Microbial Methane Oxidation in Paddy Fields

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CONTENTS

SUMMARY

Both anthropogenic and natural sources of the greenhouse gas methane (CH_4) contribute each year to the global CH_4 budget. Since natural and cultivated wetlands are the main contributors, the role of methanotrophs in these environments has been more extensively investigated. Aerobic methanotrophs are a unique subset of methylotrophic bacteria capable of utilizing CH_4 as a sole energy source. After their first discovery in 1906 by Söhngen, extensive research has been done to decipher their diversity and methanotrophic capabilities in various environments. These microorganisms were found to be ubiquitous in nature, and are currently affiliated with the *Gammaproteobacteria*, *Alphaproteobacteria*, *Verrucomicrobia* and NC10 phyla. The objectives of the research presented in this thesis were to develop new molecular methods to investigate methanotrophic diversity in different ecosystems (**chapter 2**), unravel the differences in their community composition between a source and a sink environment (**chapter 3**), use lab-scale microcosms to study the role they have on the establishment of a CH_4 and O_2 counter-gradient in wetlands and to enrich for novel methanotrophs (**chapter 4**), and to explore the phenotypical and genotypical properties of a novel isolate from a paddy field (**chapter 5**). **Chapter 1** of this thesis presents an introduction to the ecophysiology of methanotrophs and a summary of the main findings, while **chapter 6** puts the results in perspective and gives an outlook.

The **first chapter** presents an overview of our current understanding of methanotrophy, the environmental distribution of methane-oxidizing bacteria (MOB) and their global impact on the methane budget. The conversion of CH_4 to methanol in MOB takes place via a copper- and/or iron-containing enzyme called methane monooxygenase (MMO). This enzyme exists in two forms: a soluble MMO (sMMO) and a membrane-bound particulate MMO (pMMO). Although the process of methane oxidation is generally similar in MOB, other cellular metabolisms (such as carbon fixation, nitrogen fixation, etc.) and their cell structures are vastly different. This has resulted into the classification of methanotrophs. This chapter also includes a short overview on methane oxidation in wetlands and more specifically paddy fields, which is the environment that this thesis has mostly focused on.

The **second chapter** investigates the diversity of methanotrophs and aims at extending their molecular detection through the use of methane monooxygenase gene-targeted primers. A novel set of degenerate primers were designed based on the full *pmoCAB* operon sequence. The conserved regions used as target sites were found in the *pmoC* and *pmoA* genes, covering the intergenic region between those genes. The use of this primer set on various ecosystems resulted in the detection of the *pmoCA* gene fragment from methanotrophs of all phyla discovered to date. Neither ammonia oxidizers, nor the ammonia monooxygenase containing comammox *Nitrospira* were detected. Lastly, we were able to generate MOB lineage-specific fragments due to the primer binding sites immediately flanking the intergenic region. This unique property could

be further explored in future high throughput amplicon studies to distinguish different MOB lineages in various environments.

In the **third chapter** the differences in the total bacterial community composition, and more specifically methanotrophs, between a CH_4 source and a CH_4 sink environment were investigated. We found that interestingly, CH_4 fluxes can be highly variable between a wetland (traditionally known as a source) and its neighboring meadow (traditionally known as a sink) with no real statistical differences observed between the two areas. However, the total bacterial community compositions showed significant differences, with the family of *Fimbriimonadaceae* being highly enriched in the paddy field. Focusing on the MOB community, the paddy field was found to be more enriched in various methanotrophic families such as *Methylomirabilaceae*, *Methylomonaceae*, and *Methylophilaceae*. Lastly, based on the findings of this chapter and previous literature, we proposed two possible working hypotheses responsible for the observations made in this chapter.

In the **fourth chapter**, we used lab-scale microcosms for a three month incubation of methanotrophs in soil slurries, potentially enriching for previously uncultured MOB groups. The slurries became highly active, with all CH_4 provided oxidized before it reached the top compartment. The vertical oxygen profiles measured at various time points changed over time, indicating that the bacterial community is the drivers of this counter gradient occurring naturally in soil. Metagenomic analyses indicated that the orders *Methyloccales* and *Myxococcales* become highly enriched over time. The *pmoA* sequences extracted from both top and bottom layers of the soil slurries suggested that type I methanotrophs become more dominant in the top layer, while type II showed dominance in the bottom layer. Lastly, our subsequent enrichment strategies resulted in three different highly enriched methanotrophic consortia containing novel *pmoA* sequences most closely related to *Methylocystis* and *Methylomonas* genera.

The **fifth chapter** describes the phenotypical and genotypical properties of a novel methanotrophic isolate from a paddy field using microcosms as a pre-enrichment step. Based on 16S rRNA phylogeny and PLFA profiles, this strain was found to be closely related to type Ib methanotrophs and contained $C_{16:1}\omega9c$ as the major membrane phospholipid fatty acids, which has not been found in other methanotrophs previously. The draft genome obtained from this organism demonstrated a potential for metabolic diversity with enzymes involved in the sulfur cycle.

The thesis is concluded in chapter six through the integration of the results, presented in earlier chapters (**Chapter 2**, **3**, **4**, and **5**), into our current understanding of the role that methanotrophs play in cultivated wetlands. Furthermore, this chapter elaborates on uncertainties that remain to be investigated in future research. Therefore, additional experiments and recommendations are provided to aid in resolving these questions and gain further insights into the environmental impact of methanotrophs.

SAMENVATTING

Aan het wereldwijde methaan-budget dragen elk jaar zowel antropogene als natuurlijke bronnen van het broeikasgas methaan (CH_4) bij. Omdat natuurlijke en gecultiveerde draslanden de belangrijkste spelers zijn binnen dit budget is de rol van methanotrofen in deze milieus uitgebreider onderzocht. Aerobe methanotrofen zijn een unieke groep bacteriën binnen de methylotrofe bacteriën die CH_4 als energiebron kunnen gebruiken. Nadat Söhngen deze groep bacteriën ontdekte in 1906 is uitgebreid onderzoek gedaan naar het ontcijferen van hun diversiteit en methanotrofe vermogens in verschillende omgevingen. Deze micro-organismen bleken overal aanwezig te zijn in de natuur en worden op dit moment verwant aan de *Gammaproteobacteria*, *Alphaproteobacteria*, *Verrucomicrobia* en NC10 phyla.

In dit proefschrift wordt het onderzoek gepresenteerd met als doel het ontwikkelen van nieuwe moleculaire methodes om de methanotrofe diversiteit in verschillende ecosystemen te onderzoeken (**hoofdstuk 2**), het ontrafelen van de diversiteit aan soorten tussen ecosystemen die methaan produceren of consumeren **(hoofdstuk 3)**, het gebruik van microcosms om de door methanotrofen CH_4- en O_2-gradiënt een in draslanden te onderzoeken en tevens voor het verrijken van nieuwe methanotrofen **(hoofdstuk 4),** en om de fenotypische en genotypische eigenschappen van een nieuw isolaat uit een rijstveld te onderzoeken (**hoofdstuk 5**). **Hoofdstuk 1** van deze thesis zal een introductie geven op de ecofysiologie van methanotrofen en geeft een samenvatting van de belangrijkste vindingen. **Hoofdstuk 6** geeft een groter perspectief op de resultaten en geeft voorstellen voor vervolg onderzoek

Hoofdstuk 1 van dit proefschrift geeft een overzicht van de huidige kennis die we hebben over methanotrofie, hoe de methaan oxiderende bacteriën (MOB) verdeeld zijn over verschillende ecosystemen en wat hun impact is, wereldwijd, op het methaan budget. In MOB's vind de conversie van CH_4 naar methanol plaats via een koper en/of ijzerhoudend enzym dat methaan monooxygenase (MMO) wordt genoemd. Dit enzym komt in de natuur in twee vormen voor. De eerste vorm een de oplosbare vorm MMO (sMMO) en de tweede een membraan gebonden MMO (pMMO). Hoewel het proces van methaan oxidatie behoorlijk gelijk is in verschillende MOBs, variëren andere eigenschappen zoals koolstof- en stikstoffixatie en de cel structuur wel. Door deze fenotypische en genetische verschillen tussen methanotrofen is het mogelijk geweest deze in verschillende groepen op te delen. Dit hoofdstuk zal tevens een kort overzicht geven over de methaan oxidatie door methanotrofen in draslanden maar specifieker rijstvelden, hetgeen waar de focus ligt van het onderzoek in dit proefschrift.

Hoofdstuk twee richt zich op het ontwikkelen van moleculaire bio markers die de diversiteit van methanotrofen binnen verschillende ecosystemen zichtbaar maken. Een nieuwe set primers werd daarbij ontworpen gebaseerd op de gehele *pmoCAB* operon sequentie. De geconserveerde regio's die werden gebruikt als target sequentie werden gevonden in de pmoC- en pmoA-genen. Het gebruik van deze primer set op monsters uit verschillende ecosystemen resulteerde in de detectie van het methanotrofe pmoCA-gen fragment van alle tot nu toe ontdekte phyla.

Noch ammonium oxiderende bacteriën, noch de comammox *Nitrospira* (die een ammonium monooxygenase bevat), werden gedetecteerd. Ten slotte waren we in staat om MOB specifieke afstammingslijn fragmenten te genereren vanwege de primerbindingsplaatsen die onmiddellijk het intergene gebied flankeren. Deze unieke eigenschap zou verder kunnen worden onderzocht in toekomstige amplicon-studies met hoge doorvoer om verschillende MOB afstammingslijnen in verschillende omgevingen te onderscheiden.

In hoofdstuk drie wordt de diversiteit aan methanotrofe soorten in ecosystemen die methaan produceren of consumeren onderzocht. Uit de resultaten bleek dat de CH_4 stromen erg variable kunnen zijn tussen draslanden (bekend als producenten van CH_4) en de naburige grasland (bekend als consument van CH_4) met geen duidelijke statistische verschillen tussen deze verschillende systemen. De totale microbiële gemeenschap vertoonde echter significante verschillen waarbij de familie van *Fimbriimonadaceae* sterk verrijkt was in het rijstveld. Als er wordt gekeken naar de MOB gemeenschap in het rijstveld zagen we meer verrijking in verschillende methanotrofen families zoals *Methylomirabilaceae*, *Methylomonaceae*, and *Methylophilaceae*. Gebaseerd op de resultaten van dit hoofdstuk werden twee werk hypothesis geformuleerd.

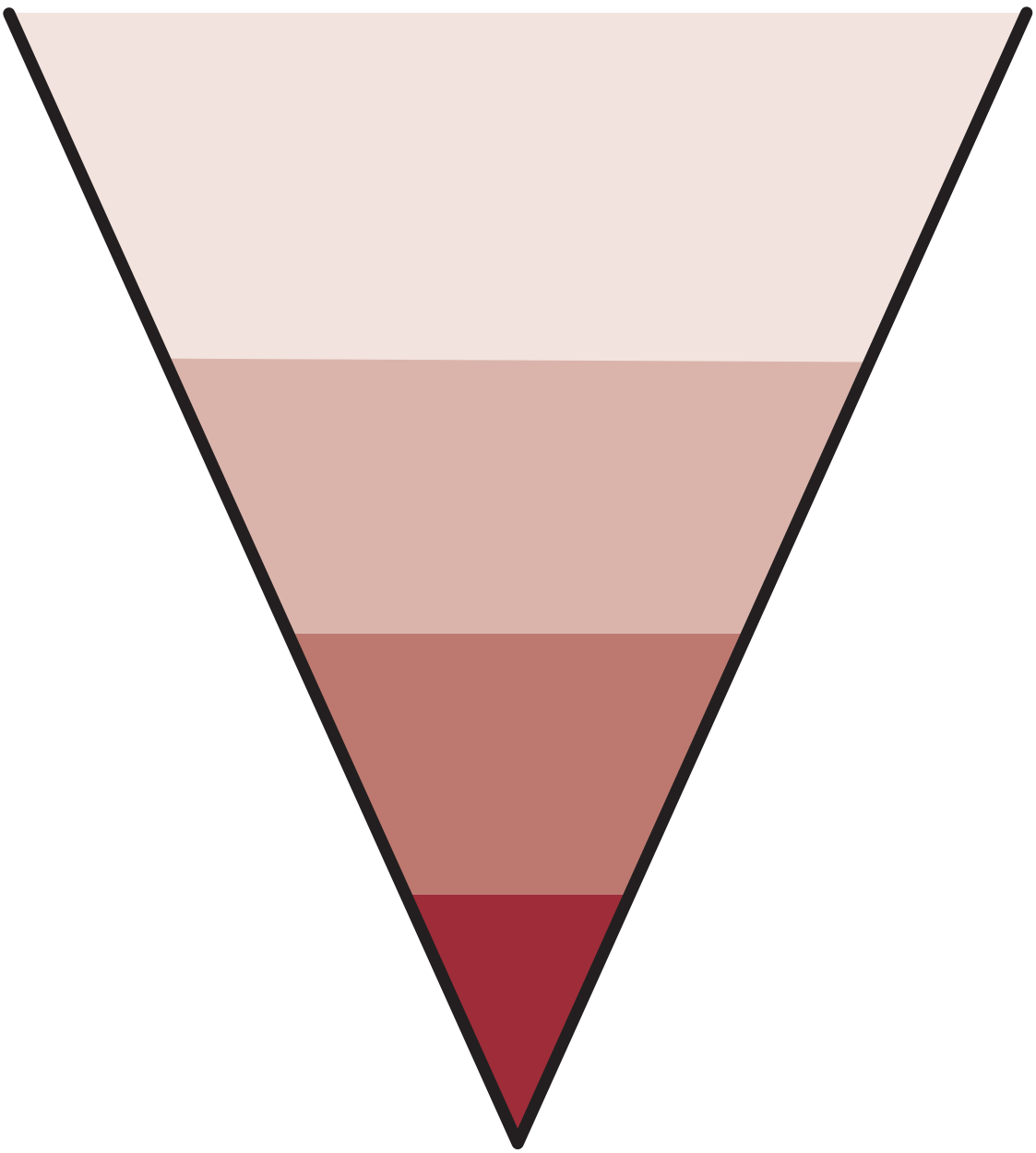
Met behulp van een drie maanden lange incubatie, van bodem monsters, in microcosms wordt in Hoofdstuk 4 geprobeerd ongecultiveerde MOB groepen te verrijken. De bodem monsters werden erg actief waarbij alle CH_4 die werd toegevoegd werd geoxideerd voordat het de bovenste compartimenten bereikten. Tijdens de verrijking werden op verschillende momenten de verticale zuurstof gradiënten gemeten, deze bleken de variëren in tijd. Een indicatie dat de microbiële gemeenschap een rol speelt in deze gradiënt. Na metagenoom analyses bleek dat de ordes *Methyloccales* and *Myxococcales* hoog verrijkt waren. De pmoA sequenties die werden geïsoleerd uit de bovenste en onderste lagen uit de bodem monsters suggereerden dat type I methanotrofen meer dominant zijn in de bovenste laag en type II methanotrofen meeer verrijkt leken te zijn in de onderste laag. Concluderend kan hiermee worden vastgesteld dat onze strategieën om te verrijken resulteerden in drie verschillende hoog verrijkte methanotrofe consortia die nieuwe pmoA sequenties bevatten het meest verwant aan *Methylocystis* en *Methylomonas* genera.

Hoofdstuk 5 omschrijft de fenotypische en genotypische eigenschappen van een uit drasland geïsoleerde nieuwe methanotroof door het gebruik van micocosms. Gebaseerd op 16S rRNA fylogenie en PLFA profielen blijkt dat deze soort dicht verwant is aan de type Ib methanotrofen. En bleek dat deze een $C_{16:1}\omega 9c$ als membraan fosfolipide heeft wat nog niet eerder is gezien in methanotrofen. Het concept genoom van dit organisme laat zien dat er een potentieel is voor diversiteit in metabolisme met enzymen betrokken in de zwavel cyclus.

Dit proefschrift wordt afgesloten met een zesde hoofdstuk waar doormiddel van de integratie van eerdere verkregen resultaten (hoofdstukken twee, drie, vier en vijf) een visie wordt gegeven op het huidige inzicht op de rol van methanotrofen in draslanden. Daarnaast wordt in dit hoofdstuk gesproken over de onduidelijkheden die in de toekomst verder onderzocht moeten worden. Daarom worden aanvullende experimenten en aanbevelingen gedaan om deze vragen op te lossen en meer inzicht te krijgen in de milieu-impact van methanotrofen.

CHAPTER 1

Introduction



BACKGROUND

A diverse group of microorganisms exist that are capable of oxidizing reduced one-carbon compounds, such as methanol, known to as methylotrophs. One unique subset of methylotrophs is further classified as methanotrophs. These are microorganisms that oxidize methane (CH_4) aerobically and use it as their sole energy source (Hanson & Hanson, 1996; Trotsenko & Murrell, 2008; Semrau *et al.*, 2010). They are ubiquitous in nature, inhabiting a wide array of environments where both oxygen (O_2) and CH_4 are available (Hanson & Hanson, 1996; Nazaries *et al.*, 2013; Knief 2015). The enzyme required for the activation of CH_4 is called methane monooxygenase (MMO). Two forms of this enzyme exist, a cytoplasmic soluble form (sMMO) and a membrane bound particulate form (pMMO). sMMO is encoded by a gene cluster *mmoXYZ* encoding for the αβγ subunits of the hydroxylase, respectively. On the other hand, *pmoCAB* gene cluster encodes for the αβγ subunits of the hydroxylase in pMMO (Murell *et al.*, 2000). According to current knowledge, the particulate form of this enzyme is much more widespread and has become the target of molecular studies.

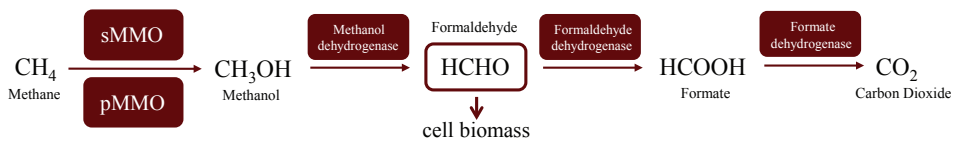


Figure 1: Methane oxidation pathway in methane oxidizing bacteria. All enzymes involved in each step of the reaction are shown. sMMO: soluble methane monooxygenase, pMMO: particulate methane monooxygenase.

Since their discovery over 100 years ago, methanotroph's number and diversity have been gradually increasing to 16 described genera affiliated with the bacterial phyla *Proteobacteria*, *Verrucomicrobia* and the candidate division NC10 (Knief, 2015). Despite their diversity, nearly all described methanotrophic isolates and cultures belong to the *Proteobacteria* and are affiliated with the classes *Gammaproteobacteria* (type I) and *Alphaproteobacteria* (type II). In fact, methanotrophic *Verrucomicrobia* were only recently discovered and are represented by a limited number of cultures (Op den Camp *et al.*, 2009). Within the NC10 phylum, the "*Candidatus* Methylomirabilis oxyfera"-like methanotrophs are able to oxidize CH_4 via an intra-aerobic pathway in anoxic habitats (Ettwig *et al.*, 2010). However, no pure isolates have been obtained from this group of methanotrophs. In this chapter, the discovery and phylogeny of methanotrophs, the process of methanotrophy, and the importance of methane oxidation in natural and cultivated wetlands are further discussed.

Discovery, phylogeny and taxonomy of methanotrophs

In 1906, Nicolas Söhngen discovered bacteria that were capable of oxidizing methane (CH_4) at the expense of oxygen (Söhngen, 1906). Finally, in the 1970s extensive isolation and characterization of these aerobic methanotrophs occurred (Whittenbury *et al.*, 1970). This gave rise to the initial three methanotroph 'Types' known as Type I, II, and X. The differentiation and characterization for these groups were based on multiple factors including intracytoplasmic membranes throughout the cell as bundles of vesicular disks (Type I) versus being aligned along the periphery of the cell (Type II), the utilization of ribulose monophosphate (RuMP) (Type I) versus serine pathway (Type II) for carbon assimilation and signature phospholipid fatty acids of 14 and 16 (Type I) versus 18 (Type II) carbons in length. However, some isolates showed characteristics of both Type I or Type II, which is why Type X was created (Hanson & Hanson, 1996). Follow-up studies proposed that *Proteobacterial* methanotrophs should be grouped as Type I or II with Type X strains being reclassified as a subset of Type I (Type Ib) methanotrophs (Bowman *et al.*, 1993). Moreover, two filamentous methane oxidizers have also been described, *Crenothrix polyspora* (Stoecker *et al.*, 2006), and *Clonothrix fusca* (Vigliotta *et al.*, 2007), possessing the *pmoA* enzyme. Both belong to the *Gammaproteobacteria* and are closely related to the Type I methanotrophs.

Methanotrophs were first grouped into six genera within the *Proteobacteria* (Hanson & Hanson, 1996). This classification was based on 16S rRNA gene sequence analyses, with the gammaproteobacterial Type I methanotrophs grouped as *Methylobacter*, *Methylococcus*, *Methylomicrobium*, and *Methylomonas*. The alphaproteobacterial Type II methanotrophs were classified as *Methylocystis* or *Methylosinus*. This classification has since been updated to 16 genera (see Figure 2 for overview): 12 within the *Gammaproteobacteria* and four in the *Alphaproteobacteria* (Bodrossy *et al.*, 1997, 1999; Bowman *et al.*, 1997; Wise *et al.*, 1999; Heyer *et al.*, 2005; Kalyuzhnaya *et al.*, 2005; Rahalkar *et al.*, 2007; Dedysh *et al.*, 2000, 2002, 2004; Dunfield *et al.*, 2003).

When independent reports of isolation and characterization of methanotrophs belonging to the phylum of *Verrucomicrobia* from volcanic ecosystems were published, our previous knowledge on methanotroph diversity greatly expanded (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). These volcanic isolates are proposed to be representatives of the genus *Methylacidiphilum* (Op den Camp *et al.*, 2009) with most lacking any intracytoplasmic membranes, possessing a complete Calvin-Benson-Bassham cycle and carboxysome-like structures, and growth stimulated by carbon dioxide (CO_2) (Dunfield *et al.*, 2007; Pol *et al.*, 2007). The growth of verrucomicrobial methanotrophs is dependent on rare earth elements (lanthanides), which are incorporated into the active center of an XoxF-type methanol dehydrogenase (Pol *et al.* 2014). Furthermore, mesophilic acidophilic isolates belonging to a novel *Methylacidimicrobium* genus within the *Verrucomicrobia* phylum have also been discovered from a volcanic soil in Italy. These strains are capable of autotrophic growth via the Calvin cycle with some containing intracytoplasmic membrane stacks (Islam *et al.*, 2008; van Teeseling *et al.*, 2014).

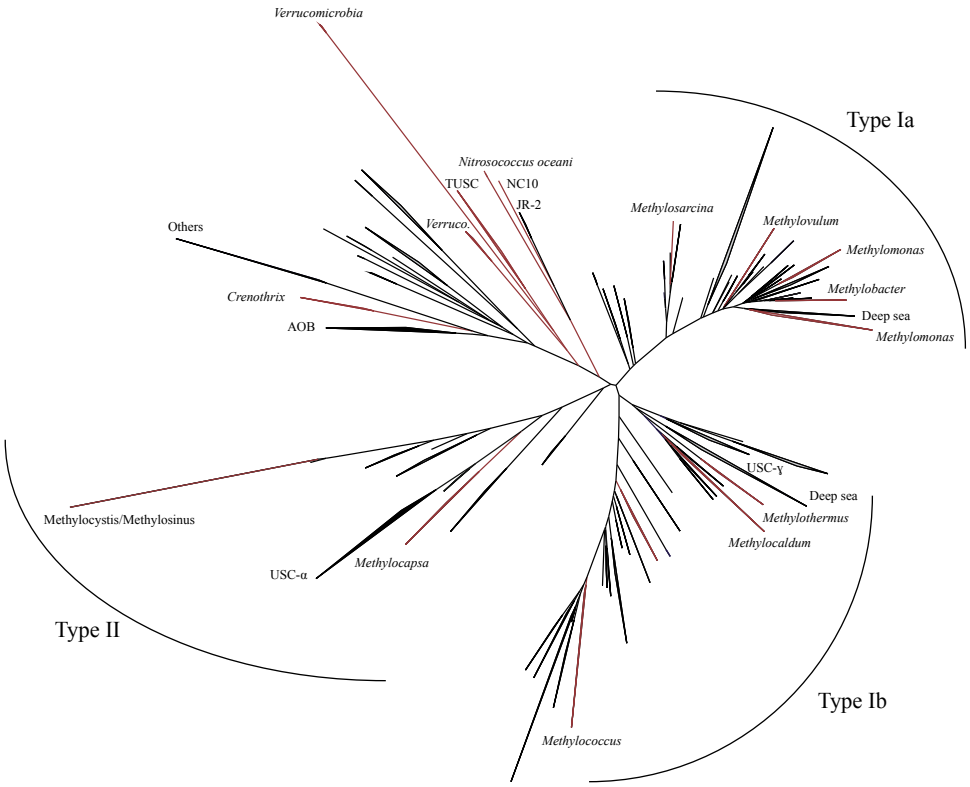


Figure 2: Representation of methanotrophic diversity with both solely environmental and isolated *pmoA* sequences. Dark branches represent only environmental sequences while branches with isolates are highlighted in red. The tree is rooted with *amoA* sequences from ammonia oxidizers (AOB). Type Ia, type Ib, and type II are clearly distinguished.

While methane oxidation has been described to proceed through an aerobic pathway in bacteria, a recently discovered bacterium *Candidatus* 'Methylomirabilis oxyfera' has greatly challenged this dichotomy (Ettwig *et al.*, 2010). This methanotrophic bacterium belonging to the NC10 phylum grows in an anaerobic condition, yet produces its own supply of oxygen by converting nitrite via nitric oxide into O_2 and N_2 gas. The produced O_2 is then used via a classical aerobic methane oxidation pathway involving a particulate methane monooxygenase to activate methane. Therefore, this bacterium performs anaerobic methane oxidation coupled to denitrification, via an intra-aerobic pathway (Wu *et al.*, 2011).

Environmental distribution of methanotrophs

Methanotrophs can be found in many environmental samples such as in wetlands, freshwater and marine sediments, sewage sludge, groundwater, paddy fields, and peat bogs (Bowman, 2006 and references therein; Hanson & Hanson, 1996 and references therein; Dedysh *et al.*, 1998b and references therein). Most isolated methanotrophs are neutrophilic and mesophilic, growing at moderate pH (5-8) and temperature ranges (20-35°C), but thermophilic, psychrophilic, alkaliphilic, and acidophilic methanotrophs have been discovered, too.

The isolation of *Methylococcus* and *Methylocaldum* species within the *Gammaproteobacteria* was the first thermotolerant MOB growing at temperatures between 30-60 and 20-47°C, respectively. *Methylothermus* was isolated from a hot spring and grows at temperatures as high as 72°C (Bodrossy *et al.*, 1999). Thermophilic strains have also been identified within the *Verrucomicrobia* with optimal growth temperatures of 55°C and above. These cells are also acidophilic with growth optima at pH 2, and were isolated from volcanic mud pots in southern Italy (Pol *et al.*, 2007), a geothermal active area in New Zealand (Dunfield *et al.*, 2007) and from an acidic hot spring in Kamchatka, Russia (Islam *et al.*, 2008).

Methanotrophy has also been observed in permanently cold areas (Omelchenko *et al.*, 1993; Berestovskaya *et al.*, 2002), which cover a significant part of the biosphere (Russwell, 1990). These areas contain substantial CH_4 fluxes (such as polar tundra regions). For example, *Methylobacter psychrophilus* isolated from a tundra soil in Russia grows at optimal temperatures ranging from 3.5 to 10°C (Omel'chenko *et al.*, 1996; Tourova, 1999). Similarly, *Methylomonas scandinavica* with optimal growth temperatures of 15°C was isolated from deep igneous groundwater in Sweden (Kalyuzhnaya *et al.*, 1999). All discovered MOB from these environments belong to the *Gammaproteobacteria* phylum. Lastly, halotolerant/-philic and alkalitolerant/-philic methanotrophs have also been discovered from marine waters, estuaries, arctic soil, groundwater and soda lakes with growth at salt concentrations between 0.15% and 4% (Sieburth *et al.*, 1987; Bowman *et al.*, 1993; Khmelenina *et al.*, 1997, 1999; Smith *et al.*, 1997; Fuse *et al.*, 1998; Kaluzhnaya *et al.*, 2001, 2008; Trotsenko & Khmelenina, 2002; Wartiainen *et al.*, 2006).

The process of methanotrophy

Despite this diverse and wide range of growth conditions, CH_4 oxidation in methanotrophs is remarkably similar with regards to the pathway they utilize to produce methanol, formaldehyde and formate as intermediates. In fact, most characterized cells to date are obligate methanotrophs capable of growing only on the C1 compounds CH_4 and methanol, with the exception of one described *Methylocella* species that can grow on organic acids (Dedysh *et al.*, 2005).

CH_4 oxidation can occur both aerobically and anaerobically, however in this thesis only aerobic oxidation is reviewed. The enzyme responsible for the conversion of CH_4 to methanol is called methane monooxygenase. To date, two different forms of this enzyme have been found. One form is called particulate methane monooxygenase (pMMO), which is a membrane-associated enzyme located in the cytoplasmic membrane encoded by the *pmoCAB* operon. Due to its

presence in most methanotrophs, pMMO has become the target of functional gene marker studies monitoring these microorganisms in a wide array of environments. The other form of this enzyme is called soluble methane monooxygenase (sMMO) and its location is within the cytoplasm. Relatively speaking, sMMO is far less ubiquitous in methanotrophs. *Methylocella* and *Methyloferata* do not have *pmoCAB* genes in their genome, but only possess sMMO. When both MMO's are present in a methanotroph, their expression is mainly regulated by the presence of copper (Cu) (Takeda *et al.*, 1976; Takeda & Tanaka, 1980; Scott *et al.*, 1981; Stanley *et al.*, 1983; Dalton *et al.*, 1984).

sMMO has been well characterized and shown to possess three components: a hydroxylase, a reductase and a regulatory protein (Colby *et al.*, 1977; Colby & Dalton, 1978, 1979; Woodland & Dalton, 1984; Green & Dalton, 1985; Fox *et al.*, 1989; Pilkington & Dalton, 1990; Wallar & Lipscomb, 1996, 2001). Electrons from NADH are transferred to the hydroxylase via the cofactors of the reductase. The hydroxylase component is composed of three subunits with a molecular structure of $(\alpha\beta\gamma)_2$. Furthermore it has been established, using spectroscopic and X-ray crystallography that the ~60 kDa polypeptide α-subunit of the hydroxylase containing an oxygen-bridged di-iron cluster is the site of CH_4 catalysis (Fox *et al.*, 1988, 1989; Rosenzweig *et al.*, 1993; Elango *et al.*, 1997). In contrast to sMMO, less is known about pMMO and its molecular properties. This is mainly a result of loss of activity occurring during enzyme preparations (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998; Takeguchi *et al.*, 1998; Basu *et al.*, 2003; Lieberman *et al.*, 2003; Choi *et al.*, 2005). What is known so far, from various studies, is that pMMO is a copper-containing enzyme with a subunit molecular structure of $(\alpha\beta\gamma)_3$. What is yet to be proven is the number, type, and function of metal centers associated with pMMO and its crystal structure (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman & Rosenzweig 2005).

Methane oxidation in wetlands

In 1982, Harris and colleagues provided the first evidence for CH_4 consumption in wetland sediments when water levels fell below the sediment surface. Since then, extensive research has been done in these ecosystems to understand the affect that microbes have on the global geochemical cycles. In natural and cultivated wetlands, CH_4 consumption is considered to be mainly performed by methanotrophs (Cicerone & Oremland 1988; King, 1992). Although determining *in situ* CH_4 oxidation on the field scale are challenging, up to 90% of the produced CH_4 in wetlands are reported to be consumed again via methanotrophs residing in the oxic top layer or in the oxic rhizosphere (De Bont *et al.,* 1978; Holzapfel-Pschorn & Seiler 1986; Schütz *et al.*, 1989; Sass *et al.*, 1990; Fechner & Hemond, 1992; Van der Gon & Neue, 1996; Schipper & Reddy, 1996; Lombardi *et al.*, 1997).

In a typical flooded soil that is fully saturated with water, the biogeochemical cycling is driven by the availability of organic compounds and oxygen along with inorganic electron acceptors (such as nitrate, sulfate, Fe(III), etc.) (Krüger *et al.*, 2002; Segarra *et al.*, 2013). Before the CH_4

produced by methanogens reaches the atmosphere, first anaerobic methanotrophs oxidize it using a suite of electron acceptors. The remaining CH_4 reaches the oxic layer of soil where it undergoes conversion by aerobic methanotrophs.

Depending on the availability of these compounds, there are different types of microbes that could be present in such environment. If sulfate is readily available, sulfate-dependent anaerobic oxidation of CH_4 (AOM) can take place (Hoehler *et al.*, 1994). In the presence of nitrite or nitrate, anaerobic methanotrophy can be coupled to nitrite and nitrate reduction and Raghoebarsing *et al.* (2006) reported the first enrichment of AOM coupled to denitrification. Surveys of both 16S rRNA and *pmoA* genes have revealed that this process can take place in wetlands (Welte *et al.*, 2016). Lastly, oxidized iron (Fe^{3+}) can be a suitable alternative electron acceptor for AOM. Studies have demonstrated the occurrence of Fe-AOM in paddy field sediments (Miura *et al.* 1992; Watanabe *et al.*, 1994; Ettwig *et al.*, 2016); however, microbial growth has not yet been demonstrated. The combination of substrate availability and its thermodynamics can result in a functioning community that is responsible for different microbial processes.

While the remaining CH_4 reaching the oxic layer of the soil can be oxidized by aerobic methanotrophs, these bacteria can also be responsible for methane oxidation at atmospheric levels (Dunfield *et al.*, 1999). Both *Alpha-* and *Gammaproteobacteria* named upland soil cluster (USC) α and α have been detected in cultured-independent studies (Knief *et al.*, 2003; Kolb *et al.*, 2005; Ricke *et al.*, 2005). CH_4 oxidation can be distinguished into two kinds of activity based on affinity: high affinity at low atmospheric CH_4 concentrations (<12 ppm) or low affinity at high CH_4 concentrations (>40ppm) (Roslev *et al.*, 1997; Gulledge *et al.*, 1997). Bacterial populations responsible for 'high affinity oxidation' are still vastly unknown, estimated to contribute to 10% of total CH_4 consumption (Topp & Pattey, 1997). Recently, Pratscher *et al.* (2018) obtained an 85% complete draft genome of a USCα genus within Beijrinckiaceae through various advanced methods. Moreover, other studies have indicated that under extremely low oxygen conditions, MOB can thrive by coupling methane oxidation to nitrate reduction (Kits *et al.*, 2015a; 2015b; Oswald *et al.*, 2016; Gilman *et al.*, 2017). On the other hand, methanotrophs responsible for 'low affinity oxidation' are more easily cultivable and much more studied (Topp *et al.*, 1991). In paddy fields, variations in CH_4 emissions have been mostly attributed to the variation in methanotrophic activity (Sass *et al.*, 1990; Schütz *et al.*, 1990).

Molecular tools for the detection of MOB

A suitable marker gene for use in molecular ecology studies of organisms is one that has sequences available in a database from which primers can be designed. This makes 16S rRNA gene an obvious choice due to the large database of sequences available and moreover, newly described organisms have their 16S rRNA gene always sequenced first and added to the database. A complementary option is the use of a functional gene that is unique to the physiology and metabolism of the organisms being studied, which has major advantages over housekeeping genes. By using a functional marker gene, researchers narrow down their investigation to the

studied functional group making their detection highly sensitive in a complex environment. Furthermore, presence of a homologous gene sequence in putative uncultivated members means that they can also be easily identified. The knowledge gained with regards to the thus inferred physiology of these novel bacteria cannot be obtained otherwise, through the use of housekeeping genes only (McDonald *et al.*, 2008).

In order to study the presence of MOB in various environments, microbiologists have used *pmoA* and *mmoX* specific primers. *pmoA* codes for the beta subunit of particulate methane monooxygenase that is shown to be highly conserved amongst MOB (Hakemian & Rosensweig, 2007). While *pmoA* genes have been sequenced from a considerable number of methanotrophs and a large data set of partial sequences is available in GenBank, *mmoX* sequence availability still remains relatively small. However, currently used primers do not always encompass all different types of MOB. For instance, recently researchers have had to come up with new and more specific primers for certain MOB such as "*Candidatus* Methylomirabilis oxyfera" (Luesken *et al.*, 2011a; 2011b). Furthermore, as mentioned previously, *Methylocella* lacks a PmoA-coding gene in its genome (Chen *et al.*, 2010; Vorobev *et al.*, 2011). Taking a closer look at the full *pmoCAB* operons from the available genomes could give us more insight with regards to putative conserved regions. This in turn would result in improved primers and better understanding of MOB communities. One such region could be present within *pmoB* sequences since it has been indicated that the active site of particulate methane monooxygenase enzyme is located within the soluble region of PmoB protein (Culpepper *et al.*, 2012a; 2012b).

Besides *pmoA* and *mmoX*, other functional gene markers that are not unique to methanotrophs, but have suitable datasets available can still be used to identify MOB. For instance, researchers have used *mxaF* coding for the large subunit of methanol dehydrogenase (McDonald & Murrell, 1997; Neufeld *et al.*, 2007), *nifH* coding for dinitrogen reductase (Auman *et al.*, 2001; Dedysh *et al.*, 2004) and *fhcD* coding for the D subunit of formyltransferase/hydrolase complex (Kalyuzhnaya *et al.*, 2004). Another technique for the molecular detection of MOB has been the use of DNA microarrays. Microchip, biochip and gene chip technology allows for the parallel analysis of highly complex gene mixtures in a single assay. While originally meant to be used for a genome-wide expression analysis, microarrays were also being developed for diagnostic applications. Strain-, subspecies-, species-, genus-, or higher taxon-specific nucleic acid probe sets could be used in microbial diagnostic microarrays (Bodrossy & Sessitsch, 2004). Nowadays however, they are being replaced by amplicon sequencing.

Lastly as an alternative, lipids can be used in molecular marker studies since methanotrophs contain unique phospholipid fatty acids (PLFAs) (Guckert *et al.*, 1991). Type I methanotrophs contain the unique PLFAs 16:1ω5t, 16:1ω6c, and 16:1ω8c, while Type II contain 18:1ω8c. The measurement of these signature PLFAs has been readily used in biomass distribution studies of both type I and type II methanotrophs in various environments (Börjesson *et al.*, 1998; Sundh *et al.*, 1995). The use of the $^{13}CH_4$ isotope to label the PLFAs of methanotrophs increases the sensitivity of detection of the PLFAs and has provided evidence of methane assimilation

at true atmospheric concentrations (Evershed *et al.*, 2006; Maxfield *et al.*, 2006; Knief *et al.*, 2003; Bull *et al.*, 2000). While these studies have had valuable findings, great care should be taken in interpreting PLFA data. First, the PLFA database for methanotrophs is much less extensive than the 16S rRNA and functional gene databases (McDonald *et al.*, 2008). Second, not all methanotrophs exhibit their corresponding PLFA profile as a recent study showed that *Methylocystis heyeri* strains (type II) contained large amounts of 16:1ω8c, a PLFA that was previously associated with type I methanotrophs only (Dedysh *et al.*, 2007).

Methanotroph population and CH_4 oxidation in paddy soils

Both type I and type II methanotrophs have been detected in rice fields (Frenzel, 2000). From different studies, it has been reported that the population of methanotrophs largely depends on the location of the experimental site (Dubey, 2001; 2003), concentration of CH_4 in soil (Bender & Conrad, 1992), and availability of ammonium (Dubey & Singh, 2000; Joulian *et al.*, 1997). Furthermore, population size of MOB tends to be higher by several orders of magnitude in rhizosphere compared to bulk soil (Dubey & Singh, 2000; 2001; Gilbert & Frenzel, 1998).
In flooded soil, oxygen diffuses from the atmosphere while CH_4 is supplied to the upper soil layer in µM concentrations from deeper layers. As a result, going down vertically from the surface would mean that there is a gradient of continuously lower oxygen concentrations, while the opposite would be observed for CH_4 concentrations (a scheme is depicted in Figure 3). Studies have shown that CH_4 and oxygen gradients in flooded soil overlap at about 2-3 mm depth from the surface (Damgaard *et al.*, 1998; Gilbert & Frenzel; 1998), meaning that beyond 3mm, there is no oxygen available via diffusion from the surface. This makes oxygen the limiting factor in the process of aerobic CH_4 oxidation by MOB in bulk soil (Krüger *et al.*, 2002).

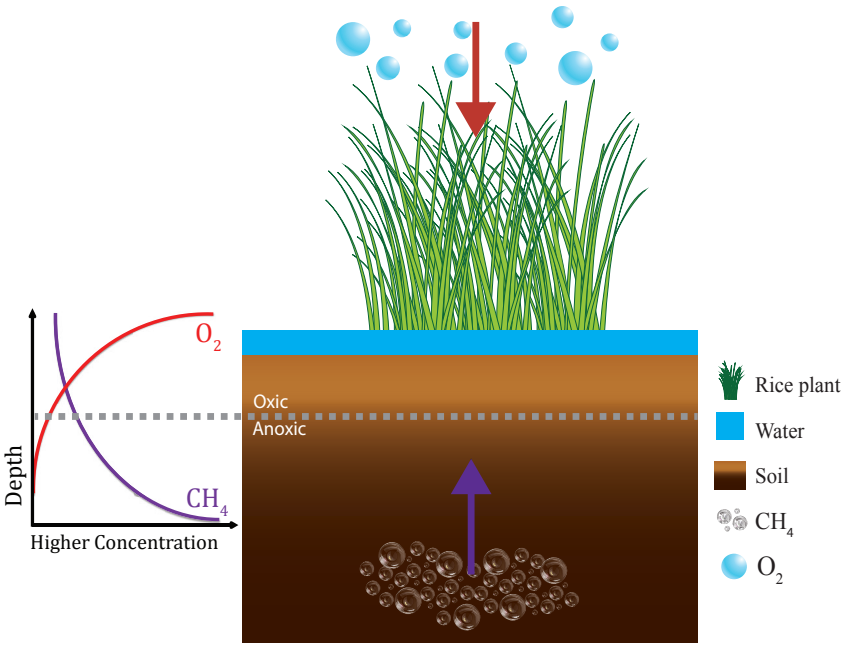


Figure 3: Cross-gradient of O_2 and CH_4 in natural and cultivated wetlands. Various mixing ratios of O_2 and CH_4 can exist depending on the depth. O_2 diffusion from the air is depicted by a red arrow while methane ebullition is depicted by a purple arrow.

Using previously discussed methods to study methanotrophic community in these environments, researchers have found more than 30 operational taxonomical units (OTUs) corresponding to the species level (Lüke *et al.*, 2010). Therefore, there is a huge diversity of MOB that exists in wetlands, most of which are still uncultivated and their niche preferences unknown. Due to different factors that can be responsible for the construction of these communities, such as CH_4 levels (Krause *et al.*, 2012), nitrogen supply (Rudd *et al.*, 1976), disturbances (Ho *et al.*, 2011) and grazing (Murase & Frenzel, 2008), researchers have tried to study these systems in laboratory scale microcosms that mimic these bacteria's natural habitat. This system allows for O_2 and CH_4 gradients to be established as previously mentioned regarding paddy soil. In a study published by Reim and colleagues (2012), they used this system to show that within soil that is only 3 mm thin, different OTUs within a single guild can share the same microenvironment. They further confirmed that CH_4 oxidation occurred at its highest rate at the oxic-anoxic interface, which separated the MOB according to substrate availability.

Thesis outline

Natural wetlands contribute 20-39% to the global emission of CH_4. This number would, however, be much higher if aerobic methanotrophs that naturally reside in these environments were not involved in CH_4 mitigation. Since the total area of cultivated wetlands has been increasing annually, the role of these MOB in oxidation of CH_4 has become increasingly important. Therefore, this thesis aimed at further expanding our understanding of methanotrophs in paddy fields. In **Chapter 2**, a molecular study was conducted to gain a better understanding of the diversity of methanotrophs. A novel degenerate primer set was designed based on full *pmoCAB* operon sequences and introduced to target methanotrophs in various environments. As a result, members of all known methanotrophic phyla, to date, could be targeted with this primer set, which has never been shown before. We were also able to generate MOB lineage specific fragments that can be used in future studies to better distinguish the different MOB lineages present in the studied sample. In **Chapter 3**, we aimed at unraveling the potential differences that exist in both total bacterial and methanotrophic community compositions between a paddy field (CH_4 source) and a meadow (CH_4 sink). This chapter illustrates that CH_4 fluxes can be highly variable, and this variability does not necessarily have a direct effect on the relative abundance of methanotrophs. Certain bacterial families such as *Fimbriimonadaceae* and various methanotrophic families such as *Methylomirabilaceae* and *Methylomonaceae* were found to be present in higher relative abundance in paddy soil. In **Chapter 4**, lab-scale microcosms together with metagenome analyses were used to follow and monitor a long-term incubation of paddy soil, and how MOB can effect the establishment of CH_4 and O_2 counter gradient in this environment. Lastly, our enrichment efforts resulted in three highly enriched methanotrophic consortia containing uncultured *pmoA* sequences distantly related to *Methylocystis* and *Methylomonas* genera. In **Chapter 5**, a novel type Ib bacterium isolated from a paddy field is introduced, obtained using the method described in chapter 4. The phenotypical and genotypical properties of this bacterium are discussed in details and compared to other well-described methanotrohpic strains. Finally, **Chapter 6** presents an overall summary of the findings together with integration and future perspectives of each of the chapters.

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Keywords: Methane, particulate methane monooxygenase, diversity, Methanotroph, Genetic marker

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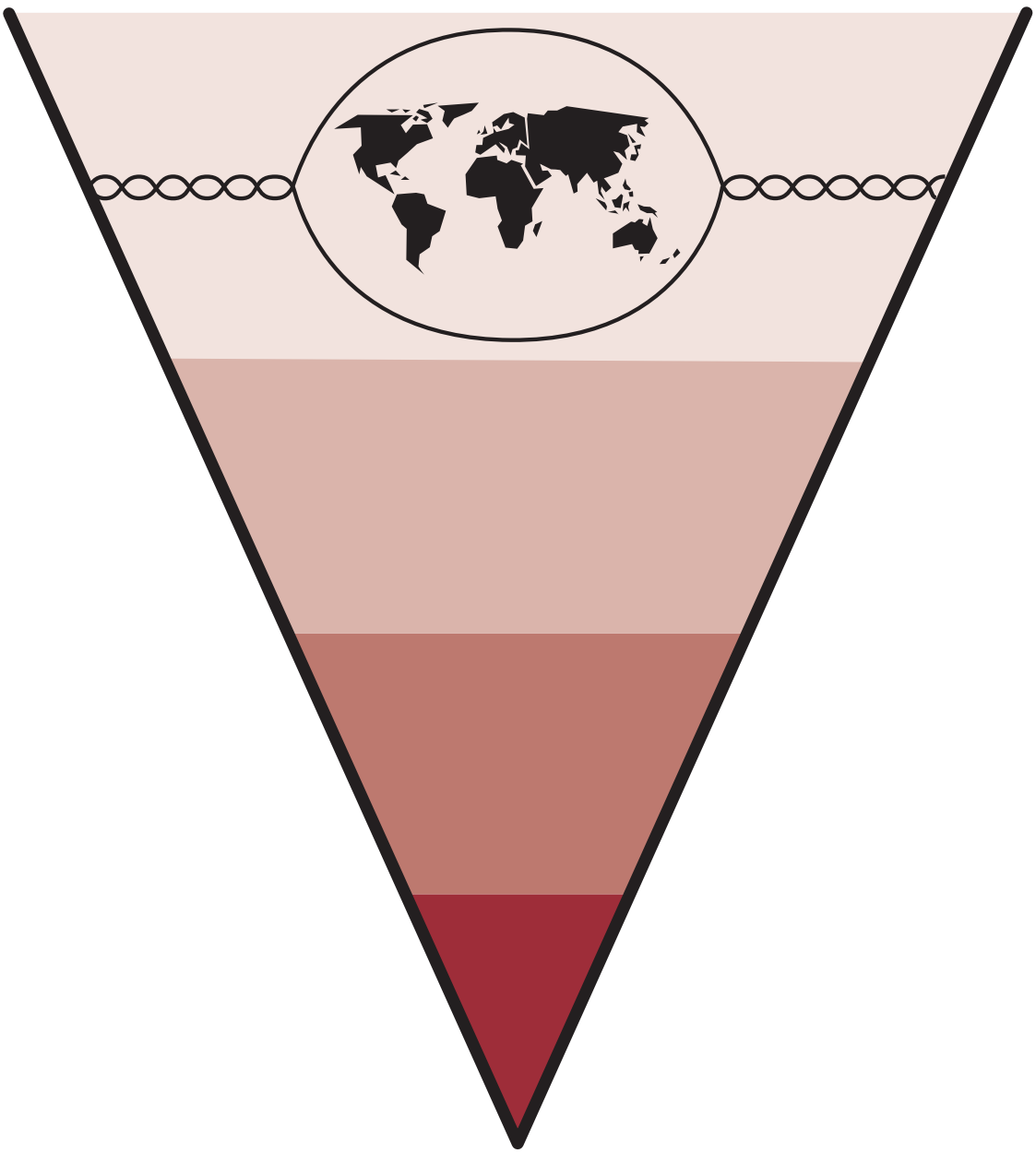
CHAPTER 2

Survey of methanotrophic diversity in various ecosystems by degenerate methane monooxygenase gene primers



ABSTRACT

Methane is the second most important greenhouse gas contributing to about 20% of global warming. Its mitigation is conducted by methane oxidizing bacteria that act as a biofilter using methane as their energy and carbon source. Since their first discovery in 1906, methanotrophs have been studied using a complementary array of methods. One of the most used molecular methods involves PCR amplification of the functional gene marker for the diagnostic of copper and iron containing particulate methane monooxygenase (pMMO). To investigate the diversity of methanotrophs and to extend their possible molecular detection, we designed a new set of degenerate methane monooxygenase primers to target an 850 nucleotide long sequence stretch from *pmoC* to *pmoA.* The primers were based on all available full genomic *pmoCAB* operons. The newly designed primers were tested on various pure cultures, enrichment cultures and environmental samples using PCR. The results demonstrated that this primer set has the ability to correctly amplify the about 850 nucleotide long *pmoCA* product from *Alphaproteobacteria*, *Gammaproteobacteria*, *Verrucomicrobia* and the NC10 phyla methanotrophs. The new primer set will thus be a valuable tool to screen ecosystems and can be applied in conjunction with previously used *pmoA* primers to extend the diversity of currently known methane-oxidizing bacteria.

INTRODUCTION

Methane is the second most important greenhouse gas contributing to about 20% of global warming (Intergovernmental Panel on Climate Change, 2014). The global methane budget is estimated to be around 600 Tg a^{-1} (Dubey, 2001) which is dominated by biogenic sources, where natural wetlands (23%), and rice fields (21%) (Frenzel, 2000) account for almost half of the total budget (Chen & Prinn, 2005). Methanogenic archaea are assumed to be the sole producers of methane that reside in these environments (Cicerone & Oremland, 1988; Conrad, 1999; Joulian *et al.*, 1997). These archaea are also present in waste treatment systems, intestines of ruminants and termites and landfills acting as additional CH_4 sources. Therefore, microbial methanogenic activity is responsible for nearly 75% of the methane emitted to the atmosphere (Chen & Prinn, 2005).

This process, is however, vastly mitigated by methanotrophic microorganisms that oxidize a large part of the produced CH_4 (Cappelletti *et al.*, 2016; Crevecoeur, 2015; Dumont & Murrell, 2005; Reeburgh *et al.*, 1993; Oshkin *et al.*, 2014). It has been estimated that of the primary productivity, roughly 1% ends up in CH_4; half of which is emitted into the atmosphere while the other half is consumed by methanotrophs (Reeburgh, 2007; Aronson *et al.*, 2013). While anaerobic methane-oxidizing archaea consume more than 75% of the CH_4 produced in certain marine sediments (Reeburgh, 2007; Beal *et al.*, 2009; Egger *et al.*, 2014), aerobic methane-oxidizing bacteria (MOB) that live at the interface between anoxic and oxic zones in marine environments (Bender & Conrad, 1992; Lüke *et al.*, 2016; Padilla *et al.,* 2016), freshwater wetlands and rice fields (Lüke *et al.*, 2014) have been estimated to consume up to 90% of the CH_4 produced in these environments (Hanson & Hanson, 1996). Alpha- and gammaproteobacterial methanotrophs have further been shown to be dominant methane consumers in acidic peatlands (Esson *et al.*, 2016; Deng *et al.*, 2013; Putkinen *et al.*, 2014). Since their discovery over 100 years ago (Söhngen, 1906), many aspects of methanotrophic bacteria have been studied (Whittenbury *et al.*, 1970; Bédard & Knowles, 1989; Hanson & Hanson, 1996; Lidstrom, 2006; Trotschenko & Murrell, 2008). At the moment, several groups of aerobic bacteria are known that convert methane by means of a copper- and/or iron-containing enzyme called methane monooxygenase (MMO) (Murrell *et al.*, 2000). Methanotrophic archaea play a prominent role in the anaerobic oxidation of methane and use methyl coenzyme-M reductase (MCR) (Knittel & Boetius, 2009; Haroon *et al.*, 2013 Welte *et al,* 2016).

Two different forms of MMO exist: a soluble MMO (sMMO) encoded by *mmoX*, *mmoY* and *mmoZ* and a particulate MMO encoded by *pmoCAB* (Lieberman & Rosenzweig, 2005). The membrane bound particulate methane monooxygenase (pMMO) catalyzes the hydroxylation of methane. It exists in virtually all methanotrophs while sMMO has only been shown in certain genera such as *Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylomonas* and *Methylocella* (Murell *et al.*, 2000). The more recent discovery of *Methylocella silvestris* (Crombie & Murrell, 2014), *Methyloferula stellata* (Dedysh *et al.,* 2015), and *Methylocella palustris* (Dedysh *et al.*, 2000)

has illustrated that some MOB do indeed possess only sMMO and would not be targeted in pMMO-focused molecular studies (Dunfield *et al.*, 2003; Dedysh *et al.*, 2000; Vorobev *et al.*, 2011; Vekeman *et al.*, 2016A). pMMO belongs to the ammonia monooxygenase superfamily and has been shown to be of high biogeochemical and chemical relevance (Bédard & Knowles, 1989, Hakemian & Rosenzweig, 2007). This is due to the tight correlation that exists between this family and the globally important methane and nitrous oxide flux (Conrad, 1996). This makes Copper containing (Cu) MMO genes extremely useful markers in biological feedback studies looking at global climate change (Singh *et al.*, 2010). Moreover, PCR-based environmental surveys have identified the ecological distribution and relevance of CuMMO-containing organisms correlated to gas flux, land use and climatic conditions (Coleman and the references within, 2012). It has also been postulated that this group of enzymes could be correlated to processes other than methanotrophy and ammonia oxidation such as butane-oxidation (Coleman *et al.*, 2012; Crombie & Murrell, 2014). Therefore molecular approaches, such as PCR with specific primer sets are a fast and convenient method to screen for the diversity of such enzymes in various environments (Murrell & McDonald, 1998; Mitsumori *et al.*, 2002; Siljanen *et al.*, 2012).

The crystal structure of pMMO has been determined to a resolution of 2.8 Å from *Methylococcus capsulatus* (Bath) and the enzyme has been found to be a trimer with an $\alpha_3\beta_3\gamma_3$ polypeptide arrangement (Lieberman & Rosenzweig, 2005). The PmoA subunit contains non-heme iron in its center and for long was proposed to be the site of substrate hydroxylation. The soluble PmoB subunit hosts two metal centers, modelled as mononuclear copper and dinuclear copper, while a third metal center occupied by zinc is located within the membrane (Lieberman & Rosenzweig, 2005). Molecular surveys showed that MOB are present, amongst others, in natural wetlands (Costello *et al.*, 2002; Samad & Bertilsson, 2017), marine ecosystems (Vekeman *et al.*, 2016b), permafrost thaw ponds (Crevecoeur *et al.*, 2015), peatlands (Lau *et al.*, 2015) and flooded rice-fields (Krüger *et al.*, 2001; Lüke *et al.*, 2009; Balasubramanian & Rosenzweig, 2007; Zheng *et al.*, 2008). Since pMMO was initially assumed to be present in all methane oxidizing bacteria, it has been used in molecular approaches to investigate methanotrophic diversity (Semrau *et al.*, 1995; Holmes *et al.*, 1999; Chi *et al.*, 2012; Saidi-Mehrabad *et al.*, 2013). More specifically *pmoA,* coding for the beta subunit of pMMO, was found to be highly conserved and as a result used as a functional gene marker (Holmes et al., 1995, Bourne *et al.*, 2001; Costello *et al.*, 1999; Kolb *et al.*, 2003 Luesken *et al.*, 2011B; Wang *et al.*, 2017).

In addition, *pmoA* amplicon pyrosequencing has been used to look at methanotrophic diversity in depth (Kip *et al.*, 2011; Lüke & Frenzel, 2011; Han & Han & Gu, 2013; Knief, 2015). For all the PCR based methods, the used primers unfortunately do not encompass all different phyla of MOB to the same extent (Bergmann *et al.*, 2011) nor do they cover new phyla such as *Verrucomicrobia* (Sharp *et al.*, 2014; Erikstad & Birkeland, 2015) and NC10. In the latter cases, more phylum specific primers had to be designed to investigate the presence of '*Candidatus Methylomirabilis oxyfera*' in various ecosystems (Luesken *et al.*, 2011B). Recently several genomes

of different MOB have been sequenced by the Omega consortium (Khmelenina *et al.*, 2013; Kits *et al.*, 2013; Khadem *et al.*, 2012; Stephenson *et al.*, 2017) and thus a much larger gene dataset is now available to design new primers to potentially cover larger methanotroph diversity. Here we introduce a new set of degenerate primers that can be used to examine the diversity of MOB in various environments with the potential ability to target all presently known methanotrophic phyla. The new primers have the capability to target *pmoC* and *pmoA* and the intergenic region in between those genes. Application of the primers to various ecosystem resulted in the detection of *pmoCA* of *Alphaproteobacteria*, *Gammaproteobacteria*, *Verrucomicrobia* and NC10 within their respective habitats. Neither ammonia oxidizers, nor the recently discovered comammox (van Kessel *et al.*, 2015; Pjevac *et al.*, 2016) were detected with these primers. Furthermore, since the binding sites of the primers immediately flank the intergenic region between the genes *pmoC* and *pmoA*, they generate MOB lineage specific fragments. This unique property could be used in high throughput sequence analysis experiments for recovering diverse lineages in further environmental studies.

MATERIALS AND METHODS

Construction of *pmoCAB* operon database and primer design:

A total of 83 different full genomic methane monooxygenase along with the isoenzyme PXM and ammonia monooxygenase gene sequences available on MaGe were downloaded (Vallenet *et al.*, 2005; Sievers & Higgins, 2014). This included *Alpha-*, *Gamma-*, and *Betaproteobacteria* (AOB), *Verrucomicrobia*, NC10, *Mycobacterium*, *Nocardia*, SAR cluster, divergent PXM operon and second operons from *Methylocystis parvus* OBBP, *Methylocystis* sp. BN69, *Methylosinus* sp. LW3, and *Methylosinus* sp. LW4 (Table 1). The genes were aligned in *pmoCAB* operon configuration. In cases where an organism's genome contained more than one copy of the operon, all copies were included in the pipeline. Sequences were aligned using MUSCLE (Edgar, 2004) and the alignment was imported into ARB (Ludwig *et al.*, 2004). Nucleotide sequences were translated into protein sequences and phylogenetic trees were constructed based on the amino acid sequences. Furthermore, using the 'Probe' tool, primers that were capable of covering all (or as much as possible) phyla were designed within ARB. The parameters for the primer design were: 18 nucleotides in length, GC content of 50 -70 %, and minimum group coverage of at least 50%. Furthermore, the primers were made specific to MOB so that they had more than five mismatches with ammonium monooxygenase *amo* gene sequences of ammonia oxidizing bacteria (AOB).

Table 1: Aligment of the new pmoCA primers on all the available genomic sequences from different phyla. Wobble positions are shown in yellow.

Gammaproteobacteria	**pmoC374**	**pmoA344**
Methylobacter tundripaldum SV96 operon 2	ACAGAGCAAGATGGTACATGGCATCA	TAAACTTCTGGGGTTGGACTTATT
Methylobacter sp. AQVZv1 operon 1	ACAGAGCAAGATGGTACTTGGCATCA	TGAATTTCTGGGGTTGGACTTATT
Methylovulum miyakonese strain HT12 operon 1	ACAGAGCAAGATGGTACTTGGCATCA	TGAACTTCTGGGGTTGGACATATT
Methylobacter luteus IMV-B-3098T operon 1	ACTGAGCAAGACGGTACATGGCATCA	TGAACTTCTGGGGATGGACATATT
Methylobacter marinus A45 operon 1	ACTGAGCAAGACGGTACATGGCATCA	TGAACTTCTGGGGATGGACATATT
Methylomicrobium alcaliphilum 20Z operon 1	ACTGAGCAAGATGGTACTTGGCATCA	TCAACTTCTGGGGATGGACATACT
Methylomicrobium buryatense 5G operon 1	ACAGAGCAAGACGGTACATGGCATCA	TCAACTTCTGGGGATGGACATACT
Methylomonas sp. M11Bv1_22234 operon 2	ACAGAGCAAGACGGTACATGGCACCA	TGAACTTCTGGGGCTGGACTTACT
Methylomonas sp. MK1 operon 2	ACAGAGCAAGACGGTACATGGCACCA	TGAACTTCTGGGGCTGGACATACT
Methylomicrobium album BG8 operon 1	ACCGAACAAGATGGCACGTGGCATCA	TCAACTTCTGGGGATGGACTTACT
Methylosarcina lacus LW14 opeorn 1	ACCGAACAAGATGGTACATGGCATCA	TCAACTTCTGGGGATGGACTTACT
Methylosarcina fibrata AML-C10 operon 1	ACAGAGCAAGATGGTACATGGCATCA	TCAACTTCTGGGGATGGACTTACT
Methyloglobulus morosus operon 3	ACAGAGCAAGACGGCACATGGCATCA	TCAATTTCTGGGGTTGGACATACT
Methylococcus capsulatus Bath A2855 operon 2	ACCGAGCAGGACGGCACCTGGCATCA	TCAACTTCTGGGGCTGGACCTACT
Methylococcus capsulatus Bath A1798 operon 1	ACCGAGCAGGACGGCACCTGGCATCA	TCATGCCATGCTCACCATGGGTGA
Methylocaldum szegediense O-12	ACCGAGCAGGATGGCACCTGGCACCA	TCAACTTCTGGGGTTGGACCTACT
Methylohalobius crimeensis operon 2	ACCGAGCAGGACGGCACCTGGCACCA	TCAACTTCTGGGGATGGACCTACT
Methylohalobius crimeensis operon 1	ACCGAGCAGGACGGCACCTGGCACCA	TCAACTTCTGGGGATGGACCTACT
Nitrosococcus watsonii	ACCGAGCAGGATGGTGCCTGGCATCA	TTAATTTCGTAGGGTTCACCTATT
Nitrosococcus oceani ATCC 19707	ACCGAGCAGGATGGTGCTTGGCATCA	TTAATTTCGTAGGGTTTACCTATT
Nitrosococcus halophilus	ACCGAGCAGGATGGTACCTGGCATCA	ATAACTTCTACGGTTTCACCTACT
Alphaproteobacteria	**pmoC374**	**pmoA344**
Methylocystis rosea SV97T operon 1	ACCGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTACT
Methylocystis rosea SB2 operon 3	ACGGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTACT
Methylocystis rosea SV97T operon 2	ACCGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTACT

Methylocystis sp. BN69 operon 2	ACGGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTACT
Methylocystis sp. BN69 operon 3	ACGGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTACT
Methylocystis parvus OBBP operon 2	ACGGAGCAGGACGGCACCTGGCACAT	TCAACTTCTGGGGCTGGACCTATT
Methylosinus sp. ATCC operon 1	ACGGAGCAGGACGGCACCTGGCATAT	TCAACTTCTGGGGCTGGACCTATT
Methylosinus sp. LW3 operon 2	ACGGAGCAGGACGGCACCTGGCACAT	TGAACTTCTGGGGCTGGACCTACT
Methylosinus sp. LW3 operon 3	ACGGAGCAGGACGGCACCTGGCACAT	TGAACTTCTGGGGCTGGACCTACT
Methylosinus sp. LW4 operon 1	ACCGAGCAGGACGGCACCTGGCATAT	TGAACTTCTGGGGCTGGACCTATT
Methylosinus sp. LW4 operon 3	ACCGAGCAGGACGGCACCTGGCATAT	TGAACTTCTGGGGCTGGACCTATT
Methylocystis parvus OBBP operon 1	ACCGAGCAGGACGGCACCTGGCATCA	ACAATTTCTGGGGTTGGACCTTCT
Methylocystis sp. BN69 operon 1	ACCGAGCAGGACGGCACCTGGCACCA	ACAACTTCTGGGGCTGGACCTTCT
Methylosinus sp. LW3 operon 1	ACCGAGCAGGACGGCACCTGGCATCA	ACAACTTCTGGGGCTGGACCTTCT
Methylosinus sp. LW4 operon 2	ACCGAGCAGGACGGCACCTGGCATCA	ACAATTTCTGGGGCTGGACCTTTT
Methylocapsa acidiphila B2	ACCGAGCAGGACGGCACCTGGCACCA	CCAATTTCTGGGGTTGGACCTATT
Verrucomicrobia	**pmoC374**	**pmoA344**
Methylacidiphilum fumariolicum SolV operon 1	ACGGAGCAAGACGGCACGTGGCATCA	GGAATTTCTGGGTTGGCACACTACC
Methylacidiphilum fumariolicum SolV operon 2	ACTGAGCAAGATGGGACATGGCATCA	GGAACTTCTGGGGTTGGGGCACTT
Methylacidiphilum fumariolicum SolV operon 3	GTTGAACAGGATGGAGTATGGCATTC	TCAATTGGTGGGGATGGTTCAGTT
Methylacidiphilum infernorum V4 operon 1	ACAGAGCAGGACGGCACATGGCATCA	GGAATTTCTGGGGTTGGACACACT
Methylacidiphilum infernorum V4 operon 2	ACCGAGCAAGATGGGACTTGGCATCA	GGAATTTTTGGGGTTGGGGGACCT
Methylacidiphilum infernorum V4 operon 3	GTTGAGCAAGATGGGGTTTGGCATTC	TTAACTGGTGGGGTTGGTTTAGTT
Verrucomicrobium sp.	ACGGAGCAGGACGGCACCTGGCACCA	TCAACTTCAATGGATGGACCCATT
Alkane monooxygenases	**pmoC374**	**pmoA344**
Mycobacterium chubuense	GCCGAAGAGGACGCCACTTGGCACCA	CGAGTTTTGATCTGTGGGCGCACC
Mycobacterium rhodesiae	GCCGAGGAGGACGCCGCCTGGCACCA	TCAACTTCGACTGGTGGGCCAACA
SAR324 cluster	**pmoC374**	**pmoA344**
SAR324 cluster bacterium	GCCTAATCTGGATGGCTCGTGGCATCA	TTCAGTGGGATGTTATGATAGGCT

NC10	**pmoC374**	**pmoA344**
Candidatus Methylomirabilis oxyfera	ACCGAGCAGGACGGGACGTGGCACCA	TTAACTTTTACTATTGGGCCTGGT
PXM (pMMO isoenzyme)	**pmoC374**	**pmoA344**
Methylomonas sp. MK1	GCCGAGCAGGACAACTCCTGGCATCA	TCGCTTACCACTACTGGAACTATT
Methylomonas sp. M11	GCCGAACAGGACAATTCCTGGCACCA	TTGCCTACCATTACTGGAACTATT
Methylobacter luteus	GCCGAGCAAGATAATTCCTGGCATCA	TTGCCTATCACTACTGGAACTATT
Methylobacter marinus	GCCGAGCAAGACAATTCCTGGCATCA	TTGCCTATCACTACTGGAACTATT
Methylobacter tundripaldum SV96	GCCGAACAGGATAATTCCTGGCATCA	TCGCCTATCATTATTGGAATTATT
Methyloglobulus morosus	GCCGAGCAAGATAACTCCTGGCATCA	TTGCCTACCACCTATGGAATTATT
Methylocystis rosea SV97T	GCGGAGCAGGACAATTCCTGGCATCA	TCGCCTATCACATGTGGAACTTTT
Methylocystis SB2	GCGGAGCAGGACAATTCCTGGCATCA	TCGCCTATCACATGTGGAACTTTT
Methylomonas sp. MK1	GCCGAACAAGATAATGCCTGGCATCA	CCGCCTATCAAATTTGGACCAATT
Methyloglobulus morosus	GGGGAACAAGACAATGCCTGGCACCA	TTGCCTACCACCTCTGGACGAATT
Betaproteobacteria	**pmoC374**	**pmoA344**
Nitrosomonas sp. AL212 operon 1	ACCGAACAGGATGCAAGCTGGCACCA	GGGGATTTTTACTGGTGGTCGCATT
Nitrosomonas sp. AL212 operon 2	ACCGAACAGGATGCAAGCTGGCACCA	GGGGATTTTTACTGGTGGTCGCATT
Nitrosomonas sp. AL212 operon 3	ACCGAACAGGATGCAAGCTGGCACCA	GGGGATTTTTACTGGTGGTCGCATT
Nitrosomonas sp. Is79A3 operon 1	ACCGAACAAGATGCATCGTGGCACCA	GGGGCTTCTACTGGTGGTCACATT
Nitrosomonas sp. Is79A3 operon 2	ACCGAACAAGATGCATCGTGGCACCA	GGGGCTTCTACTGGTGGTCACATT
*Nitrospira multiformis*operon 1	ACCGAACAGGACGCCTCCTGGCACCA	GGGGTTTCTACTGGTGGTCGCACT
*Nitrospira multiformis*operon 2	ACCGAACAGGACGCCTCCTGGCACCA	GGGGTTTCTACTGGTGGTCGCACT
*Nitrospira multiformis*operon 3	ACCGAACAGGACGCCTCCTGGCACCA	GGGGTTTCTACTGGTGGTCGCACT
Nitrosomonas europaea operon 1	ACGGAGCAAGATGCCTCCTGGCACCA	GGGGATTCTACTGGTGGTCACACT
Nitrosomonas europaea operon 2	ACGGAGCAAGATGCCTCCTGGCACCA	GGGGATTCTACTGGTGGTCACACT
Nitrosomonas eutropha operon1	ACGGAGCAAGATGCCTCCTGGCACCA	GGGGTTTCTACTGGTGGTCACACT
Nitrosomonas eutropha operon2	ACGGAGCAAGATGCCTCCTGGCACCA	GGGGTTTCTACTGGTGGTCACACT

A set of primers covering *pmoC*, the intergenic region, and *pmoA* were ultimately designed (Table 2) and ordered from Biolegio (Nijmegen, the Netherlands). The forward primer, called pmoC374, with the reverse primer, called pmoA344 resulted in product length of roughly 850 base pairs (bp) (Table 3). There are slight variations between different lineages. This is caused by variation in on average, 120 bp long intergenic region between *pmoC* and *pmoA*.

Table 2: Comparison of targeting ability between two newly designed degenerate primers and pmoA189.

	pmoC374				**pmoA344**				**pmoA189**			
Phylum	**Mismatches**				**Mismatches**				**Mismatches**			
	0	1	2	3	0	1	2	3	0	1	2	3
Gammaproteobacteria	10/18	18/18	18/18	18/18	16/18	18/18	18/18	18/18	7/18	18/18	18/18	18/18
Alphaproteobacteria	16/16	16/16	16/16	16/16	14/16	16/16	16/16	16/16	16/16	16/16	16/16	16/16
Verrucomicrobia	3/7	5/7	5/7	6/7	0/7	0/7	1/7	3/7	0/7	0/7	0/7	3/7
NC10	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1

Percent sequence coverage of all *pmoCAB* available sequences within each phylum were calculated by looking at how many sequences each primer could target. Targeting ability is also shown for zero, one, two and three mismatches within each primer.

Table 3: The new pMMO primers designed based on aligned pmoC, A, and B compared to pmoA189. MT = melting temperature. %GC = GC content in percentage.

Primers	**Sequence**	**MT**	**%GC**
PmoC374	5'-AGCARGACGGYACNTGGC-3'	42,9	56
PmoA189	5'-GGNGACTGGGACTTCTGG-3'	40,3	56
PmoA344	5'-ANGTCCAHCCCCAGAAGT-3'	42,9	50

DNA extraction and PCR conditions:

Total DNA was extracted from methanotrophic pure and enrichment cultures and from various environmental samples. Table 4 provides an overview on the cultures and samples used in this study. DNA was extracted using the PowerSoil® DNA Isolation Kit from MO BIO Laboratories (Carlsbad CA, USA) following the protocol of the manufacturer. The primers were tested using polymerase chain reaction (PCR), gradient PCR, touchdown PCR and nested PCR on all of the samples. The optimized protocol consisted of initial denaturation step at 96°C for five minutes, followed by 35 cycles at 96°C for one minute, annealing at 55°C for one minute and elongation at 72°C for two minutes. The final elongation step was done for ten minutes at 72°C.

Excision from gel after gel electrophoresis, purification, ligation and transformation of the amplified PCR products were done following the protocol described by Luesken *et al.*, 2011A. At least 20 random clones were picked for each environmental sample in a blue-white screening

and the plasmids were isolated for each PCR product with the GeneJet Miniprep Kit (Fermentas, Vilnus, Lithuania). The samples were sent to BaseClear (Leiden, the Netherlands) for sequencing of the cloned product using M13 forward primer (Luesken *et al.*, 2011A).

Table 4: Over view of the strains, enrichment culture and environmental samples tested in this study to detect pmoCA gene sequences

Name/sample	Description	Origin/Location reference
Methylocystis rosea	Pure culture *Alphaproteobacteria*	DSMZ 17621
Methylosinus sporium	Pure culture *Alphaproteobacteria*	DSMZ 17706
Methylomonas lenta	Pure culture *Gammaproteobacteria*	Hoefman *et al.*, 2014
***Methyloacidimicrobium fagopyrum* 3C**	Pure culture *Verrucomicrobia*	van Teeseling *et al.*, 2014
***Methyloacidiphilum fumariolicum* SolV**	Pure culture *Verrucomicrobia*	Pol *et al.*, 2007
***Methylomirabilis oxyfera* (DAMO)**	Enrichment culture NC10 phylum	Ooijpolder, NL Ettwig *et al.*, 2008
Sludge from waste water treatment plant (WW)	Environmental sample	Lieshout, NL Luesken *et al.*, 2011AB
Bulk soil form paddy field (BS)	Environmental sample	Vercelli, Italy Vaksmaa *et al.*, 2016
Rhizosphere of rice plants (ROOT)	Environmental sample	Vercelli, Italy Vaksmaa *et al.*, 2016
Enrichment culture with paddy field soil (RV)	Enrichment culture	Vaksmaa *et al.*, 2016
Volcanic mud (VM)	Environmental sample	Campi Flegrei caldera, Italy Pol *et al.*, 2012

Sequence analysis

The resulting sequences were checked for quality using Chromas Lite 2.1.1.0 (Technelysium Pty Ltd). Once erroneous sequences were removed, the results were blasted (BLASTx) using the publically available tools on National Center for Biotechnology Information (NCBI). Sequences matching with AMO superfamily were imported into ARB, translated into protein sequences and aligned to the previously mentioned *pmoCAB* operon dataset using ARB built-in aligner tools. Phylogenetic tree construction was performed on the amino acid alignment using maximum parsimony and maximum likelihood methods with bootstrapping of 100 times. Consensus sequences based on the fraction and frequency of residues at a specific alignment position within *pmoC* from all sequences were used to generate the tree.

Sequences are deposited in Genbank with accession numbers KY883458-KY883555 (Table S1)

RESULTS

The design of new primers was obtained by using all available *pmoCAB* operon sequences from MaGe. Interestingly, *pmoB* contained no conserved sequence stretch as a potential primer target site. Looking at the full operons, the only conserved regions resided within *pmoC* and *pmoA*. A new region at the nucleotide position 374 within the PmoC subunit of *Methylococcus capsulatus* (Bath), as a reference, was found to be highly conserved amongst all the phyla tested in this experiment. The forward primer binding site encodes for a glutamine residue at 126th base within the crystal structure of *pmoC* anchored to the membrane in *Methylococcus capsulatus* (Bath) whereas the reverse primer binding site encodes a phenylalanine residue at 107th base within *pmoA*. Our newly designed forward primer was compared to Holmes' forward primer and the results are shown in Table 1 and 2. As the tables illustrate, with zero mismatches, pmoC374 is able to target three out of seven available sequences from *Verrucomicrobia*. If a single mismatch is allowed, five out of seven sequences from *Verrucomicrobia* are targeted by pmoC374 whereas pmoA189 (Holmes *et al.*, 1995) with one mismatch still does not target any verrucomicrobial pMMO gene. The details of the novel primer set with regards to number of mismatches are listed in Table 2.

Initially, pmoA189 target region was thought to be a good matching reverse primer, however, a secondary conserved region at the 334th position within the *pmoA* gene was found. The pmoC374 with pmoA344 combination yielded a PCR product of the correct size in the samples tested, while the same could not always be observed when it was used in combination with pmoA189. In Table 1 and 2, it can be observed that pmoA344 has the ability to target 17 out of the 19 sequences belonging to *Gammaproteobacteria* with zero mismatches. Based on sequence information, pmoA334 does not have the ability to target NC10 phylum and it needs two or more mismatches to target species belonging to *Verrucomicrobia*. However, this primer improved the ability to target both *Verrucomicrobia* and the NC10 phyla in our study when pure isolates were used as positive control in the PCR reaction. The resulting sequences from the various enrichment cultures and environmental samples are depicted in Figure 1.

The *pmoCA* sequences obtained from the paddy field sample were closely related to well-known genera including *Methylosinus*, *Methylocystis*, *Methylococcus*, *Methylocaldum*, *Methylohalobius*, *Methylomicrobium*, *Methylobacter* and *Methylomonas*. Furthermore, the *pmoCA* of pure cultures of *Methylocystis rosea* and *Methylosinus sporium* belonging to *Alphaproteobacteria* and *Methylomonas lenta* (Hoefman *et al.*, 2014) belonging to *Gammaproteobacteria* could all be amplified with the new primer set. From previous studies, two isozymes of pMMO with various methane oxidation kinetics were found to be present in *Methylocystis* p. strain SC2 (Baani & Liesack, 2008), the new primers also amplified the second *pmoCA* in DNA extracted from the paddy soil. *Methylocaldum*- and *Methylococcus*-like species were also found in Waste Water samples. Furthermore, both alpha- and gammaproteobacterial *pmoCA* were found in the volcanic mud sample. Lastly, the *pmoCA* of the verrucomicrobial methanotroph *Methylacidiphilum fumarolicum* SolV could be amplified as well from a pure culture (Figure 1).

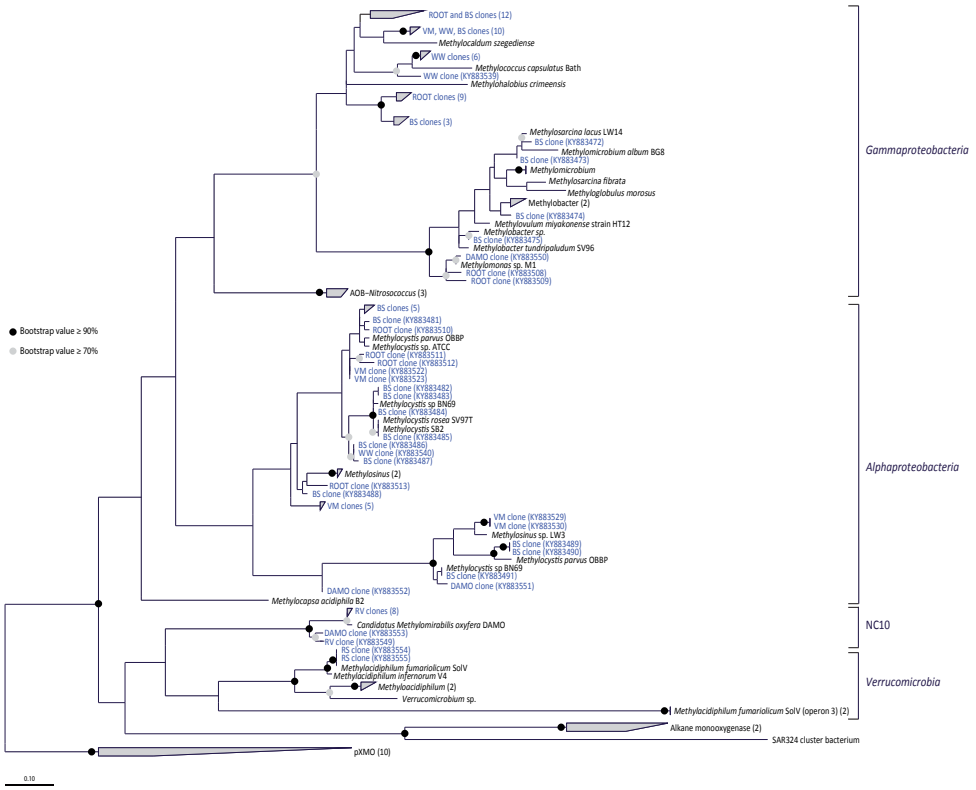


Figure 1: Representing available pMMO sequences including the sequence obtained in this study. The tree was constructed using consensus sequence, based on the fraction and frequency of residues at an alignment position chosen within *pmoC* using both ARB's PHYML (amino Acids) tool within the maximum likelihood method and Phylip PROTPARS within the maximum parsimony method. Since the two trees were highly similar, only maximum likelihood is shown here. Due to size limitation, the tree is partially collapsed for an easier illustration and pXMO is used as the out-group instead of AOB sequences that are omitted from this figure. The tree was built with 100 bootstraps and the ranges of values are shown with the respective *colored circles* at each node. Clone sequences with their respective accession numbers are *highlighted in blue* and the *numbers in the brackets* correspond to the number of sequences within a group. *Gammaproteobacteria, Alphaproteobacteria,* NC10 and *Verrucomicrobia* are clearly distinguished in the figure. Origin of clones: BS bulk soil, ROOT rhizosphere, VM volcanic mud, WW waste water sludge, RV bioreactor enrichment from vercelli, RS *Methylacidiphilum fumariolicum* SolV, DAMO *Methylomirabilis oxyfera* enrichment culture

In our experiment, only the *Verrucomicrobia* pMMO sequence most closely related to the ones in *Alphaproteobacteria* and *Gammaproteobacteria* could be detected. The new primer set was also used on a pure mesophilic *Verrucomicrobia* strain *Methyloacidimicrobium fagopyrum* 3C resulting in gene product of the correct size and gene sequence. The primers do not amplify sequences

related to the *pmoC3* group. In both anoxic enrichment cultures (DAMO and RV) tested, the *pmoCA* of NC10 phylum bacterium *Methylomirabilis oxyfera* could be amplified (Figure 1). In the case of *Methylomonas lenta* that does contain the genes for pXMO, only *pmoCA* gene sequences were detected, while the pXMO was not amplified. Lastly, no AMO (ammonia monooxygenase), PXM (alternative methane monooxygenase) or the recently discovered commamox amo were targeted nor amplified with this primer set in any of the environmental samples or the negative controls used in this study.

DISCUSSION

In the era of 'omics', molecular approaches using either specific or degenerate primers are still of high importance, especially in ecological studies where many samples need to be investigated or screened. They allow for a quick and robust detection of uncultivated microbes and aid in hypothesizing the community structure and the key processes that occur in certain environments, at the molecular level. As our knowledge and understanding of these environments expands, the tools that are used to investigate also need to be updated. More specifically, identification of the diverse organisms responsible for the oxidation of methane within various environments will help to better understand the key players involved in the methane cycle and evaluate their potential effectiveness as a biological methane filter. The currently available *pmoA* based primers are over ten years old and since known MOB diversity has since been extended, a novel primer set with broader amplification ability would be highly beneficial in molecular studies. It is also important to distinguish between copper monooxygenases belonging to the AMO superfamily to ensure the detection of MOB and not AOB or the more recently discovered comammox (van Kessel *et al.*, 2015; Pjevac *et al.*, 2016; Pinto *et al.*, 2015).

The use of all available *pmoCAB* operon sequences from MaGe allowed for the design of new primers (Table 1). Interestingly *pmoB*, which in previous studies has been suggested as the active site of the methane monooxygenase enzyme (Culpepper & Rosenzweig, 2012; Lieberman & Rosenzweig, 2005) contained no conserved sequence stretch as a potential primer target site. The only conserved regions that could be observed resided within *pmoC* and *pmoA*, both of which encode for primarily membrane bound subunits (Lieberman & Rosenzweig, 2005). Overall, PmoA is by far the most conserved subunit of this enzyme. Since for long it was thought to be the catalytic subunit as well, primers were designed based on this gene and have since become the academic standard in this line of research and used to date in many studies (Lüke & Frenzel, 2011; Rastogi *et al.*, 2009; Kip *et al.*, 2011). However, due to the two mismatches that occur at the 10th position within *pmoA* target region, previously unknown phyla (i.e. *Verrucomicrobia* or NC10) remain undetected and demand the design of phylum specific primers (Lueksen *et al*, 2011B). This variation in sequence identity is also one of the reasons why this study focused on the whole *pmoCAB* operon instead of the PmoA subunit alone (Table 2).

Previous studies have looked into analysis of MOB community in rice fields by targeting 16S rRNA, pMMO and methanol dehydrogenase (Henckel *et al.*, 1999) and observed a large variety of MOB. The new primer set used in this study was also able to detect a wide array of *pmoCA* sequences from both the bulk soil as well as the rhizosphere of an Italian rice paddy field, a waste water treatment sample, and volcanic mud samples. Further in anoxic *Methylomirabilis oxyfera* enrichment cultures started with paddy field or Ooijpolder sediment, many different *pmoCA* sequences could be retrieved (Figure 1). Furthermore, the *pmoCA* of the verrucomicrobial methanotroph *Methylacidiphilum fumarolicum* SolV could be amplified. This strain contains three complete *pmoCAB* operon structures that resemble the one observed in proteobacterial methanotrophs, plus a fourth *pmoC* copy. As expected, the primers do not amplify sequences related to the *pmoC3* group as it is further downstream in the genome and the primers do not bind there.

Most sequences from the waste water treatment plant biomass used in this study were closely related to *Methylococcus* genus as was previously observed (Luesken *et al.*, 2011A). Lastly, no AMO (ammonia monooxygenase), PXM (alternative methane monooxygenase) or the recently discovered commamox amo were targeted nor amplified with this primer set in any of the environmental samples which is an indication of the specificity. However, with some modification of the primer sequence, the same or similar sites can be used to only target AOB instead of MOB (Pjevac *et al.*, 2016; Wang *et al.*, 2017).

This study illustrates that when primer pmoC374 was used in combination with pmoA344, PCR amplification yielded the correct gene product from various environmental samples and MOB strains. Such observation could not be made when pmoA189 was used as the reverse primer. At times, there were multiple bands that occurred at the expected size within the gel. When each band was excised from the gel, all corresponded to the correct product. Since the *pmoCA* sequence covers the intergenic region, the slightly different nucleotide length observed in the PCR product is possibly due to the variation that exists in this region. This was more apparent when environmental samples were used as opposed to pure isolates, which further supports our hypothesis.

The obtained results expand our knowledge with regard to primer target ability based solely on *in silico* coverage as supposed to experimental results, since the new targeting sites would not be desirable due to occurring mismatches. Furthermore, the new pMMO primer set was able to amplify the correct product and sequence from all currently known methanotrophic phyla. If used in conjunction with Holmes' forward primer, the resulting product could be used in future next generation sequencing studies for a more extensive look at the bacterial community structure. The concurrent use of this primer set along with ones based solely on *pmoA* would allow for a much lesser bias when it comes to studies that look at the general diversity of the methanotrophic community within various environments. It also permits for the simultaneous detection of *Alphaproteobacteria*, *Gammaproteobacteria*, *Verrucomicrobia* and NC10 phyla with broader sequence variation.

DECLARATIONS:

List of abbreviations

MOB: Methane Oxidizing Bacteria
MMO: Methane monooxygenase
MCR: Methyl coenzyme-M reductase
sMMO: soluble methane monooxygenase
pMMO: particulate methane monooxygenase
CuMMO: cupper containing methane monooxygenase
PCR: Polymerase Chain Reaction
rRNA: ribosomal ribonucleic acid

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Sequences are deposited in Genbank with accession numbers KY883458-KY883555 (Table S1). The data will be publically available as of Apr 1st, 2018.

Competing interests

Mohammad Ghashghavi declares that he has no conflict of interest. Mike Jetten declares that he has no conflict of interest. Claudia Lüke declares that she has no conflict of interest.

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Author's contributions

CL conceived of the study. MG performed all experiments, computational analysis, and wrote the paper with input from CL and MSMJ. All authors read and approved the final manuscript.

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MG is a PhD candidate in the department of Microbiology at Radboud University. MG is supervised by Dr. CL, a Postdoc at the same department and promoted by Prof. dr. ir. MSMJ, the head of the Microbiology department at Radboud University.

SUPPLEMENTARY MATERIAL

Table S1: Sequences belonging to each environmental samples and their respective accession numbers from Genbank.

Sequence number	Clone ID	Accession number
Seq1	BS1	KY883458
Seq2	BS2	KY883459
Seq3	BS3	KY883460
Seq4	BS4	KY883461
Seq5	BS5	KY883462
Seq6	BS6	KY883463
Seq7	BS7	KY883464
Seq8	BS8	KY883465
Seq9	BS9	KY883466
Seq10	BS10	KY883467
Seq11	BS11	KY883468
Seq12	BS12	KY883469
Seq13	BS13	KY883470
Seq14	BS14	KY883471
Seq15	BS15	KY883472
Seq16	BS16	KY883473
Seq17	BS17	KY883474
Seq18	BS18	KY883475
Seq19	BS19	KY883476
Seq20	BS20	KY883477
Seq21	BS21	KY883478
Seq22	BS22	KY883479
Seq23	BS23	KY883480
Seq24	BS24	KY883481
Seq25	BS25	KY883482
Seq26	BS26	KY883483
Seq27	BS27	KY883484
Seq28	BS28	KY883485
Seq29	BS29	KY883486
Seq30	BS30	KY883487
Seq31	BS31	KY883488
Seq32	BS32	KY883489
Seq33	BS33	KY883490
Seq34	BS34	KY883491
Seq35	ROOT1	KY883492
Seq36	ROOT2	KY883493
Seq37	ROOT3	KY883494
Seq38	ROOT4	KY883495
Seq39	ROOT5	KY883496
Seq40	ROOT6	KY883497
Seq41	ROOT7	KY883498
Seq42	ROOT8	KY883499

Seq43	ROOT9	KY883500
Seq44	ROOT10	KY883501
Seq45	ROOT11	KY883502
Seq46	ROOT12	KY883503
Seq47	ROOT13	KY883504
Seq48	ROOT14	KY883505
Seq49	ROOT15	KY883506
Seq50	ROOT16	KY883507
Seq51	ROOT17	KY883508
Seq52	ROOT18	KY883509
Seq53	ROOT19	KY883510
Seq54	ROOT20	KY883511
Seq55	ROOT21	KY883512
Seq56	ROOT22	KY883513
Seq57	VM1	KY883514
Seq58	VM2	KY883515
Seq59	VM3	KY883516
Seq60	VM4	KY883517
Seq61	VM5	KY883518
Seq62	VM6	KY883519
Seq63	VM7	KY883520
Seq64	VM8	KY883521
Seq65	VM9	KY883522
Seq66	VM10	KY883523
Seq67	VM11	KY883524
Seq68	VM12	KY883525
Seq69	VM13	KY883526
Seq70	VM14	KY883527
Seq71	VM15	KY883528
Seq72	VM16	KY883529
Seq73	VM17	KY883530
Seq74	WW1	KY883531
Seq75	WW2	KY883532
Seq76	WW3	KY883533
Seq77	WW4	KY883534
Seq78	WW5	KY883535
Seq79	WW6	KY883536
Seq80	WW7	KY883537
Seq81	WW8	KY883538
Seq82	WW9	KY883539
Seq83	WW10	KY883540
Seq84	RV1	KY883541
Seq85	RV2	KY883542
Seq86	RV3	KY883543
Seq87	RV4	KY883544
Seq88	RV5	KY883545
Seq89	RV6	KY883546

Seq90	RV7	KY883547
Seq91	RV8	KY883548
Seq92	RV9	KY883549
Seq93	DAMO1	KY883550
Seq94	DAMO2	KY883551
Seq95	DAMO3	KY883552
Seq96	DAMO4	KY883553
Seq97	SolV	KY883554
Seq98	SolV	KY883555

BS = Bulk Soil, ROOT = Rhizosphere, VM = Volcanic Mud, WW = Waste Water sludge, RV = Bioreactor enrichment from Vercelli, DAMO = Bioreactor enrichment from Ooijplder, SolV = *Methylacidiphilum fumariolicum* SolV bioreactor.

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Keywords: Methane oxidation, CH_4 flux analysis, amplicon sequencing, statistical community analysis

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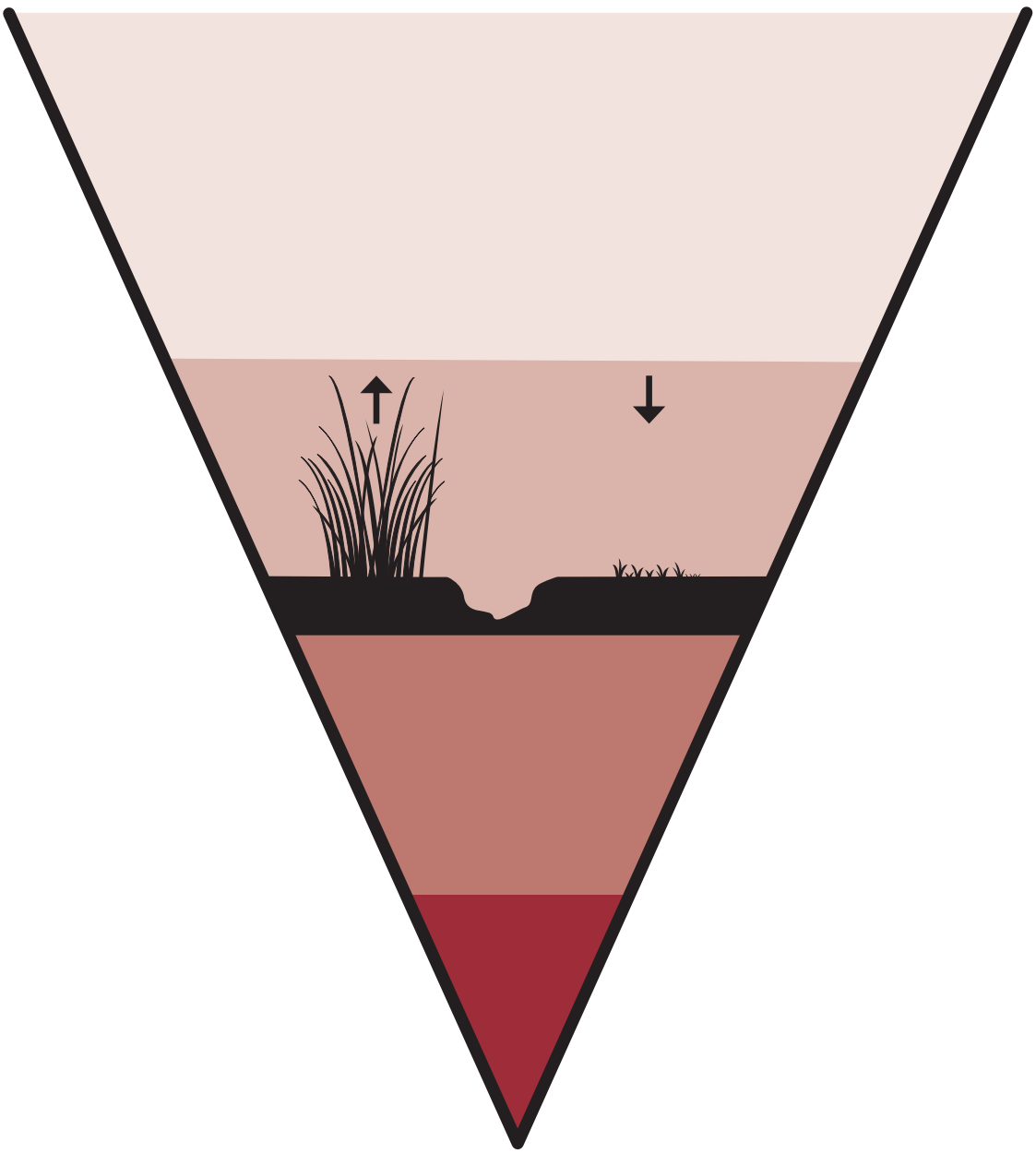
CHAPTER 3

Comparison of the bacterial and methanotrophic diversities between an Italian paddy field and its neighboring meadow



ABSTRACT

Methane is a potent greenhouse gas that contributes to global warming. However, under certain conditions, its release into the atmosphere can be mitigated by methane-oxidizing microorganisms. Typically, cultivated wetlands (i.e., paddy fields) are a major source of methane (CH_4) while forests and meadow uplands are considered to be CH_4 sinks. As the global need for rice production increases each year, more uplands are converted to inundated paddy fields. To investigate soils that may be converted into productive land for rice production, we investigated a paddy field and adjacent meadow in Northern Italy. Using a combination of 16S rRNA gene amplicon sequencing to analyze the bacterial community, and gas flux measurements to quantify CH_4 emissions, we looked for differences between classically defined CH_4 sinks (meadow soils) and CH_4 sources (paddy fields). Analysis of the total bacterial community revealed that the family Fimbriimonadaceae, which belongs to the phylum Armatimonadetes, was significantly higher in paddy field soils driving the difference between paddy and meadow soils. Whereas, we found that the methylotrophic families Methyloligellaceae and Methylomirabilaceae were also present in higher relative abundance in the paddy field. Despite these major differences, CH_4 fluxes were highly variable between the two sites with no significant differences observed. Furthermore, we found the Methylomonaceae family to be more abundant at the center of a neighboring paddy field compared to the edge of the paddy field from the current study, hinting at methanotrophic variation based on location. Taking these results into account, we propose a conceptual model to explain possible scenarios that may result in paddy and meadow fields not exhibiting classical source/sink properties. These findings call for caution when including paddy and meadow areas separately into global CH_4 flux calculations, and urge further research to discern drivers of CH_4 cycling under a range of environmental conditions rather than relying on assumptions.

INTRODUCTION

One of the most abundant greenhouse gases in the Earth's atmosphere is methane (CH_4), with its concentration steadily increasing as a result of anthropogenic activities (Wahlen, 1993; Dean *et al.*, 2018). Approximately 40% of the sources of atmospheric CH_4 are represented by natural and cultivated wetlands. In order to meet the global need for food, additional land is being converted into cultivated wetlands including inundated paddy soils. This has resulted in an increase in their total area by approximately 1% annually (IPCC, 2013; Pearman, 1986; Dlugokencky *et al.*, 1994). Heavily fertilized fields for rice production are considered to be among the highest sources of CH_4 emission on the planet (Lowe, 2006). The expansion of rice production in combination with increasing global temperatures could exacerbate CH_4 emissions from these source environments (Aselmann & Crutzen, 1989; Conrad, 2009). Understanding the potential factors that control CH_4 emissions from upland and wetland environments is necessary to accurately predict future atmospheric concentrations of CH_4.

In wetlands, CH_4 emission to the atmosphere results from a greater production of CH_4 by methanogenic microorganisms than what is oxidized by methanotrophic microorganisms (Cao *et al.*, 1998). Although some CH_4 in paddy soils can be oxidized under anoxic conditions at the expense of nitrate by *Methanoperedens* archaea (Vaksmaa *et al.*, 2016; Welte *et al.*, 2016), most methane seems to be oxidized by aerobic methane-oxidizing bacteria (MOB) (Lüke *et al.*, 2010). Known MOB belong to the *Proteobacteria*, *Verrucomicrobia* and NC10 phyla (Hanson & Hanson, 1996; Wu *et al.*, 2011; Op den Camp *et al.*, 2009). Within the *Proteobacteria*, methanotrophs are further classified into two main types known as type I and type II (Hanson & Hanson, 1996). Type I methanotrophs are affiliated with the *Gammaproteobacteria* and assimilate carbon via the ribulose monophosphate (RuMP) pathway, while type II belong to the *Alphaproteobacteria* and utilize the serine pathway for carbon fixation. These two types of MOB were shown to be the dominant methanotrophs in paddy fields, with their growth and activity influenced by soil conditions (i.e., organic content, pH, temperature), fertilizer application and vegetation cover (Hanson & Hanson, 1996; Zheng *et al.*, 2008).

Although previous studies have focused on the effect of various agricultural practices, climate change and soil features on methanotrophs, most have only included a wetland (i.e. methane source; for references refer to supplementary table S2) or an upland (i.e. methane sink; for references refer to supplementary table S2). Only few included both environments in their experimental design (Skov *et al.*, 2017; Hondula & Palmer, 2017). This has left the literature split between what main factors are influencing methanotrophic community structure within these environments. Therefore, it is important to further investigate differences in the microbial community and CH_4 fluxes of these two environments to better understand the contributions to methane emissions and global climate change.

In this study, we investigated the bacterial community from a paddy field and an adjacent meadow by 16S rRNA gene amplicon sequencing. In order to identify any influence soil cultivation

has induced on the soil bacterial community, we compared the bacterial community, with special interest in the methanotrophic community, paired with CH_4 fluxes from a neighboring meadow. We observed that the paddy field and the meadow had distinct bacterial communities being driven by the families Fimbriimonadaceae and Methyloligellaceae along with other methanotrophic groups, although the CH_4 fluxes did not differ significantly between the classical CH_4 source (paddy field) and sink (meadow) soils and were found to be highly variable across both environments. By combining the findings of this study with previous literature, we propose a conceptual model that provides several explanatory scenarios for these soils not exhibiting behaviors assumed to be universal to paddy and meadow soils.

MATERIALS AND METHODS

Study site

Field experiments took place at the rice research facility Vercelli, Italy (45°19'25.6"N 8°22'14.2"E). This field has been under rice cultivation with the rice variety *Oryza sativa* temperate japonica Onice for the last 30 years, with irrigation waters coming from the river Sesia during the growing season (May – September) and fields left fallow during the winter months. Sample acquisition took place during the maturing stage of the rice plants. The neighboring meadow has been left uncultivated for the last 30 years, contains sandy soil, and is covered with grass and small bushes. Data obtained from the study by Vaksmaa and colleagues (08°22'25.89"E; 45°19'26.98"N) is from a neighboring paddy field that has gone through the same farming practices and planted with the same rice cultivar, *Oryza sativa*.

Soil-atmosphere gas exchange and environmental variables

Soil-atmosphere CH_4 exchange was determined using a static chamber approach (Livingston *et al.* 2005) in July 2015 from the rice paddy field and meadow at the Italian Rice Research Unit in Vercelli, Italy. Measurements were made by using a 10 L volume PVC cylindrical flux chamber, covered with a gas tight lid. Chambers were fitted with small computer fans to promote even air mixing (Pumpanen *et al.*, 2004) and a small vent to prevent pressure changes inside the chamber as air was extracted (Hutchinson & Livingston, 2001). Temperature was measured by fitting a temperature probe in a small hole made at the top of the chamber. At the time of sampling, six chambers were placed carefully on the ground, where an airtight seal was created due to the permanently standing water. On the meadow site, where there was no water, a gas tight seal was created by fixing a rubber skirt to the bottom of the chambers. The headspace samples were collected from each flux chamber at five intervals over a 35-minute enclosure period using a gas tight syringe and 1 meter of tygon tubing, in order to prevent disturbance while sampling. Gas samples were stored in pre-evacuated Exetainers® (Labco Ltd., Lampeter, UK), shipped to the University of Aberdeen, UK and subsequently analyzed for CH_4 concentrations using an Agilent

6890 series gas chromatography system, with a single flame ionization detector (FID) for CH_4. Repeated analysis of standards determined that instrumental precision error was <10%.
Flux rates were determined using the HMR package (Pedersen *et al.*, 2010) in R 3.0.2 (R Core Team 2012) by plotting the best-fit lines to the data for headspace concentration (ppm) against time (minutes) for individual flux chambers. The Ideal Gas Law was used to convert gas concentrations (ppm) to molar concentrations. Fluxes were then reported in mg CH_4-C m^{-2} hr^{-1}. Soil temperature (at 10 cm) was simultaneously measured in three locations adjacent to the chambers using a type K thermacouple (Hanna Instruments Ltd., UK).

Sample acquisition and DNA Isolation:

A total of 21 soil samples were collected near the edge of the paddy field that is neighboring a meadow separated by a ditch located in the described study site. As seen in the sampling diagram (Figure S1), a total of 6 sites at the edge of the paddy field were used for samples to be collected using a 10 cm metallic core with a diameter of 8 cm. Samples were taken at three, six and nine meters from the meadow. Each row of samples was also three meters apart. To exclude rhizospheric soil, each sample site was chosen carefully to be equally distant from all neighboring rice plants, and contain no visible root material from the plants. The soil slurry from the cores was then mixed thoroughly and transferred to 50 mL falcon tubes. Lastly, 15 sampling sites within the meadow were used as described in figure S1. The samples were taken at three, six and nine meters away from the paddy field by using a 50mL falcon tube containing the top 7cm of the dry soil. Each row of samples within the meadow was also three meters apart. Soil surface was cleared before sampling to only include bulk soil. All paddy and meadow samples were stored at -20 °C for further analysis. Bulk soil cores obtained by Vaksmaa and colleagues from the center of a neighboring paddy field were done using an 80-cm soil augers at 5-m intervals (Vaksmaa *et al.*, 2017). The cores were later divided up at each 5-cm depth, however, we only compared the data obtained from the top 10-cm.
DNA from all samples was extracted using the MO BIO Power soil isolation kit following the manufacturer's protocol (MO BIO Laboratories, USA) with one modification. In the mechanical cell lysis step, the soil samples were beaten with glass beads at 30 s^{-1} frequency for 1 minute using a MO BIO 96 well plate shaker. The quality and quantity of the DNA was checked using gel electrophoresis and spectrophotometric analysis (NanoDrop 1000, Thermo Scientific, USA).

16S rRNA gene amplicon sequencing:

A two-step PCR protocol adapted from Klindworth *et al.*, 2012 and Berry *et al.*, 2012 was used to amplify bacterial 16S rRNA gene sequences using the universal bacterial primers B341f and B785r. The PCR program started an initial denaturation step at 98°C for 10 minutes, followed by 25 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 2 minutes, followed by a final extension step at 72°C for 10 minutes. The PCR products were purified using QIAquick PCR product purification kit following manufacturer's

protocol (Qiagen Inc., Germany). Per sample, a total of six PCR reactions were performed. All samples were checked for quality and quantity with gel electrophoresis and fluorescence-based analysis (Qubit 2.0, Thermo Fisher Scientific, USA) before being used in a second PCR step with barcoded B341 forward primer and reverse B785 primer containing the P1 adapter. The PCR conditions were as described above, but only 10 cycles were performed. Subsequently, all parallel reactions were pooled, purified and checked for quality again as described above. Each pooled sample was then further analyzed for quality and quantity in the last step using a Bioanalyzer (Agilent Technologies) following the manufacturer's protocol and sequenced on the Ion Torrent PGM (Thermo Fischer Scientific).

Bioinformatic analysis:

Raw reads from the Ion Torrent run were analyzed using mothur (Schloss *et al.*, 2009). The workflow consisted of eight steps: file processing, quality-based trimming, alignment based processing, pre-clustering, chimera removal, contamination removal, OTU clustering and generation of OTU files. The quality-based trimming step was done with parameters set as follows: PDIFF = 2, MAXHOMOP= 8, MAXAMBIG – 0, QWINDOWAVERAGE =20, QWINDOWSIZE = 50, MINLENGTH = 200 and MAXLENGTH = 450. In the alignment based processing step, the Silva database (v132) (Quast *et al.*, 2012) was used for the DB_ALIGN and DB_TAX function with these parameters: OPTIMIZE = "start-end"; CRITERIA = 95. Lastly, pre-clustering parameter (DIFFS = 2), contamination removal parameter (CLASS_CUTOFF = 80), distance matrix parameter (DIST_CUTOFF = 0.15), OTU clustering parameters (CLUST_ALGO = "average'; OTU_CUTOFF = "0.03"), singletons removal (NSEQ = 1; BYGROUP = false), and distance between sequences (CALC = "onegap"; COUNTENDS = "F", OUT_TYPE = "square") was set as shown. This protocol was repeated with the addition of Ion Torrent raw reads from the study published by Vaksmaa and colleagues (2017). In order to normalize for the different sequencing depths between the two data sets, the samples were subjected to a random subsampling at a depth of 10,000 reads. All OTUs with "methylo-" present in their classification were extracted and further identified as the methanotrophic OTUs in the data set.

Statistical and computational analysis:

Subsequent analysis was performed within R version 3.4.1 (R Core Team, 2012). Count data was normalized to relative abundances for all analysis. Analyses were performed with the R package *vegan* (Oksanen *et al.*, 2015). Shannon diversity was calculated using the *diversity* function and Bray-Curtis dissimilarity matrices were generated with *vegdist*. Chao1 estimates were performed using the *chao1* function from the *rareNMtests* package (Cayuela & Gotelli, 2014). Permutational multivariate analyses of variance (PERMANOVA) were performed using the *adonis2* function and classic multidimensional scaling was performed using *cmdscale*. The main drivers of differences in the microbial community composition between paddy field and meadow samples were identified using a random forest classifier from the R package *randomForest* (Liaw & Wiener, 2002).

RESULTS

CH_4 fluxes and Bacterial community structure and composition

The mean CH_4 flux from the paddy field (0.67 ± 1.76 mg C m^{-2} hr^{-2}) and meadow (-0.65 ± 1.59 mg C m^{-2} hr^{-2}) did not statistically differ from one another (t = -1.23; p = 0.251; Figure 1).

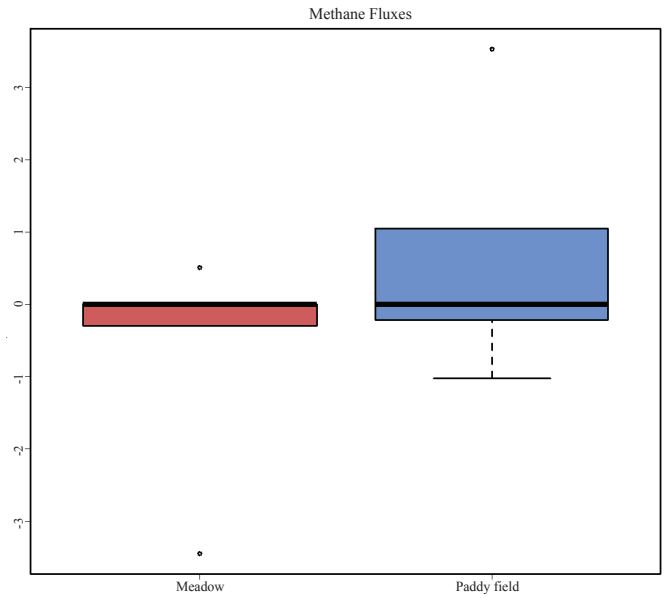


Figure 1: Box-and-whisker plot of methane fluxes in the meadow (**red**) and the paddy field (**blue**) field (t = -1.23; p = 0.251).

16S rRNA amplicon sequencing yielded a total of 958,089 reads. On average, each sample contained 12,898 ± 17,506 reads (Table S1). No significant differences were observed between paddy and meadow samples for either species richness (Chao1; t = -0.59; p = 0.574; mean in meadow = 3673 species; mean in paddy = 4260 species) or the sampling depth (t = -0.43, p = 0.67; mean in meadow = 10,179 reads, mean in paddy = 15,342 reads). However, the composition of the total microbial community in paddy and meadow soils significantly differed (PERMAnova; F = 2.27, p = 0.03; Figure 2A). A random forest classifier was used to identify the main bacterial family driving the difference between paddy and meadow soils which was the *Fimbriimonadaceae* that belongs to the phylum Armatimonadetes (formerly OP_{10}; Figure 2B). *Fimbriimonadaceae* was significantly more abundant in paddy field soils compared to meadow soils (Figure 2B; t = -4.16; p = 0.001).

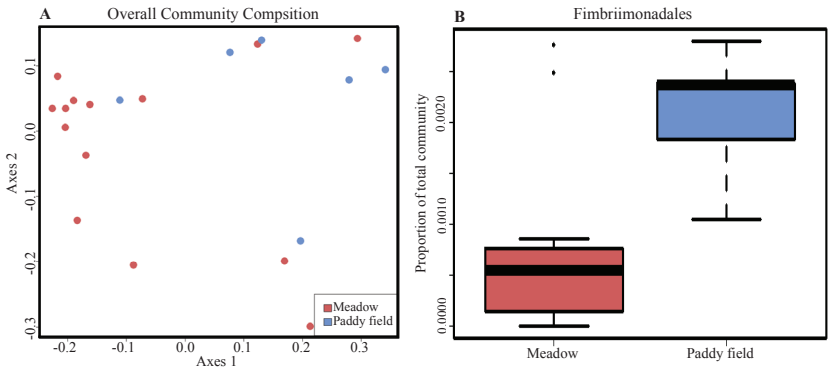


Figure 2: Total bacterial community analysis between the paddy field and the meadow. **A**: MDS plot showing the differences in total bacterial community (PERMAnova, F = 2.27, p = 0.03). Red and blue circles represent the meadow and paddy field samples, respectively. **B**: Relative abundance of *Fimbriimonadales*. Red and blue box- and whisker plots represents meadow and paddy field, respectively.

Furthermore, the bacterial community at the family level was found to be more diverse in the paddy field compared to the meadow (Shannon diversity (H'); t = -3.28; p = 0.005; mean meadow = 3.39; mean paddy field = 3.89; Figure 3).

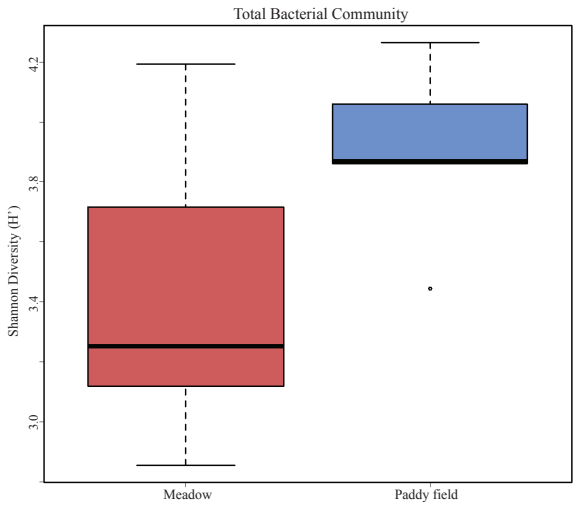


Figure 3: Box-and-whisker plot of total bacterial diversity between paddy field (**blue**) and meadow (**red**) (t-test, t = -3.28, p = 0.005).

Methylotrophic community

A total of 599 OTUs in the dataset were classified as methanotrophs originating from the *Proteobacteria*, *Verrucomicrobia* and NC10 phyla. The composition of methanotrophic community differed significantly between the paddy and the meadow soils (PERMAnova; F = 2.63, p = 0.041; Figure 4A). The most abundant methylotroph was affiliated with the family Methyloligellaceae and made up on average 21% of the methylotrophic community. Members of the families Methyloligellaceae and Methylomirabilaceae, were the top two drivers distinguishing the methylotrophic community between meadow and paddy field soils (Figure 4B-C).

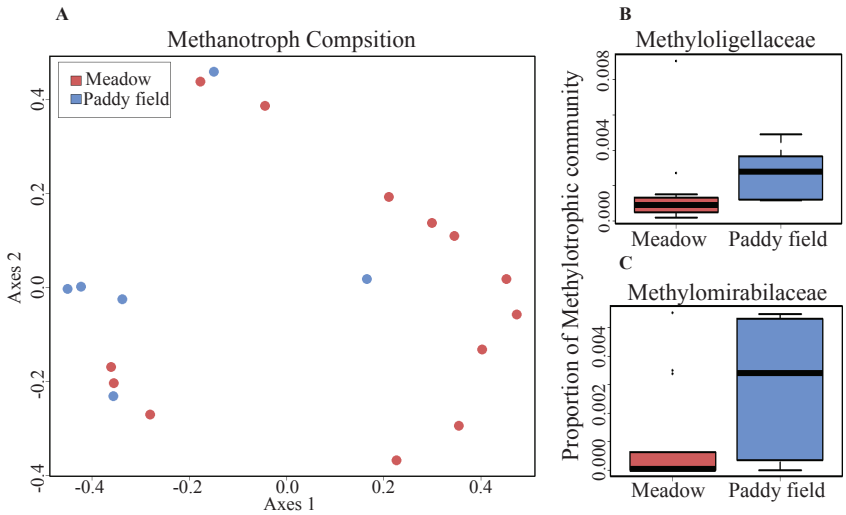


Figure 4: Methanotrophic community analysis between the paddy field and the meadow. **A**: MDS plot of each sampling site showing the difference in the methanotrophic community (PERMAnova, F = 2.63, p = 0.041). Red circles represent the samples within meadow and blue circles represent samples within paddy field. **B and C**: Box-and-whisker plot of top two families that drive the difference in the methylotrophic community between the paddy field (**blue**) than the meadow (**red**).

In general, all methanotroph family abundances were higher in paddy field soils compared to meadow (Figure 5; t = 2.53; p = 0.039).

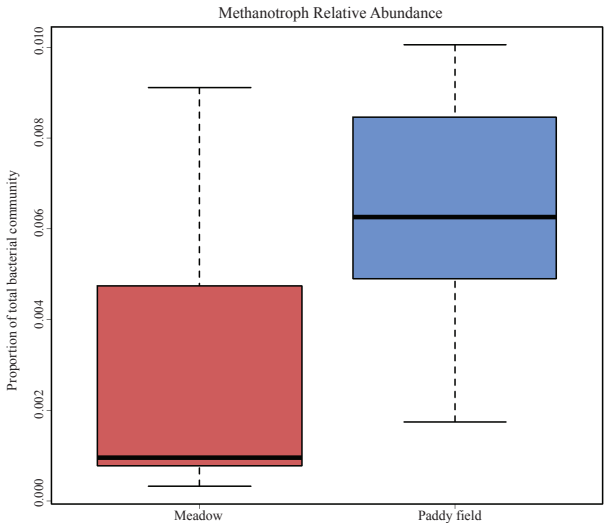


Figure 5: Box-and-whisker plot of difference in relative MOB abundance between the paddy field (**blue**) and the meadow (**red**) (PERMAnova; F = 2.63, p = 0.041).

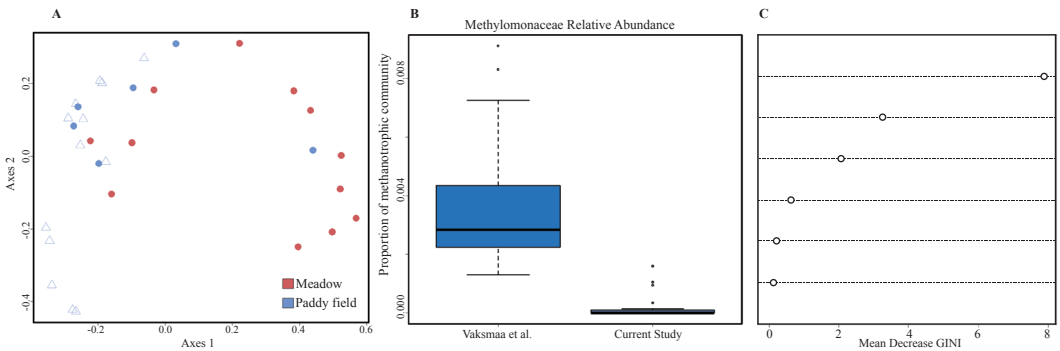


Figure 6: Investigation of spatial variation in methanotrophic community. **A:** MDS plot of each sampling site showing the difference in the methanotrophic community. The meadow (**red**) and paddy field (**blue**) samples of the current study is represented by circles while the paddy field samples from Vaksmaa *et al.* is represented by blue triangles. **B:** Box-and-whisker plot of relative abundance of Methylomonaceae family between the soil samples at the center of a neighbouring paddy field (Vaksmaa *et al.*, 2017) and the soil from the edge of the paddy field (current study). **C:** Top six families of methanotrophs driving the differences between the two data sets.

Spatial variation of Methanotrophs between paddy fields

In order to examine whether any large-scale spatial variation exists in the methanotrophic community, we compared the data obtained from this study (edge of the paddy field) to the data published by Vaksmaa and colleagues, which were obtained from samples taken from the center of a neighboring paddy field (Vaksmaa *et al.*, 2017). The methanotrophic community in the center of the paddy field more closely resembled the community at the edge of the paddy field than the meadow, however there were differences within the methanotrophic community (Figure 6A; $F = 3.91$; $p = 0.006$). Members of *Methylomonaceae* family were significantly more abundant at the center of the neighboring paddy field (Figure 6B;$t = 5.24$; $p = 0.0001$). In addition, the *Methylomonaceae* family was found to be the main driver of the differences observed between the two methanotrophic community between the two locations (Figure 6C).

DISCUSSION

The objective of this study was to survey differences in the microbial communities and more specifically the methanotrophic bacteria between a cultivated wetland and an adjacent upland meadow soil, conventionally recognized as strong sources and sinks of CH_4, respectively. Despite the traditional classification, we observed that the rice paddy field and its neighboring meadow used here are highly variable in their CH_4 fluxes (Figure 1). This, however, is a single time point and not a representative of possible seasonal fluxes from these fields. It has been reported that during the flooding period of the paddy field, in which no plants are sown, CH_4 is almost exclusively emitted into the atmosphere through ebullition with fluxes as high as 27.73 $mg \cdot g^{-2} \cdot h^{-1}$ (Yuan *et al.*, 2018; Holzapfel-Pschorn & Seiler, 1986). After rice cultivation, up to 90% of the observed CH_4 emissions into the atmosphere are due to diffusion through aerenchyma of the plants. In this study, CH_4 fluxes and samples were measured from the bulk soil of the paddy field to avoid any possible affect from rice plant or roots on the microbial community or flux data. This could potentially explain the relatively low CH_4 fluxes observed from the paddy as most of the CH_4 at the time of sampling would be emitted through the aerenchyma of the rice plants. Additionally, it has been reported that soil amendment by organic fertilization increases the availability of C substrates to methanogens and increases CH_4 production (Yuan *et al.*, 2018; Plaza-Bonilla *et al.*, 2014; Mohanty *et al.*, 2006). This effect, however, is not observed from the bulk soil in the current study. Furthermore, the paddy field was found to be low in C content (1.4 ± 0.1 %), which would be an indication of low substrate availability for microbial activities. CH_4 emissions from bulk soil during the maturing stage of rice growth, which is the stage in which the fluxes were measured in this study, are reported to be below 2 $mg \cdot g^{-2} \cdot h^{-1}$ (Yuan *et al.*, 2018; Vaskmaa *et al.*, 2016).

Despite CH_4 fluxes being similar between the two sites, we found significant differences in the total bacterial communities' structure. Since the paddy field is plowed, fertilized and planted

homogenously with rice, we hypothesized for the total bacterial composition to be established in a more homogenous manner relative to the meadow. What we found was that the total bacterial community was different from its neighboring meadow with high bacterial heterogeneity observed in both environments (i.e., highly variable; Figure 2A). Despite the variability, we found that the main family driving the difference between the two soils was Fimbriimonadaceae within the Armatimonadetes phylum (Figure 2B). Sequences classified as Armatimonadetes have been obtained by culture-independent methods from various environments including aerobic and anaerobic wastewater treatment processes, hypersaline microbial mats and subsurface geothermal water streams, as well as various rhizospheres (Portillo and Gonzalez 2009; Lee *et al.*, 2011; Tamaki *et al.*, 2011). The recently isolated *Fimbriimonas ginsengisoli* within the Fimbriimonadia class was described as strictly aerobic, Gram-negative, meso- and neutrophilic strain with the ability to grow on peptone, casamino acids and yeast extract (Im *et al.*, 2012). In another study, genera affiliated with the Fimbriimonadaceae grew better in biofilms cultured using a flow incubator with supplied inorganic nitrogen (N) conditions compared to deficient N (Li *et al.*, 2017). Since the paddy field more similarly resembles an N condition, it could explain Fimbriimonadaceae's higher abundance compared to the meadow and moreover, the higher diversity of microbial community observed in the paddy field (Figure 3). However, any role that this family of Armatimonadetes would play in paddy soil N cycling requires further investigation.

Previous research on paddy fields in Vercelli have shown that MOB communities, even in very closely located fields with nearly identical agricultural treatments show significantly different patterns (Ho *et al.*, 2011, Lüke *et al.*, 2010). This difference in the MOB community was assumed to be a consequence of variance in CH_4 fluxes (Smith *et al.*, 2016; Yuan *et al.*, 2014; Holzapfel-Pschorn & Seiler, 1986). However, difference in CH_4 fluxes could not account for the observations made in this study, particularly regarding the differences observed in the methylotrophic community in the current study (Figure 4A). Furthermore, the most abundant methylotrophs in the paddy field were classified as members of the proposed family "Methyloligellaceae" within the Rhizobiales order (Figure 4B) and Methylomirabilaceae family (Figure 4C).

To date, two separate isolates have been cultivated from Ural saline environments classified as *Methyloligella halotolerans* gen. nov., sp. nov. and *Methyloligella solikamskensis* sp. nov. (Doronina *et al.*, 2013). These obligate methylotrophic isolates within the Rhizobiales order are strictly aerobic, Gram negative, non-motile rods that utilize the serine pathway for carbon assimilation. Although environmental sequences belonging to the Methyloligellaceae family have been previously found in agricultural soil (Ceja-Navarro *et al.*, 2010), not much is known about the role these alphaproteobacterial methylotrophs play in this environment. Fertilizer application has been shown to have an inhibitory effect on type II methanotrophs in rice fields, while stimulating type I MOB (Mohanty *et al.*, 2006). In the current study, we found the Methyloligellaceae family were more abundant in the paddy field compared to the meadow. Without information about

their genetics or physiology, it is challenging to infer their possible role in carbon (C) cycling within the paddy field. However, being the most abundant OTU in our data set by an order of magnitude, it is likely that members of this group are key players in this paddy field.

Studies that investigated the presence of methanotrophs in cultivated wetlands have reported the presence of both type I and type II *Methylocaldum*-like and *Methyocystis*-like *pmoA* genes in high abundance (Collet *et al.*, 2015; Zheng *et al*, 2008; Asakawa *et al.*, 2008). Therefore, we expected the methanotrophic community to be present in higher abundance in the paddy field compared to the meadow (Figure 5). However, we did not find any classical type II methanotrophs (*Methylocystis*- and *Methylosinus*-like) reported to be present in high abundance in cultivated wetlands (Lüke *et al.*, 2010; Zheng *et al.*, 2008; Shrestha *et al.*, 2008). Although multiple factors are in play, an enriched MOB community within the paddy field could be a result of higher indigenous CH_4 available as substrate when compared to the meadow. This would in turn result in higher rates of CH_4 oxidation and overall, a higher relative abundance of methanotrophs. In future research, it would be critical to monitor the changes in the MOB community structure during the conversion of a meadow into a paddy field to see what fluctuations occur in the MOB community after a drastic change in the cultivation regiment.

Lastly, due to the highly variable CH_4 fluxes throughout the paddy field, we wanted to investigate if sampling location influenced the abundance of methanotrophs. Previous studies on the spatial variation of methane fluxes within paddy fields have demonstrated that fluxes can vary significantly even within the same field, thus making extrapolation to larger areas from point samples challenging (Oo *et al.*, 2015; Sass *et al.*, 2002; Krause *et al.*, 2009; Spokas *et al.*, 2013). In order to compare the community composition of methanotrophs between two neighboring paddy fields, we incorporated the data published by Vaksmaa and colleagues into our analyses (Vaskmaa *et al.*, 2017; Figure 6A-C). We found that while the methanotrophic community from the Vaskmaa *et al.* study resembled that of our paddy soil samples, there was a significant difference in relative abundance of certain methanotrophic families between the two sampling locations (Figure 6A-B). More specifically, members of the *Methylomonaceae* family were found in much higher relative abundance in the previous study (Figure 6B) and were found to be the main family, driving the differences observed between the two data sets (Figure 6C). Unfortunately, there is no CH_4 flux data available to compare between the two study sites and whether more indigenous CH_4 availability is the reason behind this difference. Regardless, this finding suggests that the methanotrophic community may be heterogeneously distributed across neighboring paddy fields, possibly resulting in differences in CH_4 oxidation capabilities. As indicated by others, designing a strong experiment which pairs soil samples for microbial community analysis, in situ measurements of important environmental factors (i.e., pore water pH, inorganic compound concentrations, etc) with CH_4 flux data may reveal drivers of the heterogeneity of the paddy field methanotrophic community (Hester *et al.*, 2018).

CONCLUSION

Typically, paddy fields are regarded as CH_4 sources. These CH_4 emissions stem from a combination of higher fertilizer inputs, which result in increased organic matter deposited by the rice plant into the rhizosphere, providing substrate for methanogenesis (i.e., higher methanogenic activity). In the current study, we find that the paddy field on average was a source (positive CH_4 fluxes) and the meadow a sink (negative CH_4 fluxes), though there was no statistically significant difference between the two locations due to the high variability of the flux measurements. Based on the data collected and previous studies, we propose two possible working hypotheses responsible for the observations made by this study (Figure 7).

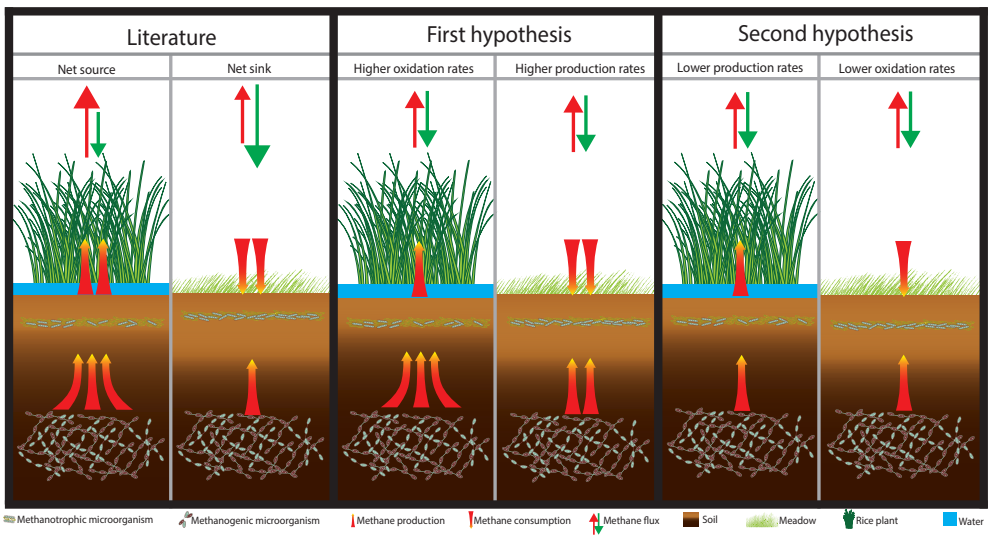


Figure 7: Hypotheses based on the data collected and previous. **A:** The paddy field and the meadow act as a CH_4 source and a sink, respectively. **B and C**: Two working models based on the findings of the current study on the paddy field and meadow not fulfilling their role as a CH_4 source and a sink.

The observed low levels of CH_4 emissions in the paddy field could be due to high turnover rates of CH_4. This is indicated by the high relative abundance of the MOB community comprised mainly of type II methanotrophs affiliated with the *Methyloligellaceae* family, and complies with previous studies (Barbosa *et al.*, 2018; Yuan *et al.*, 2018). Alternatively, the low flux could stem from a lower initial CH_4 production within the soil due to decreased various root exudates, a lower redox potential, or the provision of methanogenic substrates by heterotrophic bacteria (Mayer & Conrad, 1990; Aulakh *et al.*, 2001). Consequently, due to the high variability within

and between wetland and upland soils, caution should be exercised when making extrapolated predictions of CH_4 emissions.

ACKNOWLEDGEMENT

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SUPPLEMENTARY TABLES AND FIGURE:

Table S1: Number of sequences obtained from each sample at pre- and post-analysis.

Sample	Location	Pre-analysis	Post-analysis
1	Meadow	50470	22581
2	Meadow	47218	9273
3	Meadow	75573	30682
4	Meadow	27496	5328
5	Meadow	78818	33284
6	Meadow	87835	19081
7	Paddy	198593	77899
8	Paddy	7842	1372
9	Paddy	9433	1702
10	Meadow	28515	5130
11	Meadow	29000	5396
12	Meadow	2035	366
13	Paddy	37795	7099
14	Paddy	40746	6909
15	Paddy	30811	5974
16	Meadow	14987	2788
17	Meadow	48306	8884
18	Meadow	18682	3755
19	Meadow	21624	4440
20	Meadow	28676	6036
Total number of sequences		**958089**	**281043**

Table S2: A brief metadata of studies reporting on wetlands as a CH_4 source and uplands as a CH_4 sink environment with contradicting findings.

References	Environment	Source vs. Sink	Applied Method
Wetlands			
Feng *et al.*, 2012	Paddy field	**Source**	PCR-DGGE and qPCR
Banger *et al.*, 2012	Paddy field	**Source**	33 published papers on the subject
Ma *et al.*, 2010	Paddy field	**Source**	RFLP of 16S rRNA, pmoA
Lee *et al.*, 2014	Paddy field	**Source**	*pmoA* and *mcrA* transcripts
Shrestha *et al.*, 2010	Paddy field	**Source**	T-RFLP of *pmoA*
Ma et al., 2010	Paddy field	**Source**	qPCR and T-RFLP
Hoffmann *et al.*, 2002	Paddy field	**Source**	T-RFLP of *pmoA* and DGGE
Conrad *et al.*, 2007	Paddy field	**Source**	T-RFLP of 16S rRNA and *mcrA*
Bodelier *et al.*, 2000	Paddy field	**Source**	Radioactive fingerprinting
Noll *et al.*, 2008	Paddy field	**Source**	T-RFLP
Eller & Frenzel, 2001	Paddy field	**Source**	DGGE and FISH
Uplands			
Tate *et al.*, 2007	Shrubland	**Sink**	Flux measurements
Henckel *et al.*, 2000	Forest	**Sink**	Methane profile and DGGE
Knief & Dunfield, 2005	Upland soil	**Sink**	Molecular techniques and CH_4 flux
Benstead & King, 2001	Forest	**Sink**	Methane flux analysis
Groffman & Pouyat., 2009	Forest & Lawns	**Sink**	Methane flux analysis
Kolb *et al.*, 2005	Forest	**Sink**	Molecular techniques
Luo *et al.*, 2013	Forest	**Sink**	Methane flux analysis
Fang *et al.*, 2014	Meadow	**Sink**	Methane flux analysis
Jang *et al.*, 2011	Forest	**Sink**	Methane flux analysis and T-RFLP
Aronson & Helliker, 2010	Upland soil	**Sink**	Meta-analysis
Blankinship *et al.*, 2010	Grassland	**Sink**	Multifactor analysis

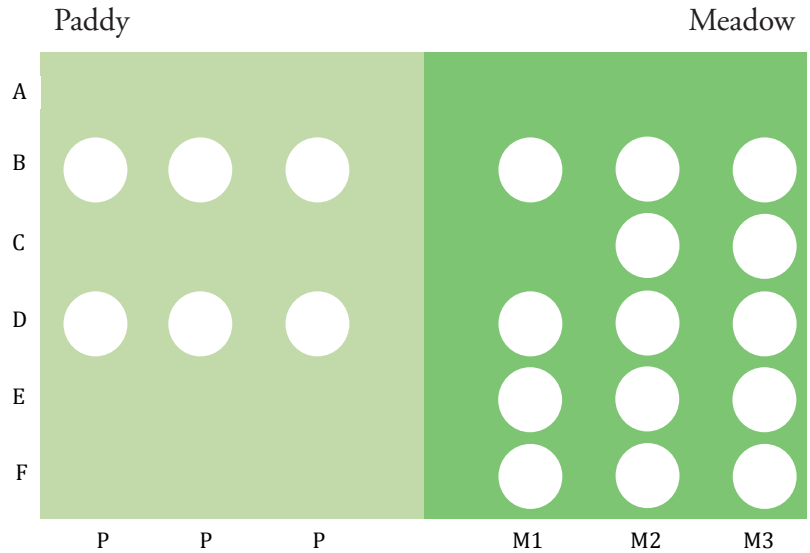

Figure S1: Sampling sites within paddy field and meadow represented by white circles. The CM1 sampling point was discarded due to poor sequence quality.

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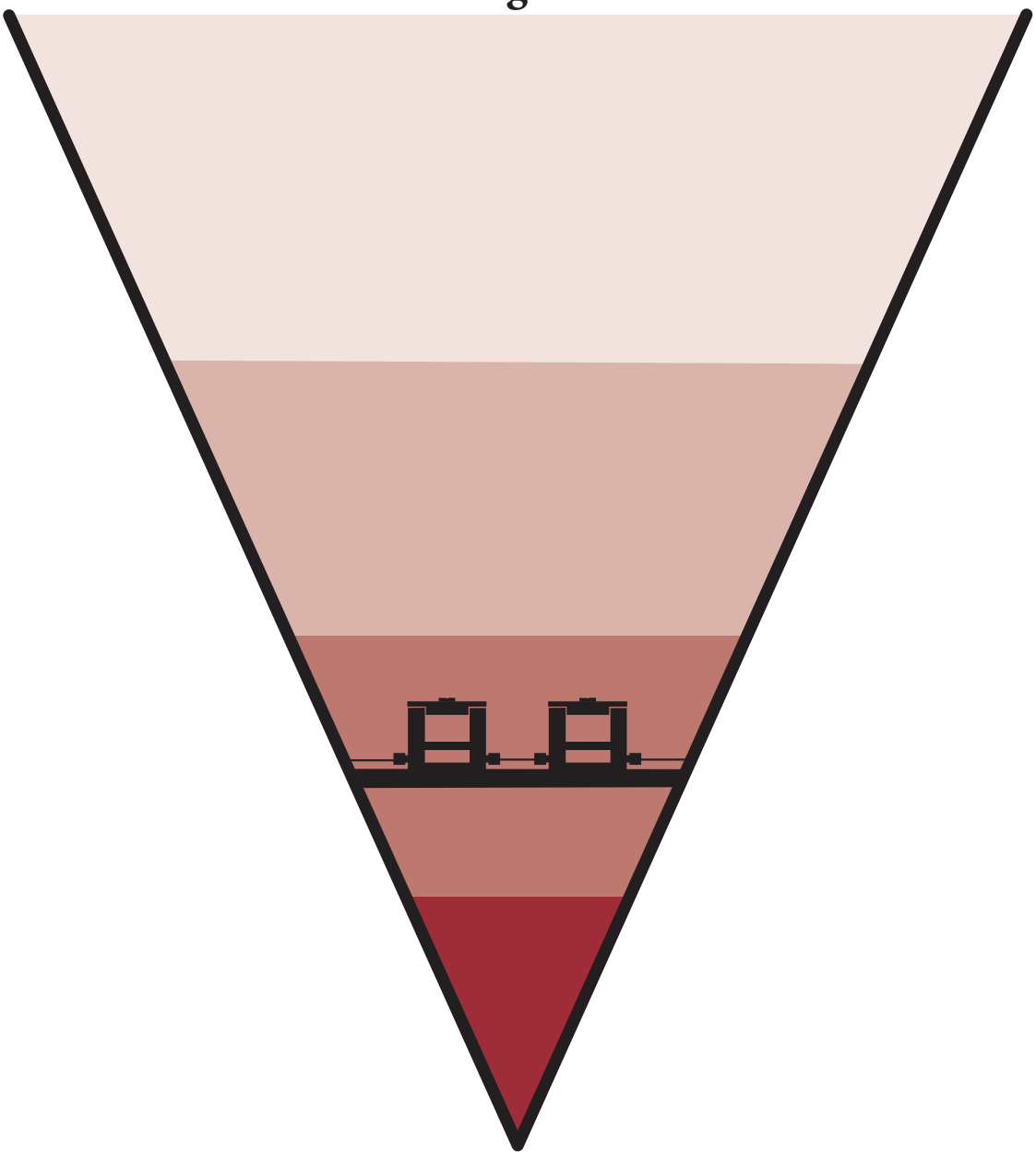
Keywords: Microcosms, CH4 and O2 counter-gradient, metagenome analysis, methanotrophic enrichment

Manuscript in preparation

CHAPTER 4

Enrichment of novel methanotrophic communities from paddy soils using laboratory scale microcosms with methane and oxygen counter-gradients



ABSTRACT

Irrigated paddy fields are one of the major sources of CH_4 with emission rates highly dependent on the balance between CH_4 production and oxidation at the oxic-anoxic interface. In the present study, we used laboratory-scale microcosms to study the methanotrophic community in paddy field soil slurries with counter-gradients of methane and oxygen. We found that the soil slurries became immediately active and that all of the CH_4 provided was oxidized. Moreover, O_2 profiles of the microcosms varied significantly over the duration of the incubations and showed that oxygen was depleted at shallower depths as enrichment continued. With increasing incubation time, 16S rRNA sequences affiliated with the gamma- and deltaproteobacterial orders *Methylococcales* and *Myxococcales*, respectively, were found in higher relative abundances. After two and three months of incubation, the enriched soil slurries from the microcosms were used as inoculum for further enrichment cultures of novel and previously uncultured type I and type II methanotrophs with *pmoA* sequences related to *Methylocystis* and *Methylomonas* species.

INTRODUCTION

Methane is known as one of the most important greenhouse gases along with water vapor and carbon dioxide (Intergovernmental Panel on Climate Change, 2015). Its emission from natural wetlands and flooded paddy fields is mitigated by methanotrophic bacteria, which act as natural biofilter by oxidizing CH_4 to CO_2 (Reeburgh *et al.*, 1993). Without these bacteria, natural wetlands and rice paddies would emit nearly 40% more CH_4 (Reeburgh *et al.*, 1993; Frenzel, 2000). Methane-oxidizing bacteria (MOB) are gram negative, aerobic microorganisms that form a subset of a functional group known as methylotrophs (Hanson & Hanson, 1996). While methylotrophic bacteria can utilize different one-carbon compounds, methanotrophs are restricted to the use of one-carbon compounds more reduced than formic acid as their source of carbon and energy (Hanson & Hanson, 1996). The oxidation of methane is catalyzed by the enzyme methane monooxygenase (MMO), which is a unique functional marker for MOB (Conrad, 1999).

In order to understand the process of methane oxidation and the adaptability and fitness of MOB in different habitats, researchers have focused on understanding their physiology and identifying and characterizing the key enzymes involved in this process. Since the oxidation of methane requires O_2 as co-substrate, but in most MOB also as terminal electron acceptor, this causes these bacteria to often be found at oxic-anoxic interfaces where both substrates are available albeit in limiting quantities (Brune *et al.*, 2000). As mentioned above, one such environment where methane oxidation is crucial to mitigate CH_4 emissions are irrigated paddy fields. Case studies have reported that depending on the season, up to 80% of the total CH_4 produced can be oxidized in paddy fields (Denier van der Gon and Neue, 1996; Kruger *et al.*, 2002; Eller *et al.*, 2005). It has been shown that agricultural practice such as fertilizer input has an impact on the MOB communities in these environments (Bodelier *et al.*, 2000; Dan *et al.*, 2001), but other factors involved in shaping MOB community structure and their effect on methane mitigation are largely unexplored.

In flooded soil, oxygen diffuses from the atmosphere while methane is supplied to the upper soil layer in micromolar (μM) concentrations from deeper layers. As a result, there is a steep gradient of oxygen from the soil surface downwards, while the opposite is observed for methane concentrations. Studies have shown that methane and oxygen gradients in flooded soil overlap at a depth of about 2 - 3 mm below surface (Damgaard *et al.*, 1998; Gilbert & Frenzel; 1998), suggesting that below this depth anoxic conditions prevail. This makes oxygen the limiting factor for aerobic methane oxidation in bulk soil (Conrad & Frenzel, 2002).

Previous amplicon sequencing-based studies have found more than 30 operational taxonomic units (OTUs) of MOB in paddy fields (Lüke *et al.*, 2010). Therefore, there is a large diversity of MOB that exists in wetlands, most of which are still uncultivated and their niche preferences unknown. Due to the different factors that can be responsible for the composition of these communities, such as methane levels (Krause *et al.*, 2012), nitrogen availability (Rudd *et al.*,

1976), disturbance (Ho *et al.*, 2011) and grazing (Murase and Frenzel, 2008), researchers have tried to study these systems in laboratory scale microcosms that mimic the natural habitat under controlled conditions. These microcosms allow for O_2 and CH_4 counter-gradients to be established similar to the situation in paddy soils. In a study published by Reim and colleagues (2012), such a system was used to show that within 3 mm thin soil layers different methanotrophic OTUs can occupy the same microenvironment. They further confirmed that highest methane oxidation rates occurred at the oxic-anoxic interface.

In this study, we used laboratory-scale microcosms with counter-gradients of methane and oxygen in combination with metagenomic sequencing to identify and enrich methanotrophic consortia from paddy fields. We used soil collected from a paddy field in Vercelli (Northern Italy) for the initial microcosm enrichment step. Using highly sensitive oxygen microsensors, we were able to follow the formation of an O_2 gradient as the soil became more enriched with gammaproteobacterial methanotrophs. Subsequently, the pre-enriched soil was used as inoculum for further enrichment cultures in agarose-containing artificial medium, also in the same microcosm setup. After transfer of the colonies obtained into liquid media, this resulted in several methanotrophic consortia that, based on their *pmoA* sequences, contain previously uncultivated MOB. These were related to members of the *Methylocystis* and *Methylomonas* genera, indicated that the microcosm set-up is a suitable tool to obtain novel methanotrophs.

MATERIALS AND METHODS

Soil sampling and microcosm incubations

The set-up and construction of the microcosms can be found in Murase & Frenzel (2007). In this study, we applied a continuous unidirectional flow of gas through four sets of two microcosms, which differed from the circular set up of the previous study. To prepare the soil slurries, 20 g of sieved dry rice field soil from Vercelli (Italy) was saturated and submerged with 12 mL of demineralized water. The slurries were incubated on a 0.1 µm gas permeable polytetrafluoroethylene membrane (RCT Reichelt Chemietechnik GmbH + Co., Heidelberg, Germany), which divided the microcosms into two compartments. The upper compartment of the microcosms received atmospheric levels of oxygen by opening the lid on a daily basis. The lower chamber received a gas mixture consisting of 20% CH_4 and 2% CO_2 mixed in Argon. During the incubation period, O_2, CO_2 and CH_4 concentrations were continuously monitored using gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS). The microcosms were incubated in the dark for a period of 90 days. In order to determine CH_4 oxidation rates, a set of two microcosms were clamped off and CH_4, O_2 and CO_2 levels were measured every 30 min for a total of seven hours. At each month, one set of two microcosms was discontinued and used for DNA extraction. Prior to sampling, vertical oxygen profiles were measured at 100 µm depth intervals over 3 mm in total using an Unisense oxygen microelectrode

OX50 and a Unisense microprofiling system (Aarhus, Denmark). Afterwards, liquid nitrogen was used to shock freeze the soil slurries. The slurries had a thickness of ~6 mm and were crudely divide into two subsamples of roughly 3 mm (top and bottom layer) using a sterile nylon string. The subsamples were stored in 15 ml Falcon tubes at -20°C for subsequent DNA extraction.

DNA extraction, sequencing and bioinformatic analyses

To reduce extraction biases, DNA was extracted using three separate methods: a classical hexadecyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof *et al.*, 1984), and the PowerSoil (QIAGEN, USA) and FastDNA (MP Biomedicals, USA) DNA extraction kits, following the manufacturer's protocols. The presence of methanotrophs was verified by PCR using two sets of primers targeting different conserved regions of the particulate MMO (*pmoCAB*) operon (Holmes *et al.*, 1995; Ghashghavi *et al.*, 2017), followed by cloning and sequencing as described elsewhere (Luesken *et al.*, 2011). The DNA samples from the separate extraction protocols were pooled and sequenced on the Ion Torrent PGM using the manufacturer's protocol (Thermo Fischer Scientific, Waltham, USA). Raw reads were checked for quality, trimmed and grouped as either top or bottom layer from first, second and third month of enrichment within CLC Workbench version 11 (QIAGEN, Hilden, Germany). Bacterial 16S ribosomal RNA together with *pmoA* gene sequences were extracted and analyzed in ARB (version 6.0.6; Ludwig *et al.*, 2004). Relative abundance and diversity of methanotrophs were estimated by mapping 16S rRNA sequences to the Silva database (version 132) (Quast *et al.*, 2012; Yilmaz *et al.*, 2013) in ARB while individual *pmoA* sequences were blasted against the NCBI database (Altschul *et al.*, 1990). Metagenomic binning was performed with MyCC (Lin & Liao, 2016), using the different layers and time points as differential coverage information (Albertsen *et al.*, 2013). The metagenome-derived genomes (MAGs) subsequently were annotated with prokka (Seemann, 2014) and checked for completeness using checkM (Parks *et al.*, 2015).

Enrichment of novel methanotrophic bacteria

The microcosms were used for further enrichment of methanotrophs in dNMS (Whittenbury *et al.*, 1970) agarose medium. The second- and third-month enrichments were chosen as inoculum. The slurry was thoroughly mixed in demineralized water before taking a 10 mL sample and mixing it with 10 mL dNMS media containing 0.5% agarose. Once solidified, the agarose layer was submerged in additional liquid dNMS media to ensure the formation of O_2 and CH_4 counter-gradients. After two weeks of incubation, single colonies were observed at various depths within the agarose. These single colonies were extracted using a sterile glass Pasteur pipette and transferred to 50 ml liquid dNMS media in 120 ml capped serum bottles containing 20% CH_4 in air as headspace. The bottles were shaken and incubated in the dark at room temperature for two weeks. After several transfers and serial dilutions, DNA was extracted from these enrichments and stored at -20°C for subsequent analysis.

Identification of methanotrophs

PCR was used to amplify *pmoA* and 16S rRNA gene sequences from the different enrichments using *pmoA* (Holmes *et al.*, 1995) and general bacterial 16S rRNA (Herlemann *et al*, 2011) gene-targeted primers, respectively. The purified PCR products (100ng) were cloned as described by Luesken *et al.* (2011) and purified with PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Further digestion were done with the restriction enzymes BamHI and EcoRI at 37°C for 180 min and samples were analyzed on 2,5% agarose gel using gel electrophoresis. Clones giving distinct restriction fragment length polymorphism patterns were sequenced at BaseClear (Leiden, the Netherlands). 16S rRNA gene sequences were analyzed using Chromas LITE version 2.1.1and blasted against the NCBI 16S rRNA database (Bacteria and Archaea). *pmoA* sequences were uploaded to ARB where phylogenetic analyses were performed. Lastly, the trimmed reads obtained from the one-, two- and three-month metagenomes were mapped to the *pmoA* sequences obtained from the enrichment cultures to check for similarity, presence and relative abundance of the enriched methanotrophs in the original microcosm incubations.

RESULTS

Microcosm monitoring

In the absence of soil slurry on the gas permeable membrane in the microcosms, it took a total of 4.5 hours for CH_4 to reach equilibrium between the two compartments. This time was extended to 16 hours when sterile soil slurry was added. However, when paddy field soil slurries were used, CH_4 levels in the top compartment never exceeded atmospheric concentrations, indicating that an active methanotrophic community was present. The average methane consumption rate was 1.0 mmol of CH_4 microcosm^{-1} hr^{-1}. As incubation time progressed O_2 and CO_2 consumption and production rates were decreasing and increasing in the top compartment, respectively.

Oxygen concentration profiles

Using microsensors, the vertical oxygen concentration profile in the soil slurry was determined at 100 µm depth intervals. O_2 concentration profiles significantly changed from the initial time point of the experiment until the end of the third month of incubation (Figure 1). After a month of incubation, O_2 concentrations were measured to be 258 µM at the surface of the soil slurry (0 mm depth), and were depleted (0 µM) at a depth of 3.2 mm (**Figure 1A**). After the second and third month of enrichment, O_2 depletion occurred at a depth of 2.5 mm (**Figure 1B**) and 2.3 mm (**Figure 1C**), respectively.

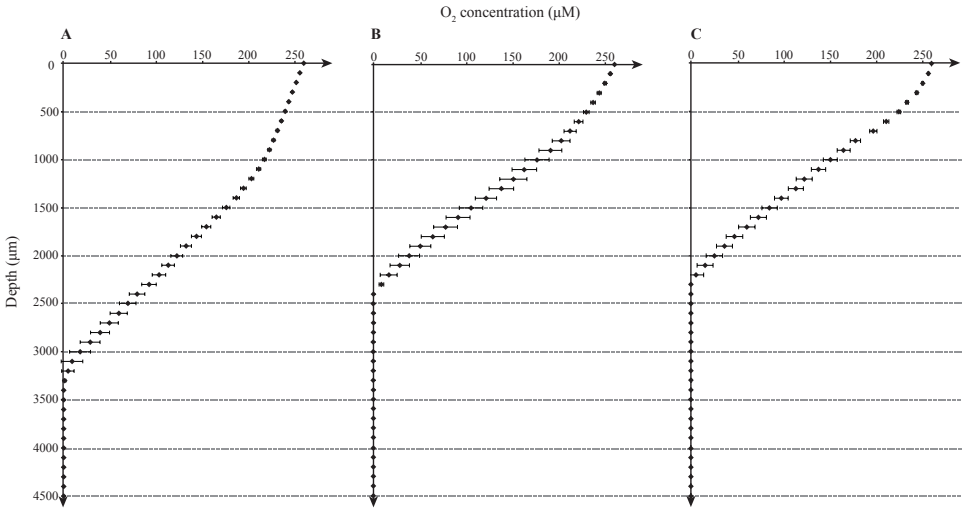


Figure 1: Oxygen profile of soil slurries from the (**A**) first (**B**) second and (**C**) third month of enrichment.

Changes in bacterial community during enrichment

We obtained metagenomes from the microcosms at different time points by IonTorrent sequencing. After quality checking and trimming, on average 2.2 million reads per sample time point (after 1st, 2nd and 3rd month) were obtained. Mapping of the extracted 16S rRNA gene sequences against the Silva database revealed *Proteobacteria* as the most abundant bacterial phylum with an average relative abundance of 65%, followed by *Acidobacteria*, which constituted 10% of all 16S rRNA sequences. Within the *Proteobacteria*, the relative abundances of the 4 most abundant classes varied between the time points. After the first month, the sample was dominated by *Betaproteobacteria* (29%), followed by *Delta-* (26%), *Gamma-* (24%), and *Alphaproteobacteria* (21%; **Figure 2A)**. However, after two months of enrichment, the *Gammaproteobacteria* became dominant (31%), followed by *Beta-* (30%), *Delta-* (27%), and *Alphaproteobacteria* (12%; **Figure 2B)**. After the third month, a similar distribution of these proteobacterial classes was observed (**Figure 2C)**.

At the order level, the most abundant groups within the *Beta-* and *Alphaproteobacteria* did not change over time. *Burkholderiales*, *Rhodocyclales* and *Nitrosomonadales* were the three most abundant orders within the *Betaproteobacteria*, and *Rhizobiales*, *Rhodospirillales* and *Sphingomonadales* within the *Alphaproteobacteria*. Here, 49% of the sequences belonged to the *Rhizobiales*. The most significant abundance shifts on the order level were observed within the *Gamma-* and *Deltaproteobacteria*. Within these, the gammaproteobacterial *Methylococcales* increased from 19% to 48% (**Figure 3**) and the deltaproteobacterial *Myxococcales* from 57% to 84% (**Figure 4**) between the first and third month of enrichment.

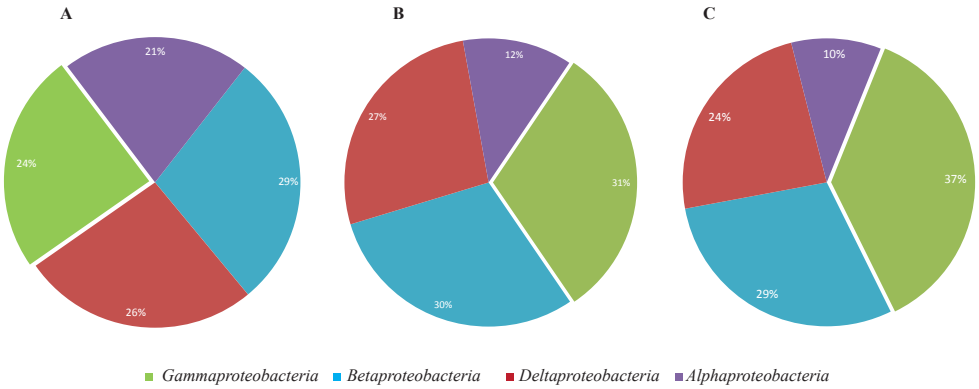


Figure 2: Relative abundance of the most abundant phyla within *Proteobacteria* based on 16S rRNA gene sequence counts after the (**A**) first (**B**) second and (**C**) third month of enrichment of soil slurries. The different phyla are distinguished by color.

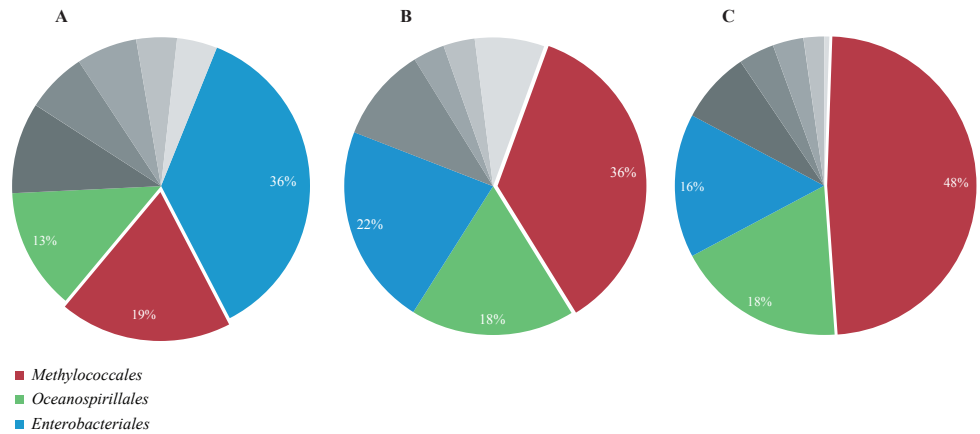


Figure 3: Top three most relatively abundant orders within the *Gammaproteobacteria* based on 16S rRNA gene sequence counts after the (**A**) first (**B**) second and (**C**) third month of enrichment. The grey areas represent minor orders within the *Gammaproteobacteria*.

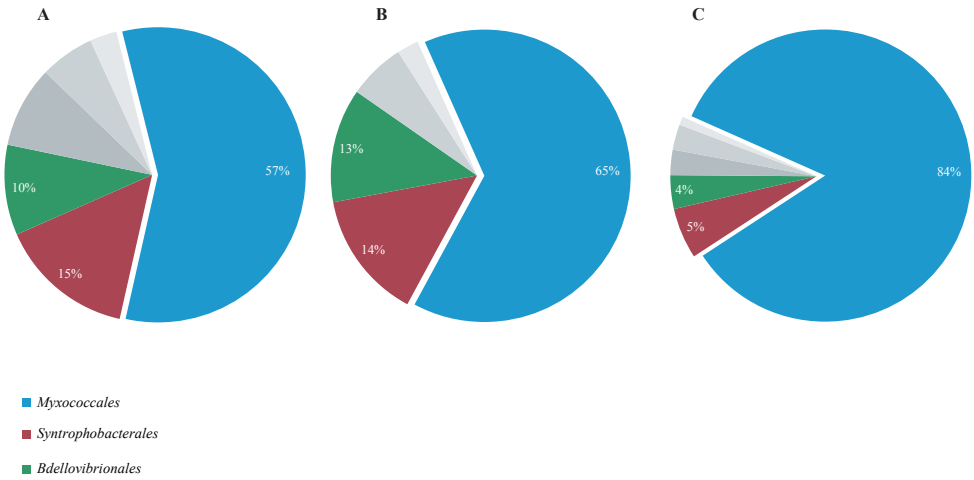


Figure 4: Relative abundance of three most abundant orders within the *Deltaproteobacteria* based on 16S rRNA sequence gene counts after the (**A**) first (**B**) second and (**C**) third month of enrichment. The grey areas represent minor orders within the *Deltaproteobacteria*.

Based on *pmoA* sequences extracted from the metagenomic data, the methanotrophic community varied between time points as well as between the top and bottom part of the soil slurry extracted from the microcosms. In total, 47, 70 and 101 different *pmoA* gene copies were extracted from the first-, second- and third-month enrichment, respectively, indicative of an active methanotrophic community. While most of the *pmoA* sequences derived from the top layers were classified as type I methanotrophs, type II was found to be most abundant in the bottom layers. After one month of incubation, *Methylobacter* (32% of all *pmoA* sequences) and *Methylocystis* (42% of all *pmoA* sequences) were the two most abundant genera from the top and bottom soil layer, respectively (**Figure 5**). Uncultured *pmoA* sequences related to both *Methylocystis* and *Methylomonas* became dominant after two months of enrichment, with 53% and 44% of the total *pmoA* sequences in the top and bottom layer, respectively (**Figure 5**). This number, however, decreased after 3 months to 18% in the top and 26% in the bottom layer.

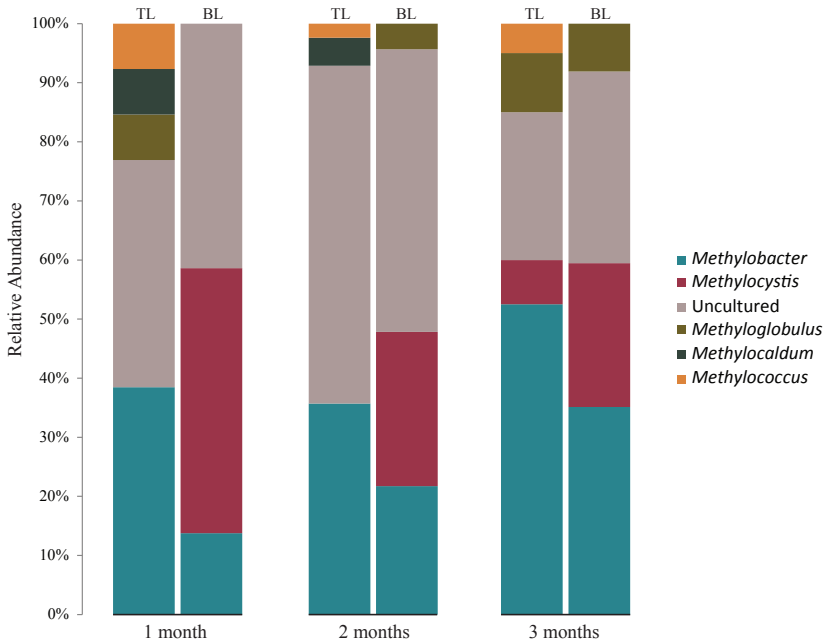


Figure 5: Relative abundance of methanotrophs based on extracted *pmoA* sequences from the metagenomic data after the first, second and third month of enrichment. TL= Top Layer, BL= Bottom Layer.

Genome binning and analysis

In the first-, second- and third-month enrichment metagenomes, roughly 2%, 3% and 7%, respectively, of the total 16S rRNA sequences extracted belonged to the order of *Methylococcales*. We were able to extract a methanotrophic genome bin from each time point with a completeness of 13%, 42% and 75%, respectively. The most complete genome bin from the third-month enrichment was analyzed, indicating the presence of multiple copies of the *pmoCAB* operon and the use of the ribulose monophosphate pathway for carbon fixation. Formate and formaldehyde oxidation pathways, along with genes for nitrogen fixation and hydroxylamine detoxification could also be identified. Based on *pmoA* (94% identity) and 16S rRNA (88% identity), the organism this genome belongs to is only distantly related to known methanotrophs, with *Methylobacter tundripaldum* SV96 as closest cultured relative.

Enriched methanotrophic consortia

Our subsequent enrichment efforts resulted in three different consortia of novel methanotrophs. Interestingly, in enrichment 2, none of the obtained 16S rRNA clone sequences matched a known methanotroph, but *pmoA* sequences were obtained. In all three enrichment

cultures, most *pmoA* clone sequences obtained were highly similar to previously uncultured environmental *pmoA* sequences. Besides these novel methanotroph types, all enrichments did also contain *Methylocystis*-like *pmoA* sequences. We could also find back the most dominant *pmoA* clone sequences from the three enrichments in the metagenome. Here, the number of reads mapping to the *pmoA* sequences from enrichment 1 and 3 increased from the first- to third-month sample from the microcosm enrichments, whereas for enrichment 2, it increased from first- to second-, and decreased at third-month sample (**Supplementary Figure 1**). The dominant *pmoA* clone sequence from enrichment 3 also corresponded to the most dominant *pmo*A sequence in the metagenome found at second- and third-month sample. Phylogenetic analyses of the *pmoA* sequences obtained from the methanotrophic enrichment cultures revealed the presence of novel, previously uncultured type I and type II methanotrophs (**Figure 6**). Furthermore, the addition of *pmoA* sequence from the binned genome showed a distant similarity to *Methylobacter tundripaldum* SV96.

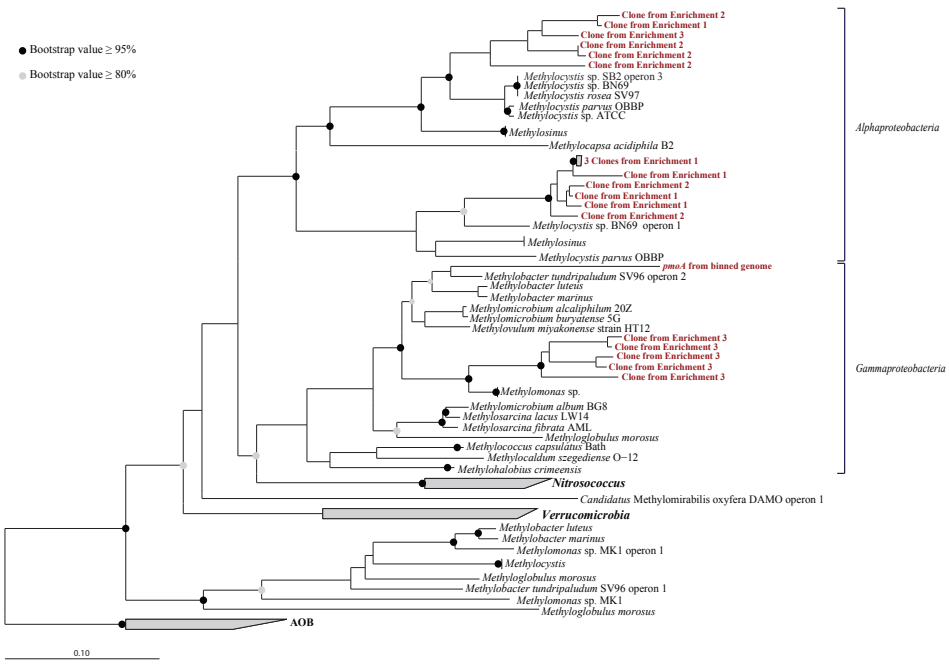


Figure 6: Representing various MOB *pmoA* sequences including the sequences obtained in this study. The tree was constructed using ARB's PHYML (amino Acids) tool within the maximum likelihood method. Due to size limitation, the tree is partially collapsed for an easier illustration and the outgroup is shown by a line. The tree was rooted using AOB sequences that are omitted from this figure. 100 bootstraps were performed and the ranges of values are shown with the respective colored circles at each node. Clone sequences are highlighted in red. Gammaproteobacteria, and Alphaproteobacteria, are distinguished in the figure.

DISCUSSION

Microcosms are an excellent tool to study methane cycling in wetland environments (Murase & Frenzel, 2007). Previous studies using this tool focused on unraveling the methane-driven microbial food web, without attempting to enrich novel methanotrophic bacteria. In this study, we report on a 90 day incubation of paddy field soil slurries in a microcosm setup, which allowed the successful pre-enrichment of novel methanotrophs. Combined with metagenomic sequencing, the microcosms furthermore enable the identification of the genomic potential of key methanotrophic players naturally occurring in soil, which can in principle be used for the design of optimized isolation strategies for yet uncultured methanotrophs.

The microcosms became immediately active after inoculation and remained active throughout the whole three month incubation period. This was achieved without the addition of medium to the soil slurries. CH_4 never accumulated in the top compartment. Therefore, the microcosms enabled us to maintain the complex food web existing in paddy soils for at least 90 days of incubation in the laboratory without loss of its high methane oxidation potential. Obtaining O_2 concentration profiles was a valuable and simple tool to follow changes in CH_4 oxidation activities, and thereby changes in the methanotrophic bacterial community. The oxic-anoxic interface shifted upwards from 3.2 mm after the first month of incubation, to 2.3 mm after three months (**Figure 1**), indicative of an increasing enrichment and activity levels of MOB.

After one month of incubation, the most abundant phylum based on 16S rRNA gene sequences were the *Proteobacteria* and their relative abundance did not vary significantly throughout the study. However, we did find that the soil slurries became more enriched in *Gammaproteobacteria* over time, with a concomitant decrease of *Alphaproteobacteria* (**Figure**). Two specific orders, the *Methylococcales* and *Myxococcales*, became highly enriched in the soil slurries in our incubations, pertaining to 48% and 84% of all the sequences within the gamma- and deltaproteobacterial classes, respectively (**Figures 3 and 4**). *Myxococcales* are known group of bacteria predominantly present in soil that feed on insoluble organic substances (Thomas *et al.*, 2008). While most members of the *Myxococcales* have been described as strict aerobes, isolates of the *Anaeromyxobacter* genus have been described to also be capable of growth under anoxic conditions by nitrate or iron(III) reduction (Treude, *et al.*, 2003).

Moreover, *pmoA* sequences extracted from the metagenome also demonstrated a change with incubation time in the methanotrophic community, in both top and bottom layers of the soil slurries. The resilience of methanotrophs to varying CH_4 and O_2 mixing ratios has been investigated, resulting in the enrichment of stable communities able to withstand these substrate fluctuations (Chidambarampadmavathy *et al.*, 2017). However, O_2 availability has been described to be a major driver for niche differentiation among methane oxidizing communities, with members of the *Methylosarcina* and *Methylophilus* genera being dominant at high (150 – 225 µM) and *Methylobacter* and *Methylotenera* at low O_2 tension (15 – 75 µM) (Hernandez *et al.*, 2015). In the current study, *Methylobacter* was found to be more dominant in the top compared

to the bottom soil layer (**Figure 5**). However, since already after two month of incubation O_2 was completely consumed at depth <2.5 mm, the 3 mm thick top soil layer will contain a mixture of methanotrophs adapted to high and low O_2 concentrations. In future studies, smaller-scale sampling and separation of the soil layers could yield more detailed results on the adaptation of different methanotrophic genera to various O_2 and CH_4 availabilities.

The metagenome also allowed us to perform genome binning of the most dominant methanotrophs in the system. This information was used to devise an isolation strategy to enrich novel methanotrophs. After three month of enrichment, 7% of all the 16S rRNA sequences extracted from the metagenomic dataset clustered with the *Methylococcales* order. This high abundance allowed us to bin out the corresponding genome with 75% completeness. The organism's closest relative based on *pmoA* and 16S rRNA gene sequences was *Methylobacter tundripaldum* SV96, a gammaproteobacterial methanotroph isolated from a soil core collected in a natural wetland, with optimal growth at 23°C, pH of 5.5 to 7.9 and optimal growth on 20% CH_4 in the headspace (Wartiainen *et al.*, 2006) (**Figure 6**). Similar parameters were used in further enrichment using diluted nitrate mineral salt (dNMS) media (Whittenbury et al., 1970). However, *pmoA* sequences related to this binned genome were not found in our consortia. Since single colonies were picked and transferred from agarose enrichments, this could explain why this organism was not found in our final enrichments.

In order to enrich for potentially novel methanotrophs, we used the second- and third-month pre-enriched soil slurries from the microcosms as inoculum, which contained several *pmoA* sequences of uncultured and unidentified methanotrophs. Due to sequencing errors and on average shorter assembled contigs, these *pmoA* sequences could not be added to the phylogenetic tree for a comparison to the clone sequences found in each of our enrichments. While classical isolation techniques tend to select for type II methanotrophs from paddy soil samples, our cultures successfully enriched previously uncultured type I and type II methanotrophs most closely related to *Methylomonas* and *Methylocystis*, respectively (**Figure 6**). In conclusion, we found that microcosms are a valuable tool for the pre-enrichment of novel, previously uncultured methanotrophic bacteria, as they are able to mimic the conditions found in wetland and paddy field soils and thus minimize cultivation biases.

CONCLUSION

The new microcosm set up in this study combined with molecular techniques allowed the long term incubation of paddy soil under near-natural conditions. Overall, our experiments provide support to previous findings that bacterial communities, and more specifically methanotrophs, play an important role in the formation of a counter-gradient between O_2 and CH_4 in flooded soils. Although type II methanotrophs have been reported to be the dominant group in paddy soils, we found that depending on the mixing ratios between CH_4 and O_2, type

I methanotrophs could also be a dominant member of the methanotrophic community. Further experiments are required to investigate in more detail MOB niche adaptation along O_2 and CH_4 gradients. Lastly, the current setup has great potential to be used as a pre-enrichment step prior to classical isolation methods as we were able to enrich methanotrophic consortia composed of previously uncultivated species.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURE

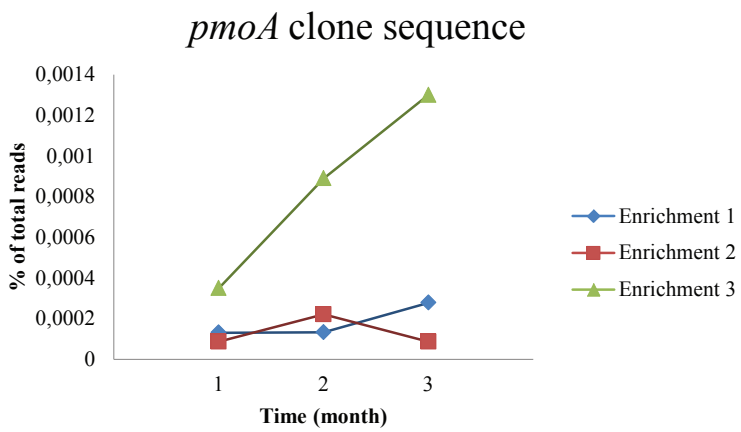

Figure S1: Relative abundance of the dominant *pmoA* clone from each of the enrichment consortia in the original metagenome after 1, 2 and 3 months of enrichment.

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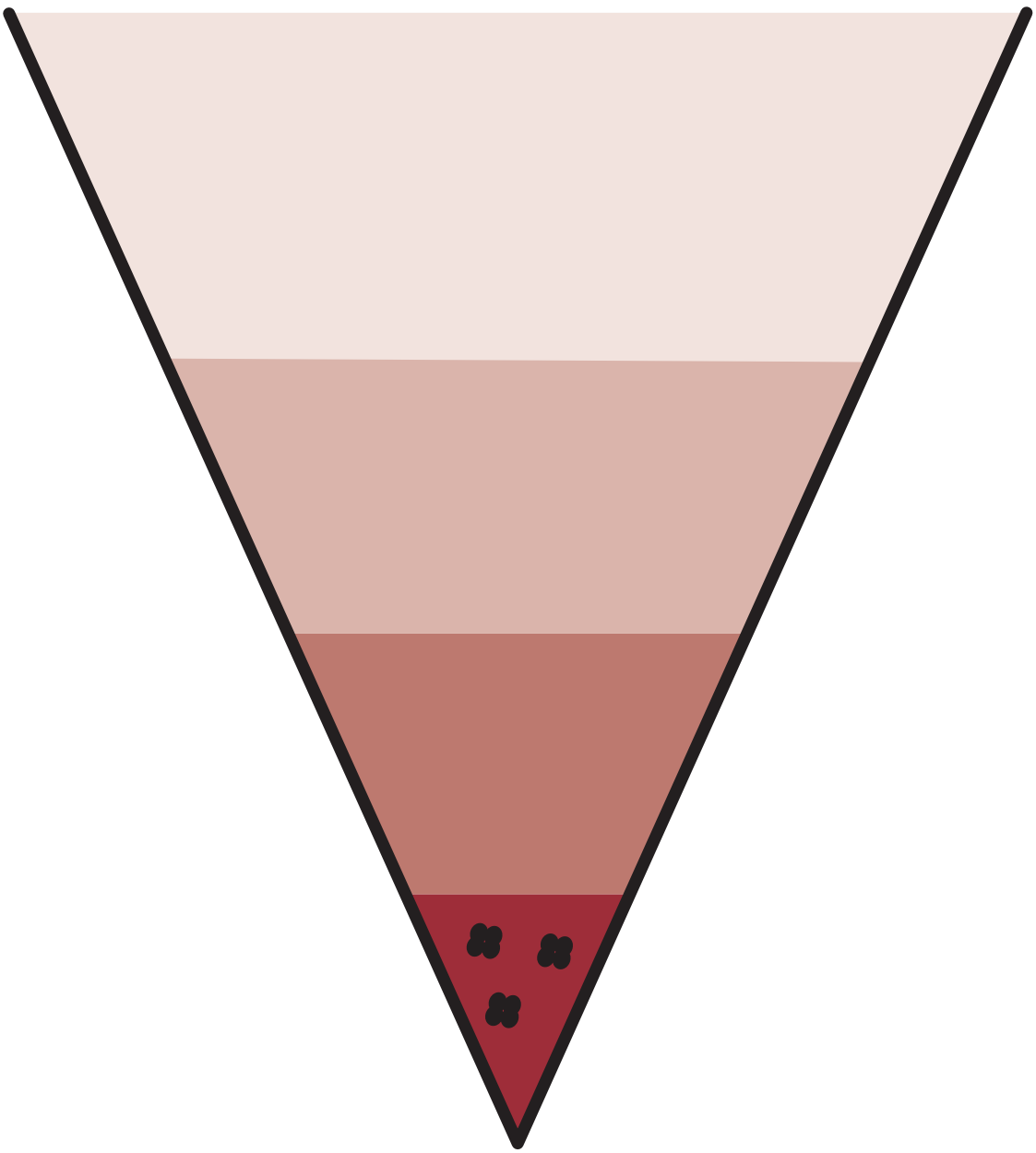
Keywords: methane, aerobic methane oxidation, paddy field, soil microbiome, comparative genomic

CHAPTER 5

A novel type Ib gammaproteobacterial methanotroph adapted to freshwater environments, *Methylotetracoccus oryzae* str. C50C1



ABSTRACT

Methane-oxidizing microorganisms perform an important role in reducing emissions of the greenhouse gas methane to the atmosphere. To date, known bacterial methanotrophs belong to the *Proteobacteria*, *Verrucomicrobia,* and NC10 phyla. Within the *Proteobacteria* phylum, they can be divided into type Ia, type Ib and type II methanotrophs. Type Ia and type II are well represented by isolates. Contrastingly, the vast majority of type Ib methanotrophs could not be cultivated so far. Here, we compared the distribution of type Ib lineages in different environments. Whereas the cultivated type Ib methanotrophs (*Methylococcus* and *Methylocaldum*) are found in landfill and upland soils, lineages that are not represented by isolates are mostly dominant in freshwater environments such as paddy fields and lake sediments. Thus, we observed a clear niche differentiation within type Ib methanotrophs. Our subsequent isolation attempts resulted in obtaining a pure culture of a novel type Ib methanotroph, tentatively named *Methylotetracoccus oryzae* C50C1. Strain C50C1 was further characterized to be an obligate methanotroph, containing $C_{16:1}\omega9c$ as the major membrane phospholipid fatty acid, which has not been found in other methanotrophs. Genome analysis of strain C50C1 showed the presence of two *pmoCAB* operon copies and XoxF5-type methanol dehydrogenase in addition to MxaFI. The genome also contained genes involved in nitrogen and sulfur cycling, but it remains to be demonstrated if and how these help this type Ib methanotroph to adapt to fluctuating environmental conditions in freshwater ecosystems.

IMPORTANCE

Most of the methane produced on our planet gets naturally oxidized by a group of methanotrophic microorganisms before it reaches the atmosphere. These microorganisms are able to oxidize methane, both aerobically and anaerobically, and use it as their sole energy source. Although methanotrophs have been studied for more than a century, there are still many unknown and uncultivated groups prevalent in various ecosystems. This study focused on the diversity and adaptation of aerobic methane-oxidizing bacteria in different environments by comparing their phenotypic and genotypic properties. We used lab-scale microcosms to create a counter-gradient of oxygen and methane for pre-enrichment, followed by classical isolation techniques to obtain methane-oxidizing bacteria from a freshwater environment. This resulted in the discovery and isolation of a novel methanotroph with interesting physiological and genomic properties that could possibly make this bacterium able to cope with fluctuating environmental conditions.

INTRODUCTION

Methanotrophs are a functional group of diverse Gram-negative bacteria that are defined by their ability to oxidize methane, which they utilize as source of carbon and energy (Hanson and Hanson, 1996; Trotsenko & Murrell, 2008; Semrau *et al.*, 2010). Since their discovery in 1906 by Soehngen, they are known to play a key role in the global methane cycle through the reduction of methane emissions to the atmosphere (Soehngen, 1906; Conrad, 2009; Chistoserdova, 2015). Aerobic methanotrophs utilize methane via a methane monooxygenase (MMO) that exists as soluble (sMMO) cytoplasmic and particulate (pMMO) membrane bound form, which both catalyse the first step of methane oxidation to methanol (Semrau *et al.*, 2010). Methane-oxidizing bacteria (MOB) are ubiquitous in nature and have been found in various environments where oxygen and methane are readily available (Bowman, 2006; Hanson & Hanson, 1996). While most grow best at moderate pH and temperature ranges, psychrophilic, thermophilic, alkaliphilic and acidophilic methanotrophs have been isolated as well (reviewed in Semrau *et al.*, 2010).

To date, the best studied methanotrophs belong to the proteobacterial classes *Alpha-* and *Gammaproteobacteria* (Bowman *et al.*, 1993; Semrau *et al.*, 2010), but MOB within the phyla *Verrucomicrobia* and NC10 (Sangwan *et al.*, 2005; Ettwig *et al.*, 2010; Op den Camp *et al.*, 2009) were recently discovered, expanding the phylogenetic diversity of MOB. Despite this diversity, MOB have remarkably similar methane oxidation pathways while incorporating different pathways for carbon fixation. Proteobacterial MOB utilize C1 compounds via the ribulose monophosphate (RuMP) or serine pathways (Trotsenko & Murrell, 2008; Whittenbury *et al.*, 1976), while verrucomicrobial MOB and NC10 bacteria use the Calvin cycle (Khadem *et al.*, 2011; Rasigraf *et al.*, 2012). After the extensive isolation and characterization of methanotrophs that took place in the 1970s, three 'types' of methanotrophs were defined (Whittenbury *et al.*, 1970; Trotsenko, 1976). The strains that incorporated carbon into biomass using the RuMP pathway, contained intracytoplasmic membranes as vesicular disks, and monounsaturated hexadecenoic (16:1) signature fatty acids were grouped under Type I. Type II strains differed from Type I by utilizing the serine pathway for carbon fixation, having intracytoplasmic membranes aligned along the periphery of the cell and monounsaturated octadecanoic acid (18:1) as major membrane lipid (Whittenbury *et al.*, 1976; Trotsenko, 1976).

In various studies, an additional group of methanotrophs has been described as type X (Whittenbury, 1981; Whittenbury & Dalton, 1981), defined originally based on genomic G+C content and intracytoplasmic membrane organisation. This group had characteristics that did not define them under one type, possessing the full RuMP pathway as well as ribulose-1,5-bisphosphate carboxylase indicative for the Calvin cycle, and at the time were considered to be adapted to higher temperatures. A combination of biochemical and molecular analyses, however, has revealed that type X strains should be reclassified under type I methanotrophs and this clade is now referred to as type Ib (Bowman *et al.*, 1993). Nonetheless, these classifications do not

encompass all isolates, with some having unexpected characteristics. For instance, a type II strain possessing signature membrane lipids that resemble type I methanotrophs (Dedysh *et al.*, 2007), and *Methylothermus thermalis*, a gammaproteobacterium that possesses both 16:0 and 18:1 fatty acids typical for type I and II methanotrophs, respectively (Tsubota *et al.*, 2005), have been reported.

Within the last 20 years, the genera containing MOB within the *Proteobacteria* have expanded to 23 (Dedysh & Knief, 2018 and the references therein). With the exception of low pH peat-adapted *Methylocella* (Dedysh *et al.*, 2000) and *Methyloferula* (Vorobev *et al.*, 2011) that only possess sMMO, all other known methanotrophs encode a pMMO (McDonald *et al.*, 1997). The genes for pMMO (*pmoCAB*), but mainly *pmoA* encoding pMMO subunit A, have been used to survey the MOB diversity in various ecosystems (Knief, 2015; Ghashghavi *et al.*, 2017; Sengupta & Dick, 2017). These studies have shown remarkable environmental diversity, even within the comparably well studied proteobacterial clades. Although within the *Gammaproteobacteria* there have been 12 genera for both type Ia and Ib that contain cultivars, isolates are lacking for the many uncultivated environmental sequence clusters (Semrau *et al.*, 2010).

Type Ib methanotrophs are known to possess a high metabolic diversity (Wise *et al.*, 1999; Madigan *et al.*, 2017). However, this diversity is still to be fully explored due to the many clades of environmental sequences lacking any isolate. These sequences cover a vast variety of natural habitats such as peat, upland and wetland soil, hot springs, lakes, rivers, ground water, and deep sea, potentially representing highly diverse metabolic capabilities (Hanson & Hanson, 1996; Murrell, 2010; Kip *et al.*, 2010). The presence of multiple pathways for carbon and nitrogen fixation and assimilation and of both soluble and particulate MMOs make it difficult to generalize when discussing physiological abilities of type Ib methanotrophs or any other type of MOB.

Methylococcus capsulatus is the only well-described type Ib organism, which has since become the model organism for the entire group (Ward *et al.*, 2004). However, sequences from this group are mostly found in upland soil (Knief *et al.*, 2003). Presently, most known type Ib organisms seem to occur in freshwater environments, but only few isolates have been described. These have a tendency to live very close to a methane source and under oxygen-limited conditions (Islam *et al.*, 2015; Danilova *et al.*, 2016). In this study, we isolated a novel type Ib methanotroph, tentatively named *Methylotetracoccus oryzae* strain C50C1, from a freshwater ecosystem and performed physiological and genomic characterization. Based on observations from electron microscopy and sequence analyses it belongs to a novel genus that is widely distributed in paddy fields and lake ecosystems, making it a potential model representative for this group. We furthermore compared different physiological aspects of this isolate (habitat distribution, optimum growth temperature and pH, and key enzymatic activities) to the other known isolates within the type Ib methanotrophs.

MATERIALS AND METHODS

Enrichment conditions and isolation approach

Enrichments of methane oxidizing bacteria were started from a paddy field soil sample in Cixi, Zhejiang Province, China (N 30°11.066'; E 121°21.351'). Soil characteristics and sampling procedure are described in detail elsewhere (Ho *et al.*, 2011). Pre-enrichment was carried out for 14 days in gradient microcosms supplied with 15% methane from the bottom compartment and ambient air from the top (Murase & Frenzel, 2007). After pre-incubation, the soil was harvested, diluted in NMS medium (Whittenbury *et al.*, 1981) and plated onto solid NMS medium containing 2% agarose. Plates were incubated in air-tight jars supplemented with ambient air and 20% methane. Selected colonies were streaked onto fresh plates to obtain single colonies. The latter, however, were composed not only of methanotrophic bacteria but also of satellite heterotrophic microorganisms. Selected colonies that contained a lowest number of satellite cells were picked and used to inoculate 30 ml serum vials containing 10 ml of two-fold-dilute NMS medium. After inoculation, the vials were sealed with rubber septa and methane was added aseptically to attain a final mixing ratio of approximately 20% (v/v). The inoculated vials were then incubated at 24°C and 100 rpm. The cultures were examined by phase-contrast microscopy and, if morphologically uniform, the cells were transferred to fresh medium and grown again under the same growth conditions. This process of serial dilutions was repeated over 6 months until the target isolate, designated strain C50C1 was obtained in a pure culture. Once isolated, this methanotroph was maintained in two-fold dilute NMS medium and was sub-cultured in 4 week intervals.

Phase contrast and electron microscopy

Morphological observations and cell-size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss, Jena, Germany). Cells morphology was examined by using batch cultures grown to the early-exponential, late-exponential and stationary growth phases. For preparation of ultrathin sections, cells of the exponentially growing culture of strain C50C1 were collected by centrifugation and pre-fixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4°C and then fixed with 1% (w/v) OsO_4 in the same buffer for 4 h at 20°C. After dehydration in an ethanol series, the samples were embedded into Epon 812 epoxy resin. Thin sections were cut on an LKB-4800 microtome (LKB-Produkter AB, Stockholm, Sweden) and stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol. The specimen samples were examined with a JEM-100B transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Growth experiments

Physiological tests were performed in liquid two-fold dilute NMS medium with methane. Growth of strain C50C1 was monitored by measuring OD_{600} for 2 weeks under a variety of

conditions, including temperatures of 2-37°C, pH 4.0-8.5 and NaCl concentrations of 0-4.0% (w/v). Variations in the pH were achieved by mixing 0.1 M solutions of H_3PO_4, KH_2PO_4, K_2HPO_4, and K_3PO_4. The utilization of potential carbon sources was examined using 0.05% (w/v) concentrations of the following compounds: formate, glucose, sucrose, galactose, lactose, fructose, citrate, succinate, pyruvate, acetate, and ethanol. The ability to grow on methanol was tested in NMS medium containing 0.01–5% (v/v) methanol.

Nitrogen fixation activity was assessed by monitoring growth in nitrogen free medium. Incubations were performed in batch in triplicates. Bottles of 120 ml were sterilized and aseptically supplied with 17 ml of liquid five-fold dilute sterilized ammonium mineral salts (AMS) medium or five-fold dilute nitrogen-free mineral salts medium (MS). The headspace contained either ambient or low O_2 atmosphere (2% v/v). Low O_2 concentrations in the headspace were achieved by 5 rounds of applying vacuum to the bottles, followed by flushing with N_2/CO_2 (90/10% v/v). Subsequently, 2% O_2 (v/v) was added aseptically. All bottles received 10% CH_4 (v/v) aseptically. Prior to inoculation, biomass from 3 batch incubations pre-grown on five-fold diluted AMS, NMS and MS medium, respectively, to mid-exponential phase was pooled. Cells were washed twice to remove any remaining nitrogen source by pelleting the biomass in 50 ml tubes at 1000 × *g* for 10 minutes (Eppendorf 5810 centrifuge, Hamburg, Germany). Subsequently, the supernatant was removed and replaced with nitrogen-free five-fold dilute MS. Cells were dissolved in five-fold dilute MS medium. All bottles were inoculated with 3 ml of the washed cells at a starting OD_{600} of 0.05. OD_{600} was measured using a spectrophotometer (Spectronic200, Thermofisher Scientific, Waltham, MA, USA). The CH_4 concentrations in the headspace were measured by injection of 50 µl gas samples into a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, USA) equipped with a Porapaq Q 100/120 mesh (Sigma Aldrich, Saint Louis, USA) and a flame ionization detector (FID), O_2 concentrations using an Agilent 6890 series gas chromatograph coupled to a mass spectrometer (Agilent, Santa Clara, USA) equipped with a Porapak Q column heated at 80°C with Helium as the carrier gas as described previously (De Jong *et al.*, 2018).

Molecular analyses

Extraction, analysis and identification of phospholipid-derived fatty acids (PLFA), including DMDS-derivatization to determine double bond positions, was performed as described by Dedysh *et al.* (2007). DNA was extracted from 2 mL liquid culture using the PowerSoil DNA isolation Kit (MoBio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol. The genomic DNA was sequenced on the Illumina MiSeq platform, with MiSeq Reagent Kit v3 (600 cycles, yielding 2x 300 bp paired end sequencing reads; Life technologies, Carlsbad, USA). For genomic library preparation using the Nextera XT kit (Illumina, San Diego, USA.), in total 5 µl gDNA normalized to 0.2 ng/µl were used. Fragmentation was performed enzymatically, followed by incorporation of the indexing adapters and amplification of the library as described by the manufacturer. Purification of the amplified library was performed

using AMPure XP beads and quality and size distribution of the library was checked using the Agilent 2100 Bioanalyzer and the High sensitivity DNA kit (Agilent Technologies, Santa Clara, USA). Fluorimetric quantitation of the library was performed by Qubit using the dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA). For normalization of the library, the concentration measured by Qubit and the average fragment size obtained with the Agilent 2100 bioanalyzer were used. After dilution to 4 nM end concentration, the library was denatured and diluted using the Denature and Dilute Libraries Guide (Illumina, San Diego, USA), loaded in the cartridge and the sequence run was started using the Illumina Miseq platform (Illumina, San Diego, USA).

Bioinformatic analysis

Illumina raw sequencing reads were imported into CLC Genomics Workbench (v11.0.2, Qiagen/CLCbio, Aarhus, Denmark) and trimmed on quality and length (≥100 bp), resulting in nearly 11.5 million reads which were used for subsequent analyses. Reads were assembled using CLC Genomics Workbench (assembly parameters: Word size=20, Bubble size=50, Minimum contig length=200; mapping parameters: Mismatch cost=2, Insertion cost=3, Deletion cost=3, Length fraction=0.5, Similarity fraction=0.8). As a slight contamination in the culture used for DNA extraction was observed, metagenomic binning was performed based on CG content and sequencing depth (Albertsen *et al.*, 2013). The assembled genome of strain C50C1 was composed of 42 contigs with an N50 of 199.476 bp, an overall genome size of 4.8 Mbp and an average GC content of 63%. Genome completeness and contamination were estimated by CheckM (Parks *et al.*, 2014) to be 99.1% and 3.3%, respectively. Binned contig sequences were submitted to the RAST automated annotation pipeline (Overbeek *et al.*, 2013), which includes genomic object prediction (CDSs and RNA genes), sequence homology searches, prediction of protein localization and reconstruction of metabolic networks. Subsequently, the annotation was refined manually and compared to publicly available genomes of aerobic MOB.

Data availability

The high quality draft genome of strain C50C1 is available at NCBI under BioProject accession number PRJNA361434.

RESULTS AND DISCUSSION

Isolation of a gammaproteobacterial methanotroph from paddy soil

Incubation of paddy field soil in a methane/oxygen counter-gradient microcosm and further purification of enriched bacteria on NMS medium resulted in three gammaproteobacterial methanotrophs that were classified as type Ib. One strain (referred to as strain C50C1) was further purified via several transfers in liquid NMS medium until a pure culture was obtained.

Strain C50C1 was represented by Gram-negative and non-motile cocci or coccoids (1.1-1.4 × 1.3-1.8 μm in size), which reproduced by binary fission and occurred singly, in pairs, in tetrads or formed large cell clusters in old (2 and more weeks) cultures (**Figure 1A-C**). Examination of thin-sectioned cells of strain C50C1 revealed a typical Gram-negative structure of the cell wall and the presence of intracytoplasmic membranes, arranged as stacks of vesicular disks (**Figure 1D**), which is characteristic of type I methanotrophs. Globular structures apparently representing an S-layer were observed on the cell surface (**Figure 1E**). Although the presence of S-layers is highly characteristic for many type I methanotrophs including *Methylococcus* species (Khmelenina et al., 2013), this type of S-layer symmetry has not been reported for any of the previously described methanotrophs.

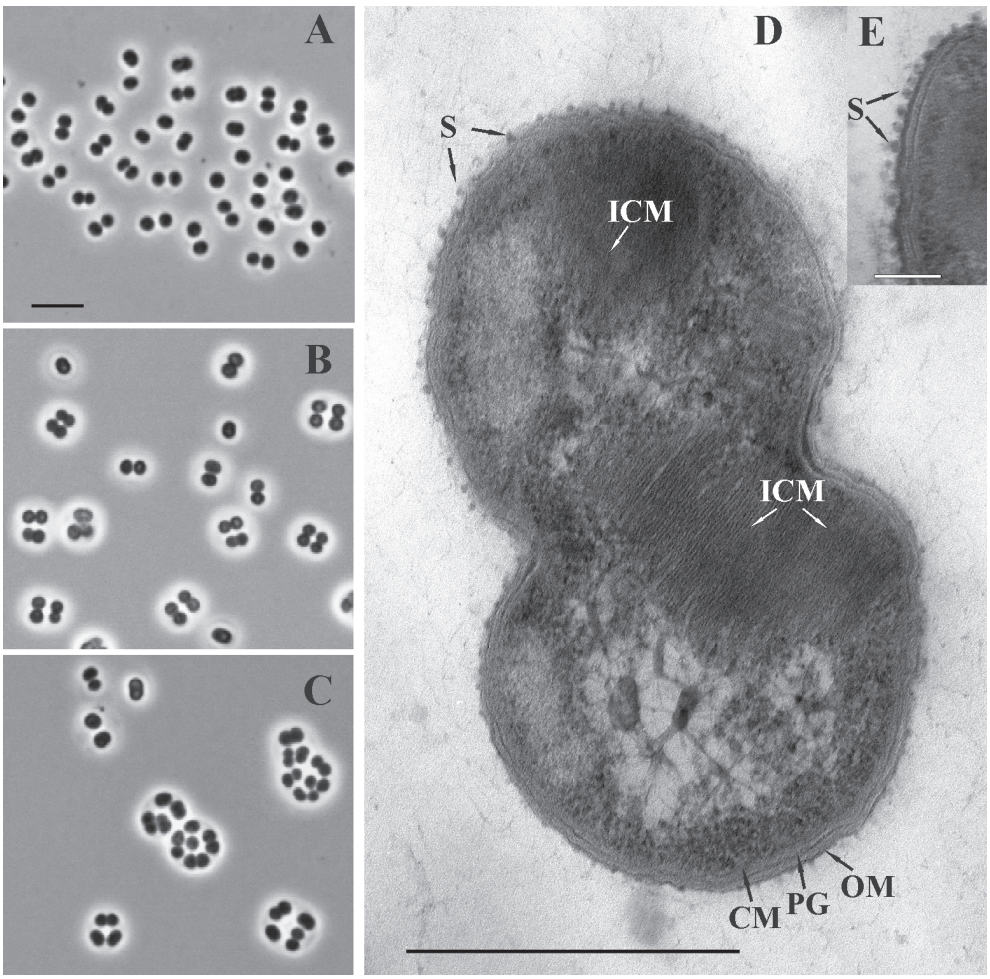


Figure 1: (**A**, **B**, **C**) Phase-contrast micrographs demonstrating cell morphology of strain C50C1 in 4-, 7- and 14-days old cultures. Bar, 5 μm. (**D**, **E**) Electron micrograph of an ultrathin section of a cell. ICM, intracytoplasmic membranes; CM, cytoplasmic membrane; OM, outer membrane; PG, peptidoglycan layer; S- S-layer. Bars, 0.5 μm (**D**) and 0.1 μm (**E**).

Strain C50C1 was able to grow only on methane and methanol. Methanol supported growth in the concentration range of 0.1-4% (v/v); highest growth rates (doubling time 21 h) occurred at 3% (v/v). No growth was observed on multicarbon compounds. Strain C50C1 grew in the pH range of 4.8-8.3, with the optimum at pH 6.8-7.5. The temperature range for growth was 4-30°C, with the optimum at 18-25°C. The doubling time on methane and methanol under optimal growth conditions was 16 and 21 hours, respectively. Strain C50C1 was highly sensitive to salt stress and growth was inhibited at NaCl concentrations above 0.3% (w/v).

Based on 16S rRNA and *pmoA* gene-based phylogeny, strain C50C1 could be classified as type Ib methanotroph affiliated with the Rice Paddy Cluster 1 (RPC1; **Figure 2**). RPC1 forms a monophyletic lineage, containing *pmoA* sequences that were mostly retrieved from freshwater environments such as lakes, groundwater and paddy fields (Lüke *et al.*, 2010; Lüke & Frenzel, 2011; Knief, 2015). So far, few members of type Ib methanotrophs have been characterized, resulting in the description of five genera. However, most clusters contain environmental sequences only and lack cultured representatives (**Figure 2**). Closest cultivated relatives of strain C50C1 include *Methylococcus capsulatus*, *Methylocaldum gracile* and *Methyloparacoccus murrellii* (94% 16S rRNA gene identity to each species and 92% amino acid identity to the *pmoA* gene of *M. capsulatus*).

Phenotype and growth characteristics of strain C50C1

We made a phenotypic comparison between strain C50C1 to other type Ib isolates (**Table 1**). C50C1 grows on methane and methanol as sole energy sources (**Table S1**), is able to fix N_2 (**Figure S1**), and grows at temperatures between 4 and 30°C, which is a much larger range than other characterized type Ib methanotrophs (**Table 1**). Similar to other MOB, it prefers pH values between 6 and 8, and is sensitive to 0.3% NaCl. Major phospholipid-derived fatty acids (PLFAs) in strain C50C1 are $C_{16:1}\omega9c$, $C_{16:1}\omega7c$, and $C_{16:0}$. $C_{16:1}\omega9c$ is highly unusual for type Ib methanotrophs, but low amounts have also been detected in *Methylogaea* and *Methyloparacoccus* (**Table 1**, **Table S2**). High amounts of this PLFA have so far been only detected in MOB belonging to *Alphaproteobacteria* (Lüke & Frenzel, 2011), and its presence in strain C50C1 gives it a specific signature. The recently described *Methyloterricola oryzae* belonging to the RPC1 possesses mainly $C_{16:0}$, $C_{16:1}\omega6c$ and $C_{16:1}\omega7c$, typical of type Ib methanotrophs (Frindte *et al.*, 2017). Based on the complete PLFA profile, however, C50C1 is most closely related to *Methyloterricola oryzae,* strengthening the placement in RCP1 (**Figure S2**). Furthermore, PmoA (**Figure 2**) and 16S rRNA gene-based phylogeny (**Figure 3)** both show a clear affiliation of strain C50C1 with the type Ib MOB.

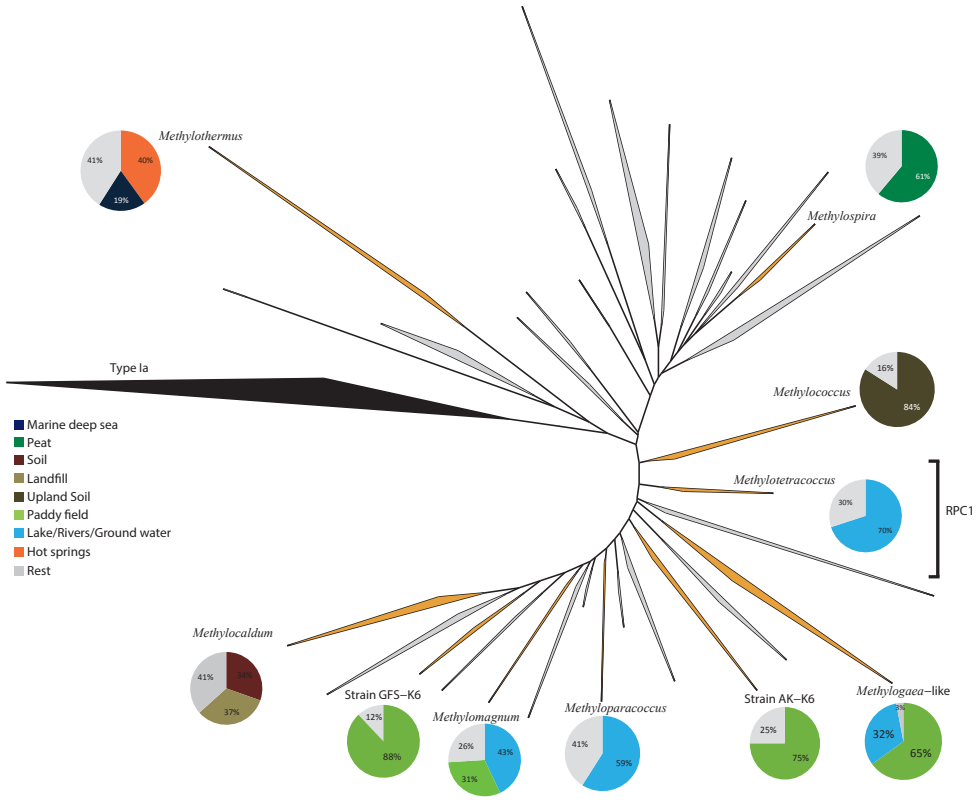


Figure 2: Phylogenetic inference of methane monooxygenase (PmoA) protein sequences of type Ib methanotrophs. The tree is constructed using ARB's neighbour joining method. Type Ia sequences were used as outgroup. Clades coloured in orange are represented by isolates, clades in grey by environmental sequences only. All clusters that contain isolates are accompanied by a pie chart with colours representing the environments the majority of sequences belong to. RPC, Rice Paddy Cluster 1. The bar indicates 0.1 substitutions per amino acid position.

Table 1: Comparison of strain C50C1 growth characteristics to other type Ib methanotrophs. 1: *Methylotetracoccus oryzae* C50C1 (this study), 2: *Methyloparacoccus* (2 strains), 3: *Methylococcus capsulatus* (2 strains), 4: *Methylocaldum* (4 strains), 5: strain GFS-K6, 6: *Methylogaea oryzae*, 7: *Methylomagnum*, 8: strain AK-K6, 9: *Methylospira* cluster. ND= no data.

	1	2	3	4	5	6	7	8	9
Isolation source	Rice field, Italy	Pond water, South Africa and Japan	thermal bath water, UK	Marine sediment	Terrestrial methane seep pond sediments, Bangladesh	Rice field, Uruguay	Rice fields, Bangladesh and Japan	Warm spring sediments , Armenia	Acidic sphagnum peat bog, Russia
PmoA cluster	Freshwater sediment-2; RPC-1	Freshwater sedment-2: RPCs	*Methylococcus*-like	*Methylocaldum*-like	*Methylococcaceae* family	JRP-4	*Methylococcus-Methylocaldum-Methyloparacoccus-Methylogaea* clade	*Methylococcaceae* family	OSC
Major habitat	Freshwater lake	Freshwater lake	Meadow/shrubs	Soil	Rhizosphere/ root	Paddy field	Lake sediment/soil	Paddy field	Peat
Growth temperature (°C)									
range	4-30	20–37	28–55	20-62	8-35	20–37 30–35	20-37	8-35	8-25
Optimum		25–33	37–50		25-28		31-33	25-28	14-25
pH									
range	6-8	5.8–9	5.5–9.0	5-9	5.0-7.5	5–8	5.5-9.0	5.0-7.5	4.2-6.0
Optimum		6.3–6.8	NR	6-8	6.4-7.0	6.5–6.8	6.8-7.4	6.4-7.0	6.0-6.5
Tolerence to 1% NaCl	no	no	yes	ND	no	no	no	no	no
Key enzyme activities									
sMMO	-	-	+	-	-	-	+	-	-
Nitrogenase	+	-	+	-	+	-	-	+	+
RubisCO	-	-	+	+	+	-	-	+	+
Cell morphology	Cocci	Cocci	Cocci-rods	Rods–pleomorphic	Rods	Curved rods	Rods	Rods	Curved rods (spiral)
motility	none	none	variable	+	none	none	+	none	+
Major fatty acids	C16:1ω9c, C16:1ω7c, and C16:0	C16:1ω7c	C16:0, C16:1ω7c	ND	C16:1ω7c	C16:0	C14:0, C16:0, C16:1ω7c*	C16:1ω7c	ND

Table 1 continued

Cell size (µm)	1.1-1.4 × 1.3-1.8	0.8-1.5	0.8-1.5 × 1.0-1.5	0.6-1.2 × 1.0-1.8	1.5-2.2 × 0.5-1.5	0.5-0.7 × 2.0-2.2	1,5-2.0 × 2.0-4.0	1.5-2.2 × 0.5-1.5	1.0-1.5 × 2.0-2.5
Pigmentation	White to brown	White	White to brown	Brown	White	White	White	White	ND
Cyst formation	–	–	+	+	–	–	+	–	–
Chain formation	+	–	+	+	–	–	–	–	–
DNA G+C content (mol%)	62.77	65.6	59-66	56.5-57.2	ND	63.1	64.1	ND	ND
Reference	Current study	Hoefman *et al.*, 2014	Data from Bowman *et al.*, 1993	Takeuchi *et al.*, 2014, Bodrossy *et al.*, 1997	Islam *et al.*, 2015	Geymonat *et al.*, 2011	Islam *et al.*, 2015, Khalifa *et al.*, 2015	Islam *et al.*, 2015	Danilova *et al.*, 2016

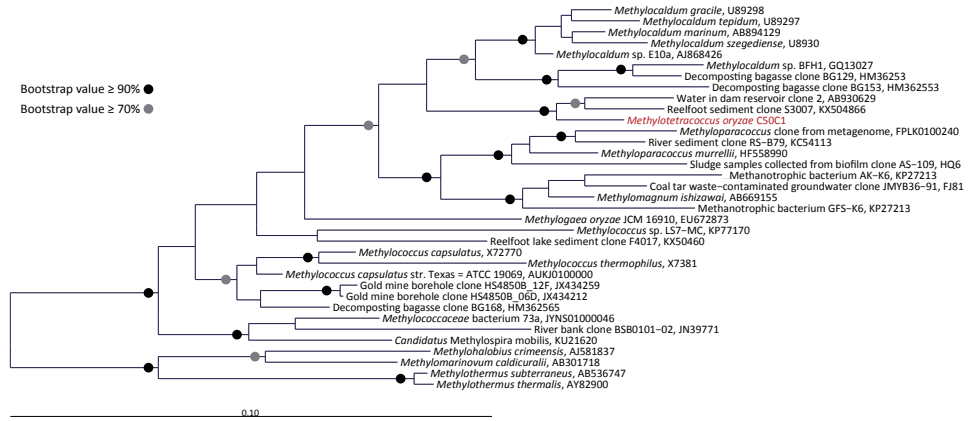


Figure 3: 16S rRNA gene-based phylogenetic analysis of a subgroup of closely related type Ib methanotrophs to strain C50C1 (in red), including isolates and environmental clones. Selected members of the *Methylothermaceae* were used to root the tree. Black and grey circles at the nodes indicate bootstrap support values ≥90% and ≥70%, respectively. The bar indicates 0.1 substitutions per amino acid position.

Diversity and ecological niches of type Ib methanotrophs

To gain an overview of diversity and habitat preference of cultivated and uncultivated type Ib methanotrophs, we performed a phylogenetic analysis of approximately 2800 publicly available *pmoA* sequences from various environments. We classified the habitat information into eight environmental categories and compared the *pmoA* diversity to the environmental origin of the sequences (**Figure 2**). Sequences could be grouped into 32 major sequence clusters. For a long time, only the genera *Methylococcus* and *Methylocaldum* were represented by isolates, however, recently several additional type Ib methanotrophs were obtained in pure culture (**Figure 2**, **Table 1** and the references within). *Methylomagnum*, *Methylogaea*, and strains SK-K6 and GFS-K6 all belong to clusters containing environmental sequences derived mainly from paddy fields. These isolates grow in similar pH ranges, but *Methylogaea* and *Methylomagnum* possess a slightly higher optimum growth temperature of 30-35°C.

Methyloparacoccus and the tentatively named *Methylotetracoccus* clades have most sequences derived from freshwater ecosystems. Since these strains have been isolated from similar environments, their growth parameters and genome-inferred physiological capabilities are highly similar. Contrastingly, both *Methylococcus* and *Methylocaldum* have been isolated from sources that differ from the major habitat of their respective sequence clade, based on environmental sequences. The former was isolated from a roman thermal bath, the latter from marine sediment (Bowman et al., 1993; Bodrossy et al., 1997; Takeuchi et al., 2014). Lastly, *Methylospira mobilis* appears to be an accurate representative for its clade of mainly peat-derived environmental clones, as it is adapted to acidic conditions (Danilova et al., 2016). Although type Ib MOB have shown

to be diverse with regards to their environmental adaptability, they seem to play a very minor role in marine ecosystems, where most sequences belong to type Ia.

Genome sequencing of strain C50C1

To gain further insights into the metabolic potential of strain C50C1, we sequenced and analysed its genome. Assembly and binning resulted in a 4.83 Mbp draft genome consisting of 42 contigs longer than 1 kb. Based on single copy marker gene analysis, the genome was predicted to be 99.1% complete, with 3.3% of contamination. The overall GC content is 63%. In total, the genome was predicted to contain 4302 protein coding sequences (CDSs) and one copy of the ribosomal RNA operon. Genome size and GC content are comparable to the four other sequenced type Ib methanotrophs, which range from 3.3 to 5 Mbp and 57 to 63%, respectively (**Table 2**). The rRNA operon copy numbers in bacterial genomes can vary from 1 to as many as 15 copies and a correlation of copy number with resource availability has been hypothesised (Klappenbach *et al.*, 2000). Most other type Ib genomes also harbour only one single copy, with the exception of *Methylococcus capsulatus* Bath that contains two (**Table 2**). Thus, MOB appear not to be in need of multiple rRNA copies for rapid adaptation to substrate availability, but this requires further analyses once more genomes of type Ib and other types of methanotrophs are sequenced.

Methane oxidation

Based on the genomic information, the metabolic pathways for methane oxidation and energy conservation in strain C50C1 were reconstructed (**Figure 4**). The genome includes two copies of the *pmoCAB* operon encoding the membrane bound pMMO and four additional copies of *pmoC*, which are scattered throughout the genome. However, none of the two *pmoCAB* operons encodes the high affinity pMMO-2 isoenzyme described in *Alphaproteobacteria*, which has been shown to be responsible for oxidation of methane at low mixing ratios (Baani and Liesack, 2008). Since the concentrations of CH_4 and O_2 that strain C50C1 would be exposed to in its natural environment are not comparable to the ones experienced by atmospheric methane oxidizers, possessing a high-affinity pMMO would not necessarily be an advantage in a wetland. Neither the distinct *pmoABC* operon encoding the so-called pXMO (Tavormina *et al.*, 2011), nor genes for the sMMO were identified in the genome, although the latter have been found in *Methylococcus capsulatus* (Bowman *et al.*, 1993) and in several *Methylomagnum* strains (Islam *et al.*, 2015, Khalifa *et al.*, 2015; **Table 1** and references therein). According to recent studies, sMMO seems not to play a role in methanotrophy in paddy fields, as it was found to be absent in all rice field isolates and PCR-based studies only detected *mmoX* genes related to *Methylocystis*/*Methylosinus* species (Reim *et al.*, 2012).

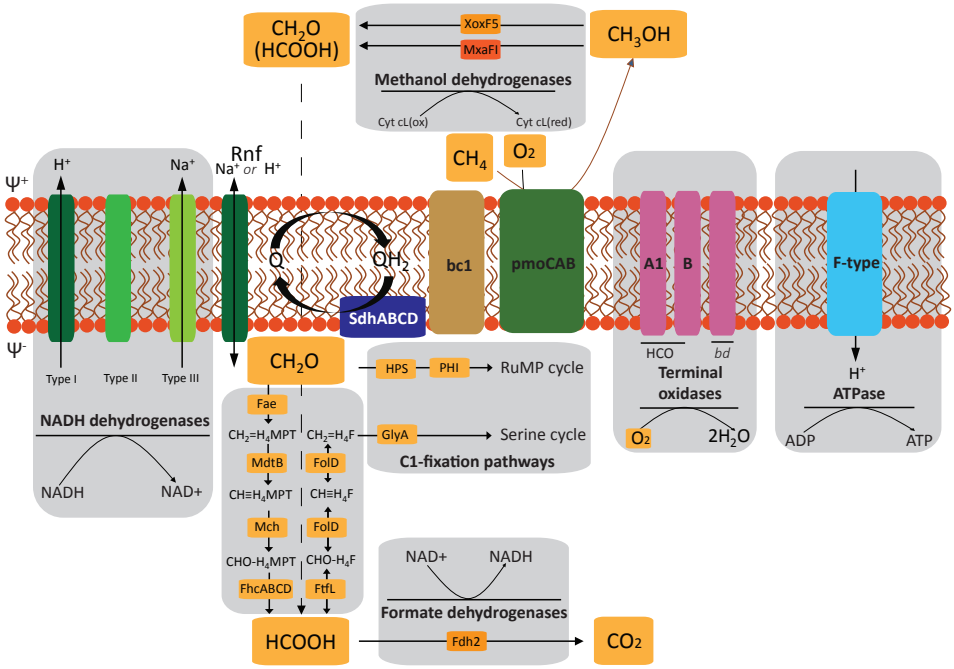


Figure 4: Predicted energy metabolism of strain C50C1. SdhABCD, Succinate dehydrogenase; Rnf, NAD-ferredoxin reductase; Q, quinone; bc1, cytochrome bc_1 complex; fae, Formaldehyde-activating enzyme; Mch, methenyl-H_4MPT cyclohydrolase; FhcABCD, Formyltransferase/hydrolase complex; FtfL, Formate-tetrahydrofolate ligase; FolD, Methylene-H_4F dehydrogenase/cyclohydrolase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase; GlyA, serine hydroxylmethyl transferase.

Methanol and Formaldehyde oxidation

For the subsequent oxidation of methanol to formaldehyde, the C50C1 genome encoded both the lanthanide-dependent XoxF5-type (Pol *et al.*, 2014; Martinez-Gomez *et al.*, 2016) and the calcium dependent MxaFI-type methanol dehydrogenase (MDH). The XoxF5-type MDH has been shown to have a higher affinity than MxaFI and, unlike the MxaFI-type enzyme, to directly convert methanol to formate in *Methylacidiphilum fumariolicum* SolV, which lacks a dedicated formaldehyde dehydrogenase (Keltjens *et al.*, 2014). However, XoxF-type enzymes also were shown to efficiently oxidize formaldehyde (Wilson et al. 2008). In accordance with the dependency of XoxF-type MDHs on pyrroloquinoline quinone (PQQ), strain C50C1 also encoded genes for PQQ biosynthesis. Electrons from the oxidation of methanol are transferred to cytochrome c_L, which serves as the primary electron acceptor for MDH. In the periplasm, cytochrome c_L is oxidized and the electrons end up at typical membrane-bound terminal oxidases by way of class I *c*-type cytochromes (Hanson & Hanson, 1996).

Most of the reducing equivalents required for the metabolism of methane are produced by the oxidation of formaldehyde (Hanson & Hanson, 1996; Trotsenko & Murrell, 2008). Formaldehyde is an important intermediate as it forms the branching point for anabolic carbon fixation via the serine or RuMP cycle, and catabolic substrate oxidation to CO_2. However, this compound also is highly toxic and its production and consumption consequently need to be tightly regulated (Attwood & Quayle, 1984).

A variety of enzymes have been shown to catalyse formaldehyde oxidation. Based on their electron acceptor, they can be grouped into NAD(P)$^+$-dependent and dye (cytochrome)-linked formaldehyde dehydrogenases (FalDH). Based on the genomic data, strain C50C1 possesses a homolog (74% amino acid identity) to a membrane-associated dye-linked PQQ-dependent FalDH putatively catalysing formaldehyde oxidation. This enzyme has been characterized in *Methylococcus capsulatus* Bath (Zahn *et al.*, 2001) and was shown to be a member of the Sulfide:quinone oxidoreductase enzyme family. Under high-copper growth conditions, this enzyme was found to be the major formaldehyde dehydrogenase. Additional homologs are present in *Methylocaldum* and *Methylohalobius*, however with much lower identity (≤ 40%) and potentially different functions within the Sulfide:quinone oxidoreductase family. C50C1 is lacking homologs of S-(hydroxymethyl) glutathione dehydrogenase (EC 1.1.1.284), which provides an alternative route from formaldehyde to formate in all other type Ib MOB.

Similar to other type Ib species, C50C1 has tetrahydrofolate (H_4F) and 5,6,7,8-tetrahydromethanopterin (H_4MPT)-linked C1 carrier pathways. H_4MPT is the archaeal analogue of H_4F and can transfer formyl-, methenyl-, methylene- and methyl-groups (Mashhadi *et al.*, 2010). These two pathways were regarded as redundant. However, more recent observations have shown that formate might be a branching point for anabolic and catabolic reactions making these two pathways function in parallel (Crowther *et al.*, 2008). The generation of methylene H_4F and its subsequent entry to the serine pathway is done through direct condensation of formaldehyde with H_4F. Alternatively, methylene H_4F can be formed from formate in the tetrahydromethanopterin pathway from H_4MPT. The latter seems to occur in a facultative methylotrophic, non-methane oxidizing *Methylobacterium* (Marx *et al.*, 2003), thus making it likely to occur in strain C50C1 as well. In contrast to *Methylobacterium*, C50C1 furthermore possesses FolD, a bifunctional methylene-H_4F dehydrogenase and methenyl-H_4F cyclohydrolase instead of the usual *mtdA*/*fch* gene pair encoding enzymes catalysing the separate reactions, respectively. In *Methylobacterium chloromethanicum* CM4, FolD has been shown to be specifically involved in dissimilation of the methyl-H_4F (Studer *et al.*, 2001). Although this process varies within MOB, all type Ib genomes analysed to date with the exception of strain C50C1 encode for the MtdA/Fch couple and lack FolD.

Formate oxidation

In *Methylococcus capsulatus* Bath and *Methylobacterium extorquens*, two isoenzymes have been characterized to be involved in formate oxidation (Dalton, 1979; Christoserdova *et al.*,

2004). The first of these formate dehydrogenases (FDH-1) has been characterized as a tungsten-containing enzyme in M. extorquens and is arranged in a fdhABC gene cluster (Chistoserdova et al., 2004). While this enzyme has been identified in Methylococcus capsulatus Bath and M. capsulatus Texas, it is not present in other type Ib species including strain C50C1. Contrastingly, the second FDH-2 is a molybdenum (Mo)-depending enzyme encoded by the fdhCBAD gene cluster. This enzyme is found in all other type Ib including strain C50C1 making it much more widespread than its tungsten-containing counterpart. In general, tungsten enzymes seem to be mostly present in anaerobic microbes, which could be a direct result of its availability and its higher redox properties relative to Mo in anoxic ecosystems (Kletzin & Adams, 1996). Functionally speaking, the two FDHs are virtually identical when their respective cofactor is present (Chistoserdova et al., 2004).

Energy conservation and respiration

The draft genome of strain C50C1 encodes a complete electron transport chain, including a proton or sodium ion-translocating NAD-ferredoxin reductase (Rnf) complex, NADH:ubiquinone reductases (H^+ and Na^+-transporting types; Complex I), succinate dehydrogenase (Complex II), cytochrome bc_1 complex (Complex III), quinone-reducing cytochrome *bd*-type and putatively cytochrome *c* reducing heme-copper (HCO; Complex IV) terminal oxidases and a F_oF_1-type ATPase (Complex V) (**Figure 4**).

The Rnf (*Rhodobacter* nitrogen fixation) complex is a novel ion-motive electron transport chain found in phylogenetically diverse prokaryotes. In *Acetobacterium woodii*, the Rnf complex catalyses oxidation of Fd_{red} with concomitant reduction of NAD^+ (Biegel *et al.*, 2011). The soluble B subunit (RnfB) of the complex is proposed to be the entry point for electrons from reduced ferredoxin. The C subunit (RnfC) mediates NADH reduction, thus serving as exit point of electrons. The free energy of this reaction is conserved in the electrogenic transport of protons or sodium ions across the membrane, thus establishing an electrochemical potential (Biegel *et al.*, 2011). The genomes of *Methylobacter* and *Methylotenera* encode for this complex as well (Hernandez *et al*, 2015). Complex I transfers electrons from NADH into the quinone pool, coupled to the translocation of four protons across the inner membrane, which further contributes to the formation of a proton motive force (*pmf*) that can be used to synthesise ATP by complex V. Complex II links the tricarboxylic acid (TCA) cycle to the respiratory chain by transferring the electrons derived from succinate oxidation into the quinone pool.

Previous studies have indicated that pMMO also is coupled to the electron transport chain at the level of quinone, with inhibitor studies providing additional evidence of this link (Zahn *et al.*, 2001 and references therein). The oxidation of methane by the pMMO requires the additional activation by oxygen. As one oxygen atom of O_2 is reduced to H_2O and the second is incorporated into methane to form methanol, this results in a net consumption of two electrons per methane oxidized. Electrons from the subsequent oxidations of methanol and formaldehyde either end up in a membrane bound class I *c*-type oxidase or directly enter the quinone pool, respectively. The

reduced quinol then transfers the electrons to the cytochrome bc_1 complex, where the reduction of cytochrome *c* is linked to formation of *pmf* via the so-called Q-cycle. Complex IV finally uses the electrons obtained from cytochrome *c* to reduce O_2 to H_2O. This reaction also is linked to active translocation of protons, thus contributing to *pmf*.

The genome of strain C50C1 contains all of the subunits of two members of the heme-copper oxidase (HCO) superfamily, encoding one A-family and one B-family terminal oxidase. B-family enzymes have been shown to be adapted to lower concentrations of oxygen than the A-family, resulting in a higher affinity for O_2 but fewer protons pumped per electron (Han *et al.*, 2011). Possession of both A- and B-family HCO types may allow strain C50C1 to respire using a wide range of oxygen concentrations. This is further supported by the presence of a cytochrome *bd* oxidase, a respiratory quinol:O_2 oxidoreductase with a very high O_2 affinity (Borisov *et al.*, 2011). However, enzymes of the *bd* oxidase family conserve less energy than HCOs, as they derive electrons for O_2 reduction directly from quinol and lack conserved channels for proton pumping, thus bypassing energy conservation at complexes III and IV (Han *et al.*, 2011; Borisov *et al.*, 2011).

C1 fixation, denitrification and sulfur metabolism

Fixation of carbon and subsequent assimilation of formaldehyde occurs through the RuMP pathway in strain C50C1, which is typical for type Ib methanotrophs. Additionally, strain C50C1 also encodes the serine cycle enzymes serine hydroxymethyl transferase (GlyA), phosphoenolpyruvate (PEP) carboxylase (Ppc), and malate dehydrogenase (Mdh). PEP carboxylase, which is a key enzyme of the serine cycle, is missing in both *Methylococcus* and *Methylocaldum* genera. The PEP carboxylase encoded by C50C1 belongs to the "non-regulated" group of PEP carboxylases (Anthony, 1982) with their activity not controlled by intermediates of the TCA cycle or glycolysis/gluconeogenesis (Newaz & Hersh, 1975). Whether these additional enzymes give strain C50C1 an advantage over other type Ib remains to be investigated. Furthermore, all the enzymes for gluconeogenesis, the TCA cycle, and the non-oxidative pentose phosphate pathway are encoded in strain C50C1's genome. In contrast to *Methylocaldum marinum* (Takeuchi *et al.*, 2014), *Methylococcus capsulatus* Bath (Bowman *et al.*, 1993) and strain GFS-K6 (Islam *et al.*, 2015), ribulose-1,5-bisphosphate carboxylase/oxygenase is not encoded in the genome of strain C50C1 (**Table 1**).

A possible side reaction of the pMMO in MOB is the oxidation of ammonia to hydroxylamine (NH_2OH). Subsequently, hydroxylamine is detoxified to produce nitrite and nitrous oxide (N_2O), apparently without linking this reaction to energy conservation (Campbell *et al.*, 2011). Strain C50C1 possesses genes encoding for cytochrome cd_1 nitrite reductase (NIR), a NnrS protein involved in response to nitric oxide (NO), NO reductase (NOR) and lastly a NnrU family protein required for NIR and NOR expression. However, hydroxylamine oxidoreductase (HAO) or hydroxylamine reductase is missing from the genome of strain C50C1. As in other MOB, no chemolithotrophic growth was observed on ammonium in strain C50C1 and the

apparent lack of hydroxylamine detoxifying enzymes might contribute to an inability to cope with nitrogen stress caused by nitrification intermediates. However, it has been reported that *M. denitrificans* strain FJG1 under extreme hypoxia couples CH_4 oxidation to nitrate reduction (Kits *et al.*, 2015), which can be an explanation for the presence of denitrification genes in strain C50C1.Similar to other methanotrophs such as *Methylosarcina lacus* and *Methylocaldum szegediense*, strain C50C1 possesses the full *soxYZ* operon for sulfur oxidation along with sulfite dehydrogenase SoxD and the sulfur oxidation molybdopterin protein SoxC. However, whether this genomic potential corresponds to an environmental relevance of strain C50C1 in the sulfur cycle remains to be investigated.

Description of *Methylotetracoccus* gen. nov.

Methylotetracoccus [Me.thy.lo.tet.ra.coc'cus]. N.L. n. methylum (from French me´thyle), the methyl group; N.L. pref. methylo, pertaining to the methyl radical; N.L. masc. subst. from Gr. adj. *tetra*, four; N.L. masc. n. coccus (from Gr.n. *kokkos*), a grain or berry; N.L. masc. n. *Methylotetracoccus*, referring to a methyl-using organism with tetrad-forming coccoid cells.
Gram-stain negative, non-motile cocci or coccoids, which reproduce by binary fission and occur singly, in pairs, in tetrads or form large cell clusters in old cultures. Cells contain intracytoplasmic membranes, arranged as stacks of vesicular disks. Strictly aerobic, neutrophilic, mesophilic and non-thermotolerant. Members of the genus are obligate utilizers of C1 compounds, such as methane and methanol. Methane is oxidized by pMMO, with sMMO and pXMO being absent. Cells are capable of dinitrogen fixation. The major PLFAs are $C_{16:1}\omega9c$, $C_{16:1}\omega7c$, and $C_{16:0}$. The most closely related genera are *Methyloparacoccus*, *Methylocaldum*, and *Methylomagnum* within the family *Methylococcaceae* in the class *Gammaproteobacteria*. Known habitats are freshwater ecosystems such as paddy fields and lake sediments.

Description of *Methylotetracoccus oryzae* sp. nov.

Methylotetracoccus oryzae (O'ryzae N.L. masc. adj. *oryzae*, pertaining to a paddy field). Description is as for the genus with the following amendments. Cells are 1.1-1.4 µm wide and 1.3-1.8 µm long. Growth occurs only on methane and methanol. Methanol supports growth in the range of concentrations 0.1- 4% (v/v); highest growth rates with specific generation times of 0.033 h^{-1} (doubling time 21 hours) are observed at 3% (v/v). Optimal growth occurs at 18-25°C and pH 6.8-7.5. Highly sensitive to salt stress; growth is inhibited at NaCl concentrations above 0.3% (w/v). The type strain C50C1^T was isolated from a paddy field in Cixi, Zhejiang province, China. The G+C content of the type strain is 63 mol% (genome sequence).

CONCLUSION

In this study we isolated a novel type Ib methanotroph that can serve as a representative organism for the type Ib freshwater lineage. We report the high-quality draft genome of strain C50C1, which can help designing further research to study the role of these MOB in the environment. Based on growth experiments along with genomic data, C50C1 seems to be an obligate methanotroph able to fix nitrogen. The draft genome indicates a potential for metabolic flexibility, with genetic modularity including multiple methanol dehydrogenases, several pathways for formaldehyde oxidation, all enzymes of one and several enzymes of another pathway for C1 fixation, and several terminal oxidases. These genomic potentials could allow strain C50C1 to adapt to various environmental conditions, as already seen in its growth temperature range. The potential for sulfur oxidation within strain C50C1 and its environmental relevance needs to be further investigated.

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SUPPLEMENTARY MATERIALS

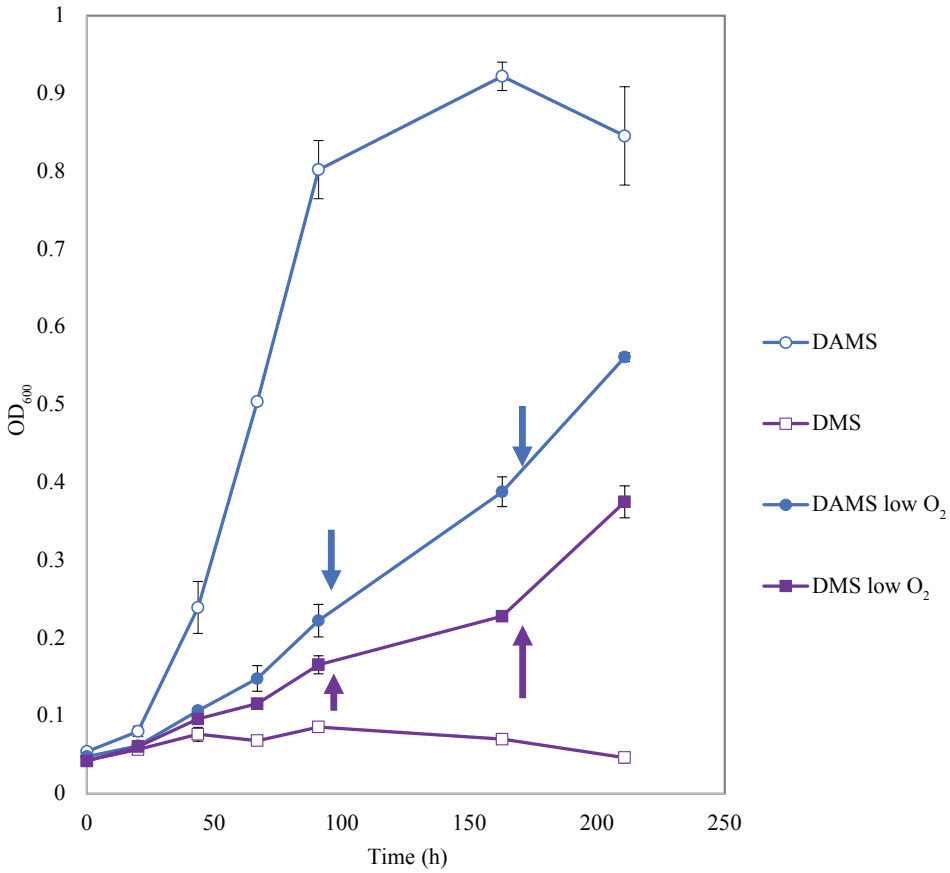


Figure S1: Growth dynamics of strain C50C1 in nitrogen-sufficient (DAMS;circles) or nitrogen-free (DMS;squares) medium under atmospheric (open symbols) and low (solid symbols) O_2 levels. Optical density was measured at 600 nm. Arrows represent O_2 replenishment.

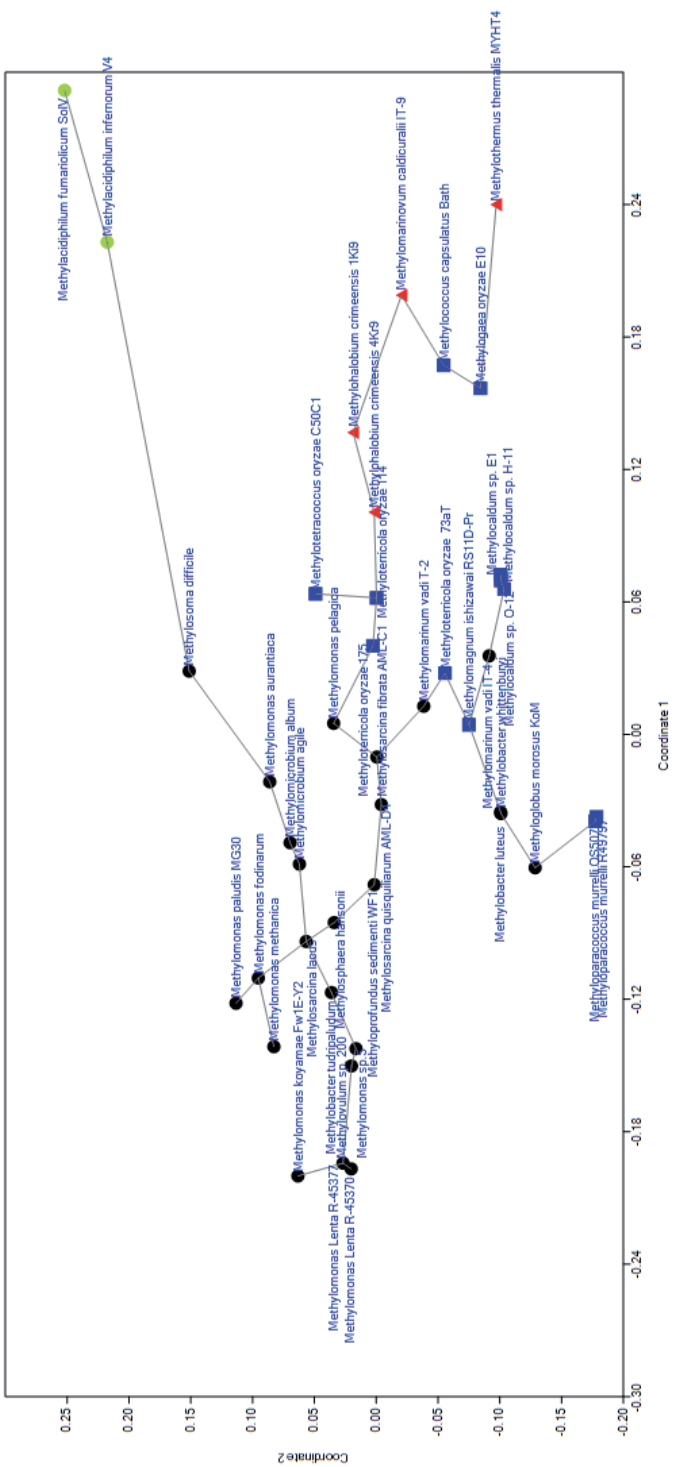


Figure S2: PLFA profiles of type I and verrucomicrobial MOB as compared by non-metric multi-scale dimensional scaling (NMDS) of PLFA profiles of methanotroph cultures (expressed as percentage of total PLFA content). The two-dimensional distances between samples in the NMDS plot show the relative similarity between samples. Closest matching PLFA profiles are indicated with a minimum spanning tree analysis displaying the shortest distance (i.e., similarity) to connect all PLFA profiles, resulting in relating to the nearest neighbor for every sample. The analyses was carried out with the software PAST. Black dots = type Ia; blue squares = type Ib; red triangles = *Methylothermaceae* (type Ic); green dots = *Methylacidiphilaceae* (type III).

Table S1: Substrate utilization pattern of strain C50C1. This bacterium can only utilize methane and methanol as energy source.

Substrate	**OD_{600} ***
Methane	1.10 ± 0.15
Methanol	0.09 ± 0.01
Acetate	0.03 ± 0.005
Pyruvate	0.04 ± 0.01
Succinate	0.03 ± 0.005
Malate	0.03 ± 0.005
Ethanol	0.04 ± 0.005
Glucose	0.03 ± 0.005
Fructose	0.03 ± 0.005
Sucrose	0.03 ± 0.005
Formate	0.03 ± 0.005
Formaldehyde	0.02 ± 0.01
control	0.03 ± 0.005

*OD_{600} = maximal optical density reached.

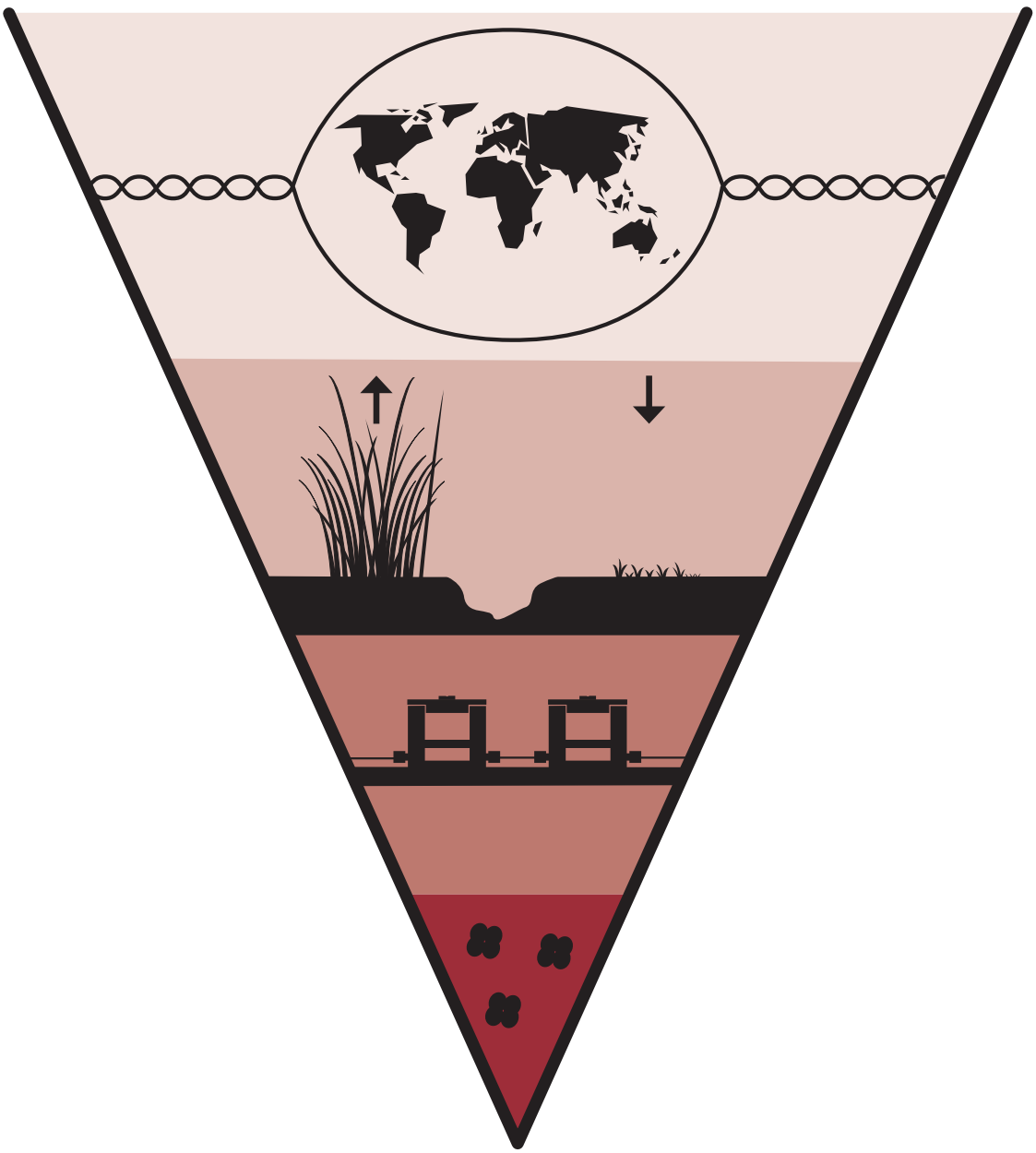
Table S2: Phospholipid Fatty Acid (PLFA) profiles of MOB strain C50C1. The values are the average of two technical replicates.

PLFA	% of total PLFA	
	Average	**SD***
C14:0	0.34	±0.01
C15:0	1.12	±0.06
C16:0	17.73	±0.77
C16:1w9t	3.91	±0.05
C16:1w9c	33.01	±2.66
C16:1w5t	0.19	±0.02
C16:1w7c	18.13	±14.82
C16:1w6c	8.67	±0.03
C16:1w5c	5.95	±0.27
C16:1	0.80	±0.02
C17:0	0.15	±0.00
C17:0	0.26	±0.03
C17:1w8c	0.16	±0.01
C18:1	0.11	±0.02
C18:0	0.53	±0.00
C18:1w7c	0.93	±0.18

*SD = standard deviation.

CHAPTER 6

Integration and Outlook



Methane (CH_4), the most reduced carbon compound in the atmosphere, is considered to be a potent greenhouse gas along with water vapour and CO_2 (Intergovernmental Panel on Climate Change 2015). It is produced in many natural and anthropogenic ecosystems that are vulnerable to climate change, making predictions for future shifts in CH_4 highly uncertain. Wetlands are considered to be major sources of CH_4 and are predicted to form the majority of the CH_4 in our atmosphere by 2100 (McNorton *et al.*, 2016). Increased CH_4 emissions from these ecosystems would in turn have an exacerbating climate feedback, resulting in further warming of our planet. Multiple factors, however, such as physical, geochemical and microbial inter-linkages are involved in this process complicating the understanding of it (Rigby *et al.*, 2017; Turner *et al.*, 2017). This thesis has focused on the involvement of aerobic methanotrophs as biofilter for the produced methane. The results of this thesis provide an outlook for research on the important role that aerobic methanotrophs play in mitigating methane emissions and thus global climate change.

Identification of methanotrophic diversity

Microorganisms are recognized as the engines behind Earth's biogeochemical cycles (Falkowski *et al.*, 2008). Specifically in the case of the global CH_4 cycle, microbes are involved in both production and consumption of this greenhouse gas. Most of the CH_4 produced gets oxidized by methanotrophs before it reaches the atmosphere in many environments (Semrau *et al.*, 2010; Dedysh & Knief, 2018 and references therein). Aerobic methane oxidizing bacteria (MOB) initiate CH_4 oxidation via an enzyme called methane monooxygenase (MMO), utilizing O_2 to activate CH_4 to methanol (Hanson & Hanson, 1996). Due to its ubiquity in MOB and high degree of conservation, the *pmoA* gene encoding for one of the subunits of this enzyme has been used as a biomarker for investigation of aerobic methanotrophy in the environment. Although methanotrophs have been found in various environments, including extreme habitats (Op den Camp *et al.*, 2009), their diversity and phylogeny have been expanding only in recent years.

Considering all the novel isolates and the recently obtained genomic information from these pure cultures, we wanted to introduce novel primer sets that can be used in future molecular studies to investigate MOB communities. In **Chapter 2**, we used publically available full MMO gene operons (*pmoCAB*) to design novel degenerate primers. Interestingly, *pmoB*, which is proposed to encode for the active site of the MMO protein, was the least conserved of all genes (Lieberman & Rosenzweig, 2005). Whether different MMO enzymes with similar affinities for CH_4 have a higher conservation in their *pmoB* subunit is a question that needs to be further investigated. In our experience, *pmoA* was still the most conserved gene of this operon. However, since the intergenic regions between the three genes were found to be highly variable in the methanotrophic lineages, this posed the possibility to target lineage specific sequence fragments. For this reason, we designed degenerate primers that target conserved regions in *pmoC* and *pmoA*, in positions flanking the intergenic region.

With the introduction of the new primers, future research of the diversity of methanotrophs in the environment can more robustly target these organisms. Furthermore, with the advancement in sequencing technology to produce longer reads, this primer set would be a great candidate for the generation of amplicon libraries. This would greatly aid in phylogenetic analysis of methanotrophs and expand our understanding of their diversity in various ecosystems.

CH_4 sources and sinks can be highly variable

As the human population on earth rises, our food production needs to increase to meet the demands. This translates to increasing transformation of wetlands into cultivated agricultural land (IPCC, 2013; Pearman, 1986; Dlugokencky *et al.*, 1994). To date, there have been many studies that focus on the effect of methanotrophs on CH_4 fluxes in either wetland or upland environments. These studies reach contradicting conclusions on various factors in soil (such as temperature, pH, fertilizing, O_2 availability, etc.) effecting the methanotrophic community with some showing a positive correlation and others having a negative one (see, i.e., Supplementary Table S1 in Chapter 3). What is not so often considered in the experimental design is the inclusion of both a sink and a source environment along with CH_4 flux data to investigate the methanotrophic community composition in both environments in more detail. This is what was done in Chapter 3 of this thesis to understand whether variations in CH_4 fluxes affect the establishment of native bacterial communities in soil.

Our findings suggest that one should exercise caution when making assumption that every cultivated wetland is a source and upland is a sink for CH_4. These fluxes can be highly variable, which could be influenced by multiple factors (such as season, sampling site, water temperature, etc.). Moreover, our results with regards to the methanotrophic diversity and relative abundance in a cultivated wetland were in consent with literature reports, observing a higher and more diverse MOB community in a cultivated wetland compared to a non-cultivated meadow. In future research, it would be highly relevant to follow possible shifts in the total bacterial community in soil when the soil is changed from an upland to a wetland used for cultivation. 'Soil transplant' experimental designs, which displace soil from one environment to another, would shed light on how and if bacterial communities change to be more consistent with the surrounding community. Lastly, spatial variations of methanotrophs need further investigation to deduce possible trends in the community based on location within a studied site.

Microcosm as a system to study methanotrophs

In **Chapter 4** of this thesis, we transplanted soil samples from a paddy field into lab-scale microcosms in order to investigate the role microbes play in the establishment of CH_4 and O_2 counter-gradients. This microcosm system has been used previously to illustrate that even a millimeter of soil can make a huge difference with regards to the community structure of methanotrophs in paddy fields (Reim *et al.*, 2012). We found that this system could be used for long term (3 month) incubation of soil to allow enrichment of slow growing methanotrophs. The

continuous monitoring of the enriched soil slurries together with metagenome sequencing revealed that the bacterial community was apparently driving the establishment of the O_2 profile.
Generally speaking, classical methods of isolation used to obtain pure cultures from paddy field samples result in type II methanotrophs being isolated. This is due to faster growth rates and reported higher abundance of type II methanotrophs compared to type I in this type of environment (Hanson & Hanson, 1996; Henckel *et al.*, 2000; Macalady *et al.*, 2002). An advantage of using microcosm systems in future isolation experiments is that a pre-enrichment with slow-growing methanotrophs that are present in lower relative abundance can be obtained. Therefore, the microcosm set-up opens up new opportunities to study previously uncultured lineages of methanotrophs that are highly prevalent in wetland ecosystems based on environmental sequences.

Importance and relevance of pure isolates

We are currently living in what can be called the "omics" era where many studies are designed to be culture-independent with vast amount of data recovered from oftentimes minute sample volumes. These metagenome and transcriptome studies, along with proteome and metabolome analysis have allowed us to gain great insights into how bacteria function in a community at different levels of the cellular machinery, both in the environment and engineered systems. While many ground-breaking discoveries have been made using such methods, many hypotheses have also been raised with regards to the function of specific organisms observed in those microbial consortia. Hence, cultivation, and isolate of pure cultures is necessary for the study of these specific physiological traits.
In **Chapter 5** of this thesis, various phenotypical and genotypical aspects of pure methanotrophic isolates were compared with focus on a novel type Ib isolate introduced in this chapter. The detailed comparisons of physiological properties described would not have been possible if the isolates were not obtained in pure cultures. Moreover, a closer look into the different isolates with regards to their physiology and genome analysis gives us more insights to the adaptability of different methanotrophs to the environment they are isolated from. In conclusion, this study illustrated that obtaining novel isolates will always be at of central importance in studying the role microbes play in various ecosystems.

Concluding remarks

More than a century of research on methanotrophs so far has undoubtedly proven them to be an intriguing group of microbes to study from an ecological, physiological, biochemical and application point of view. While these microorganisms come in a great variety of shapes and sizes with countless genomic potentials and physiological traits, they all share the process of CH_4 oxidation. This one crucial step in these microorganisms has an enormous global impact on the earth's carbon cycle. Judging by trends seen in recent years of increasing global temperatures and climate change, this specialized group of bacteria will only become more important for the future of our planet. Therefore, methanotrophs will undeniably be the subject of research tackling to unravel their global impact on earth's biogeochemical cycles.

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Mohammad Ghashghavi was born on September 21st, 1986 in Tehran, Iran. In 2005, he completed his secondary education at the International School of Helsinki, in Finland, obtaining an International Baccalaureate (IB) diploma. In September of 2005 he moved to Canada where he started his Bachelors' degree at the University of Alberta (U of A). He majored in Biological Sciences and minored in Psychology. After finishing his bachelors' degree in 2010, he moved to Portugal where he started his Masters' in Applied Microbiology at the University of Lisbon. In 2012, he transferred back to the U of A, where he obtained his Master's degree in Microbiology and Biotechnology. During this time, he was recognized and granted several awards as an outstanding graduate Teaching Assistant. Shortly after his MSc defense in August of 2014, he moved to the Netherlands where he started his PhD project at the Microbiology department at Radboud University. The findings of his PhD work resulted in this thesis.