
		19.58	WD147972-MHERY	ARB_DDB70611	
		18.45	C11-79	ARB_F8998D78	landfill simulating lysimeter,
		17.55	SIP CM48	EU131051	coal mine soil
		16.98	RS-8-156	AM849634	methanotroph pmoA clone from rhizosheric soil of flooded rice microcosms
		16.59	uncultured_putative_methanotroph	AY597822	Sphagnum peat bog soil
		15.62	18DN-A10	ARB_D641E4E9	Alpine meadow
		15.07	M2+75	ARB_6BA81373	landfill simulating lysimeter,
		15.03	C11-13	ARB_1793DC78	landfill simulating lysimeter,
		13.66	C11-91	ARB_1F731396	landfill simulating lysimeter,
		12.55	uncultured_methanotrophic_proteobacterium	AY488081	Lake Konstanz littoral lake fr
		12.27	RSM86	AM910143	rhizosphere

					field
		10.91	uncultured_Methylocystis_sp.	AY195661	landfill site cover soil
		10.40	GP18	EU244579	tropical agricultural soil
		1853 4.68	uncultured Methylocystis	DQ379514	Soil
Environmental_clones_alpine_meadow	9	7929. 35	GIS12H4_P12	AY080955	Gisburn oak forest soil microc
		2304. 00	20DN-G6	ARB_8A126 CCE	Alpine meadow
		1888. 38	1		
		1449. 02	18DN-F87	ARB_A9373 CD2	Alpine meadow
		1422. 68	20DN-E1	ARB_178743 60	Alpine meadow
		1273. 73	No_Na243	ARB_4C767 C14	Alpine meadow
		930.1 3	20D1-C4	ARB_A38BF 86B	Alpine meadow
		927.2 4	No_Na276	ARB_5E1256 04	Alpine meadow
		819.1 0	MHPCr49	EF644613	peatland with pH 4.5
		463.6 5	20D1-C9	ARB_70D2F E69	Alpine meadow
		462.9 9	No_Na221	ARB_28E61 C28	Alpine meadow
		446.3 8	20DN-G9	ARB_615D1 B80	Alpine meadow
		446.3 8	20DN-H5	ARB_C26202 BB	Alpine meadow
		422.6 0	L4Apom	AY781164	Finnish raised bog complex; pe
		407.8 3	uncultured_eubacterium_PD_1	AF006046	Peat acidic soil
		390.3 3	20DN-A4	ARB_BB167 3AA	Alpine meadow
		331.8 6	No_Na205	ARB_37177B F3	Alpine meadow

		283.54	20D1-D1	ARB_810524BB	Alpine meadow
		274.44	20D1-C6	ARB_3B595579	Alpine meadow
		272.08	*Methylocystis*_sp_H2s	FN422005_	
		264.78	No_Na208	ARB_C65327D3	Alpine meadow
		241.02	No_Na287	ARB_6DD424C8	Alpine meadow
		240.30	No_Na185	ARB_38913B05	Alpine meadow
		233.40	20DN-G5	ARB_8DB42DB	Alpine meadow
		230.27	20D1-G4	ARB_B85349CA	Alpine meadow
		172.55	20D1-H5	ARB_FE25FE85	Alpine meadow
		171.16	uncultured_bacterium	AY309213	Northern peatlands peat soil a
		166.95	20D1-A9	ARB_E1E4EFA5	Alpine meadow
		165.36	No_Na288	ARB_E7730472	Alpine meadow
		160.35	No_Na256	ARB_7A0E6AA1	Alpine meadow
		144.86	20D1-F2	ARB_368DF0B1	Alpine meadow
		141.91	20DN-E5	ARB_E412CE16	Alpine meadow
		110.79	No_Na292	ARB_CE57857E	Alpine meadow
		108.03	No_Na277	ARB_B3E86279	Alpine meadow
		105.82	peat11-2	ARB_204E48B4	Moorhouse Peat
		102.56	20DN-H1	ARB_26949437	Alpine meadow
		100.95	20DN-F6	ARB_BCCD9C56	Alpine meadow
		93.68	No_Na158	ARB_BA6761F7	Alpine meadow
		92.47	20DN-G2	ARB_5FF35FAC	Alpine meadow

		91.17	18DN-C3	ARB_40D1D145	Alpine meadow
		82.87	No_Na211	ARB_8F31E058	Alpine meadow
		81.58	No_Na169	ARB_105B0E07	Alpine meadow
		80.47	uncultured_eubacterium_PD_2	AF006047	Peat acidic aci soil
		79.95	No_Na279	ARB_56138A26	Alpine meadow
		76.43	20D1-E3	ARB_19A8E435	Alpine meadow
		74.60	No_Na248	ARB_6CF435C4	Alpine meadow
		72.78	20DN-D6	ARB_A7F7AA5E	Alpine meadow
		66.20	uncultured_eubacterium_PD_4	AF006048	Peat acidic aci soil
		60.83	18DN_E9 18DN_B10		
		60.73	No_Na242	ARB_5BE29BAB	Alpine meadow
		59.05	20D1-E7	ARB_C98F1118	Alpine meadow
		57.96	20DN-A9	ARB_52141EA7	Alpine meadow
		57.68	No_Na259	ARB_9F8DEE4D	Alpine meadow
		52.44	20DN-F4	ARB_75884CEF	Alpine meadow
		43.38	No_Na190	ARB_FC4217E6	Alpine meadow
		40.10	20DN-B10	ARB_484D4CC	Alpine meadow
		40.10	20DN-C4	ARB_80B4AE42	Alpine meadow
		38.23	peat1-2	ARB_4D45B710	Moorhouse Peat
		37.65	20DN-D1	ARB_61BD3A3	Alpine meadow
		37.27	20DN-H7	ARB_4A6E524B	Alpine meadow
		37.00	20DN-H10	ARB_54B0C715	Alpine meadow
		35.59	MHPSr12	EF644626	peat pH4.5
		35.01	MHrXyw10		

			MHrYyy11		
		33.88	20D1-A1	ARB_4F8EABBB	Alpine meadow
		32.61	20DN-H9	ARB_5F094BD7	Alpine meadow
		29.93	No_Na262	ARB_4FB11E53	Alpine meadow
		29.90	20DN-C1	ARB_28CEB23F	Alpine meadow
		29.84	20DN-A2	ARB_5468EAD	Alpine meadow
		27.71	No_Na231	ARB_AF386EF8	Alpine meadow
		26.77	18DN-F4	ARB_CDF7F8AB	Alpine meadow
		26.60	20DN-B9	ARB_2FDCFCEB	Alpine meadow
		25.47	20D1-H2	ARB_C96515D9	Alpine meadow
		24.60	20DN-H4	ARB_E0C3E78A	Alpine meadow
		24.11	No_Na299	ARB_31AB04CF	Alpine meadow
		22.58	No_Na255	ARB_B1A83271	Alpine meadow
		21.89	MHPCr2	EF644604	peat pH4.5
		19.23	18D1-E1-100	ARB_8261274D	Alpine meadow
		17.49	No_Na266	ARB_F885ACA4	Alpine meadow
		17.46	20DN-H6	ARB_DE77DAD2	Alpine meadow
		17.32	20DN-F3	ARB_7C7C09A6	Alpine meadow
		16.87	18D1-C2	ARB_C06176A6	Alpine meadow
		16.34	No_Na180	ARB_FDFE0174	Alpine meadow
		15.89	Methylocystis_sp._W31	ARB_5D7DF7B1	empty
		15.38	GISH4_12P	ARB_B641D56E	Gisburn oak forest soil microc
		15.38	uncultured_bacte	AY080951	Gisburn oak

					microc
		15.16	No_Na230	ARB_7C140CEC	Alpine meadow
		15.11	peat11-6	ARB_33DE4622	Moorhouse Peat
		14.86	20D1-D4	ARB_694CDF77	Alpine meadow
		14.73	No_Na228	ARB_8C773594	Alpine meadow
		14.10	No_Na269	ARB_86FE7470	Alpine meadow
		14.00	No_Na183	ARB_11D9DF41	Alpine meadow
		13.13	No_Na227	ARB_77CA5B67	Alpine meadow
		13.08	No_Na268	ARB_9A3082AB	Alpine meadow
		12.84	No_Na188	ARB_4CF8FFF8	Alpine meadow
		12.81	GIS12H4_P8	ARB_6C57FE5B	Gisburn oak forest soil microc
		12.50	20D1-G5	ARB_49EA2CC	Alpine meadow
		11.20	No_Na258	ARB_9C7FB9E	Alpine meadow
		11.08	20DN-B3	ARB_5718988	Alpine meadow
		11.00	MHPCr39	EF644612	peat pH4.5
		10.95	20D1-C1	ARB_7574315A	Alpine meadow
		10.85	No_Na201	ARB_4D946AF5	Alpine meadow
		10.46	20DN-E9	ARB_BA32599D	Alpine meadow
		450.53	No_Na294	ARB_C77E0009	Alpine meadow
Environmental_clones_rice_field_China_AM910123	2	156.08	No_Na172	ARB_F3E663A4	Alpine meadow
		147.30	18DN-F2	ARB_E3A834FD	Alpine meadow
		103.22	18DN-D10	ARB_990E5CF4	Alpine meadow
		76.25	18DN-H9	ARB_E27C1E97	Alpine meadow

		54.34	No_Na191	ARB_F65F84E3	Alpine meadow
		38.27	No_Na301	ARB_FC4A2564	Alpine meadow
		37.13	20DN-H8	ARB_933EC2CA	Alpine meadow
		13.62	O45W-e	AJ868255	Various upland forest soils so
		1448.68	RSM52	AM910123	rhizosphere soil from rice field
Methylocystis_heyeri_Sakb2s_and_H2t_AM283546	2	35.50	RSM46	AM910119	rhizosphere soil from rice field
		671.69	methanotrophic alpha	AJ868287	Soil
Environmental_soil_clones	2	140.02	Methylocystis heyerii	AM283546	peat bog lake Teufelssee
		17.81	uncultured_bacterium	AY662380	Managed and highly degraded wa
Methanotroph C9 species MphC9	1	13.00	uncultured_bacterium	AY662389	Managed and highly degraded wa
		22.78	methanotroph C9	EU359001	
Methylosinus_trichosporium_M23_UMTRA_6M23	1	18.83	*Methylosinus_trichosporium_M23*	AJ459037	Coleroon Delta (India), coast
Methylosinus_sporium_strains_8_SD56_44_2_SC8_AJ459005	4	98.50	Methylosinus_sporium_SD56	AJ459005	Saale polluted river freshwate
Methylosinus_sporium_strains_LW8_LC3_and_environmental_clones_DQ119048	4	92.67	Methylosinus_sporium_44/2	AJ458997	Rugen, Nardevitz (Germany), wa
		18.43	Methylosinus_sporium_8	AJ459018	Hiddensee, Bessin (German Balt
		18.34	Methylosinus_sporium_SC8	AJ459011	Saale polluted river freshwate
		21.51	Methylosinus_sp._LW8	AY007284	Lake Washington

					sediment fresh
		12.22	Methylosinus sp.	DQ078510	Lake Sediment
		12.22	Methylosinus sp.	DQ078509	Lake Sediment
		6.48	Methylosinus sporium	DQ119048	Lake Sediment
Methylacidiphilum_spp_PmoA_1_2_3	10	0.00	Methylacidiphilum_fumariolicum_PmoA2	EF591086	
		0.00	Methylacidiphilum_kamchatkense_PmoA4	FJ462791	
		0.00	Methylacidiphilum_kamchatkense_PmoA3	FJ462790	
		0.00	Methylacidiphilum_fumariolicum_PmoA3	EF591087	
		0.00	Methylacidiphilum_infernorum_PmoA3	CP000975	
		0.00	Methylacidiphilum_kamchatkense_PmoA2	FJ462789	
		0.00	Methylacidiphilum_fumariolicum_PmoA1	EF591085	
		0.00	Methylacidiphilum_infernorum_PmoA2	CP000975	
		0.00	Methylacidiphilum_kamchatkense_PmoA1	FJ462788	
		0.00	Methylacidiphilum_infernorum_PmoA1	CP000975	
Nitrosococcus_oceanus_and_C113_amoA_ATCC19707	2	0.00	Nitrosococcus_oceanus	ATCC19707	
		0.00	Nitrosococcus_sp_c113		

Methylococcus_ capsulatus_YP_ 115248	2	16.50	P4C32	AM713081	soil
Environmental_c lones_ AY488067	2	0.00	??		
		583.2 9	uncultured methanotroph	DQ235464	Lake Sediment
uncultured bacterium JH-TY23	1	340.3 5	uncultured methanotroph	AY488067	Lake Konstanz littoral lake fr
		86.50	JH-TY23	EU193288	paddy field soil
Environmental_c lones_rice_padd y	6	37.33	PS.I.3	AB222876	Japanese rice field soil
Environmental_c lones_EU19328 0	20	31.20	RS.H4.1	AB222919	Japanese rice straw incorporat
		29.37	RS.H4.2	AB222920	Japanese rice straw incorporat
		12.18	RS.H3.1	AB222912	Japanese rice straw incorporat
		12.18	RS.H3.4	AB222915	Japanese rice straw incorporat
		11.09	RS.H2.1	AB222909	Japanese rice straw incorporat
		5150. 38	uncultured methanotroph	AY488072	Lake Konstanz littoral lake fr
		1497. 11	uncultured bacterium	DQ067083	Lake sediment
		936.0 8	uncultured_bacte rium	AY662376	Managed and highly degraded soil
		833.3 5	uncultured methanotroph	AY488074	Lake Konstanz littoral lake sediment
		441.8 6	S14m-62	EF623770	14m depth in water column

		350.25	uncultured_bacterium	AY662387	Managed and highly degraded wa
		249.00	1		
		151.23	uncultured bacterium	DQ067069	Lake sediment
		116.67	JH-TY15	EU193280	paddy field soil
		100.75	uncultured methanotroph	AY488075	Lake Constance littoral sediment
		96.25	uncultured methanotroph	AY488076	Lake Constance littoral sediment
		93.83	uncultured methanotroph	AY488073	Lake Constance littoral sediment
		80.42	L6pol	AY781167	peat soil
		34.50	L4dol	AY781163	peat soil
		27.50	RSM24	AM910107	rhizosphere soil from rice field
		27.00	RSM40	AM910116	rhizosphere soil from rice field
		25.33	uncultured bacterium	DQ067072	Lake sediment
		18.58	L8pol	AY781168	peat soil
		17.83	L5pol	AY781166	peat soil
		11.00	uncultured methanotroph	AY488077	Lake Constance littoral sediment
ORI_Methylocaldum_szegediense_789	1	0.00	Methylocaldum_szegediense_789	U893...	
ORI_Methylocaldum_tepidum_2911792	1	0.00	Methylocaldum_tepidum	MTU89304	soil

MctepLK6					
ORI_Methylocaldum_gracile_2911786 Mcgrac	1	0.00	Methylocaldum_gracilis	MGU89301	
ORI_EU275141_Methylocaldum_sp_05J_I_7_pmoA	1	0.00	Methylocaldum_sp_05J_I_7_pmoA	EU275141	
ORI_EU275142_Methylocaldum_sp_0510_P_2_pmoA	1	0.00	Methylocaldum_sp_0510_P_2_pmoA	EU275142	
Methylomonas_sp_83_LW15_LW13_LC1_and_environmental_samples_AM910134	24	2478.38	Methylomonas_sp._ML64	AF510080	landfill
Methylomonas_strain_M5_and_environmental_clones_EU358985	8	686.84	1HA_4	AY550696	Transbaikal soda lake (Suduntu
		397.75	Methylomonas_sp._LW15	AF150795	Lake Washington sediment fresh
		233.96	9_661_9	DQ008363	Eastern Snake River Plain Aqui
		161.86	1		
		130.47	W9_661_5	DQ008403	Eastern Snake River Plain Aqui
		121.78	en1.1w.txt	ARB_40802EAD	marine or estaurine mar/est
		70.27	uncultured methanotrophic	DQ119042	Lake Sediment
		59.68	Methylomonas sp.	DQ119046	Lake Sediment
		32.17	Methylomonas_sp._IMET_10556	ARB_76FA20E3	empty

		27.22	1		
		23.28	1		
		17.71	P7m-03	EF623665	7m depth in water column
		17.50	1HM_33	AY550701	Transbaikal soda lake (Suduntu
		15.83	RSM72	AM910134	rhizosphere soil from rice field
		14.39	I13	EU358990	soil-water interface from temperate rice fields
		14.07	RSM29	AM910109	rhizosphere soil from rice field
		12.97	JH-TY02	EU193267	paddy field soil
		12.71	Methylomonas_sp._LW13	AF150793	Lake Washington sediment fresh
		10.96	JH-TY11	EU193276	paddy field soil
		1.50	Methylomonas sp.	DQ078501	Lake Sediment
		0.00	Methylomonas_methanica	U31653	empty
		0.00	Methylomonas_sp._D1a	AJ544092	empty
		0.00			
		2787 6.70	MHPSr2	EF644619	peatland with pH 4.5
Environmental_clones_EU193270	11	2275 2.73	uncultured_bacterium	AY309206	Northern peatlands peat soil a
		5159. 50	uncultured bacterium	DQ202261	Peatlands
		964.8 9	peat1-3	ARB_746BB899	Moorhouse Peat
		470.5 0			
		362.0	uncultured_bacte	AY309207	Northern

					peat soil a
		296.45	I37	EU358985	soil-water interface from temperate rice fields
		21.14	peat11-3	ARB_8BD11070	Moorhouse Peat
		667.37	JH-TY05	EU193270	paddy field soil
		135.23	18DN-F10	ARB_E58EBA09	Alpine meadow
		125.75	18DN-B1	ARB_1A515361	Alpine meadow
		56.83	18DN-F1	ARB_D830B6A4	Alpine meadow
		30.90	18DN-D8	ARB_929CF196	Alpine meadow
		27.54	18DN-G4	ARB_BD721563	Alpine meadow
		23.33	IB76	EU358982	soil-water interface from temperate rice fields
		22.54	No_Na305	ARB_302C7D09	Alpine meadow
		21.66	18DN-D7	ARB_70781463	Alpine meadow
		15.73	18DN-D1	ARB_74191A72	Alpine meadow
		14.85	18DN-G1	ARB_A7C92EE0	Alpine meadow
Methylobacter_tundripaludum_LW_12_and_environmental_clones_incl_Moor_house_peat_MHPSr6_	12	944.36	20DN-E3	ARB_CC67CD18	Alpine meadow
Alpine meadow , austria	1	318.18	1		
		181.95	No_Na213	ARB_76100C9A	Alpine meadow
		26.18	L2_2-81	ARB_CF520331	

		22.95	LP19	AB064376	Sediment samples in TCE-contam
		22.00	13RNMSmb9	EF625914	landfill cover soil
		18.67	MHPSr6	EF644623	peatland with pH 4.5
		17.50	No_Na226	ARB_66BBA285	Alpine meadow
		10.93	LP1	AB064369	Sediment samples in TCE-contam
		4.83	1		
		2.00	Methylobacter_sp._LW12	AY007285	Lake Washington sediment fresh
		0.00	1		
		21.42	No_Na198	ARB_385C8083	Alpine meadow
ORI_AJ868410_Methylobacter_sp_5FB_pmoA MhbSpec2	1	0.00	Methylobacter_sp._5FB	AJ868410	Upland soils, 20% CH4 enrichme
Environmental_clones_AY550728	8	609.20	Methylobacter_sp._D3a	ARB_E90D89F3	
ORI_AF016982_Methylobacter_sp_BB5_1_pmoA Mb_BB_51 j10b_txt	1	346.61	MLM46	AY572001	Soda lake
		202.04	*Methylobacter*_sp._MALY4	ARB_DC2A4D57	empty
		154.87	SL_4.121	AY550719	Transbaikal soda lake sediment
		144.53	SL_5.24	AY550728	Transbaikal soda lake sediment
		31.04	Sp-8	ARB_2CEE0E27	source of pmoA clone
		19.04	RB-16	AY236517	Roman Bath hot freshwater fw
		18.38	japan_yumoto_6-	ARB_36C267	Japan

					spring freshw
		4.71	Methylobacter_sp_BB5_1_	AF016982	marine or estaurine mar/est
ORI_DQ119047_Methylosoma_difficile_LC_2	1	1.00	Methylosoma_difficile_LC_2_pmoA	DQ119047	
Methylovulum miyakonense	1	0.00	*Methylovulum miyakonense*	AB501285.1	
ORI_strain_M200_pmoA_consensus	1	1.50	strain_M200_pmoA_consensus		
Methylomicrobium_spp	2	0.00	Methylomicrobium_buryatense_5B_		
		0.00	Methylomicrobium_japanense	AB253	
Clonothrix_fusca	2				
		45.50	Clonothrix_fusca	gi119352924	
		45.50	*Clonothrix fusca*	DQ984192	
Environmental_clones_EU071118	2				
Methylosarcina_spp_and_environmental_clones_EF62592	10	17.67	Der23	EU071118	landfill cover site
		17.67	Der1	EU071088	landfill cover site
		95.67	Der25	EU071119	landfill cover site
ORI_FJ529779_Michigan_soil_Late_DF_51_B06	1	49.33	13RH2Omb10	EF625924	landfill cover soil
		44.83	13RNMSmb15	EF625913	landfill cover soil
ORI_DQ367738	1	21.50	13Wytnmc_mb18	EF015807	Wytham soil
		10.75	1		

_Lake_Washington UncGamma		10.75	12WHmb8	ARB_9AA1CE34	
		0.50	Methylosarcina_lacus MeoSpec5	AY007286	
		0.00	methylobacter_albus MetAlbu3	U31654	
		0.00	Methylosarcina_fibrata	AF177325	
		0.00	Methylosarcina_quisquiliarum	AF177326	
		0.00	Michigan_soil_Late_DF_51_B06	FJ529779	
		0.00	Lake_Washington UncGamma	DQ367738	
ORI_AJ579663_German_upland_soil_E5FB_fE5FB_f	1	0.00	German_upland_soil_E5FB	AJ579663	
ORI_EU275141_Methylocaldum_sp_05J_l_7_pmoA	1	0.00	Methylocaldum_sp_05J_l_7_pmoA	EU275141	