ANAEROBIC OXIDATION OF METHANE IN ITALIAN PADDY FIELDS

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Outline of the thesis

The aim of this thesis is to obtain a better understanding of the diversity, distribution and role of microorganisms mediating anaerobic oxidation of methane in paddy fields, with an emphasis on the recently discovered Methanoperedens archaea.

In **Chapter 1**, a general introduction into the topic of the PhD thesis is provided.

The initial feasibility studies to detect nitrate-dependent anaerobic oxidation of methane (N-AOM) microorganisms in Italian paddy fields are described in **Chapter 2**. The chapter describes the abundance and distribution of two anaerobic oxidation of methane (AOM) microorganisms: '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria '*Candidatus* Methylomirabilis oxyfera' in bulk soil and rhizosphere of a paddy field. Community analysis based on 16S rRNA gene amplicon sequencing and qPCR of the water-logged soil and the rhizosphere showed that AOM-associated archaea (AAA), including '*Candidatus* M. nitroreducens', comprised 9% (bulk soil) and 1% (rhizosphere) of all archaeal reads. The NC10 phylum bacteria made up less than 1% of all bacterial sequences. In addition to detecting the N-AOM microorganisms, we showed with slurry incubations amended with nitrate and ^{13}C-CH_4, that this paddy field soil has significant activity for N-AOM (79.9. nmol g^{-1}_{dw} d^{-1}). The qPCR with newly developed 16S rRNA gene primers demonstrated that AAA ranged from 0.28 to 3.9 x 10^6 copies g^{-1}_{dw} in bulk soil and 0.27 to 2.8 x 10^6 in the rhizosphere. The abundance of NC10 phylum bacteria was an order of magnitude lower. Prior to this study, sequences of '*Candidatus* M. nitroreducens' were detected but their abundance had not been linked to nitrate dependent AOM in paddy fields. This chapter was published as '**Distribution and activity of the anaerobic methanotrophic community in a nitrogen-fertilized Italian paddy soil**' in FEMS Microbial Ecology 2016.

After the characterization and genome assembly of the first characterized '*Candidatus* M. nitroreducens', published in 2013, specific molecular detection tool could be designed to quantitatively and sensitively detect methyl-coenzyme M reductase (*mcrA*) gene sequences of this important N-AOM archaeon. **Chapter 3** reports the design of two novel *mcrA* primer combinations that specifically target the *mcrA* gene of '*Candidatus* M. nitroreducens'. The first primer pair results in fragments of 186bp which can be used to quantify '*Candidatus* M. nitroreducens' cells, while the second primer pair yields an 1191bp amplicon that is well suited for detailed phylogenetic analysis. Six different environmental samples were tested with the new qPCR primer pair, and abundances were compared with 16S rRNA gene primers. The qPCR results indicated that highest numbers of '*Candidatus* M. nitroreducens' *mcrA* gene copies were found in rice field soil with 5.6±0.8*10^6 copies g^{-1} wet weight, while Indonesian river sediment had only 4.6±2.7*10^2 copies g^{-1} wet weight. Besides freshwater environments,

sequences were also detected in marine sediment of the North Sea, which contained about 2.5±0.7*10^4 copies g^{-1} wet weight. Phylogenetic analysis of the amplified 1191bp *mcrA* gene sequences from the different environments indicated that all clustered together with available genome sequences of *mcrA* of known '*Candidatus* M. nitroreducens' archaea. The research of this chapter resulted in the article '***McrA* primers for detection and quantification of the anaerobic archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens**' in Applied Microbiology and Biotechnology 2017.

After developing a functional gene-based molecular tool and having shown that '*Candidatus* M. nitroreducens' is present in many ecosystems and highly abundant in Italian paddy field bulk soil, we set out to investigate the stratification patterns of microbial community and AOM potential in the same paddy field. **Chapter 4** focuses on microbial diversity and methane metabolism in the upper 60 cm of a paddy soil by qPCR, 16S rRNA gene amplicon sequencing and anoxic ^{13}C-CH_4 activity tests with nitrate, nitrite and iron as electron acceptors. The bacterial community consisted mainly of *Acidobacteria, Chloroflexi, Proteobacteria, Planctomycetes* and *Actinobacteria*. Among archaea, *Euryarchaeota* and *Bathyarchaeota* dominated over *Thaumarchaeota* in the upper 30 cm of the soil. *Bathyarchaeota* constituted up to 45% of the total archaeal reads in the top 5 cm. In the methanogenic community, *Methanosaeta* were generally more abundant than the versatile *Methanosarcina*. The measured maximum methane production rate was 444 nmol $g_{dw}h^{-1}$. qPCR confirmed the results of chapter 2, and throughout the soil core '*Candidatus* M. nitroreducens' was more abundant than NC10 phylum bacteria at all depths, except at 60cm where both were very low in abundance. We characterized the AOM potential with a suite of electron acceptors, with maximum rates of nitrate-, nitrite- and iron-dependent anaerobic oxidation of methane of 57, 55 and 56 nmol $g_{dw}h^{-1}$, respectively, at different depths. These results demonstrated that there is substantial AOM potential in fertilized paddy fields, with '*Candidatus* M. nitroreducens' archaea as the main contributor, possibly not only with nitrate but as well as with iron as electron acceptor. This chapter was published as '**Stratification and diversity of methane-oxidizing microorganisms in a nitrogen-fertilized Italian paddy soil**' in Frontiers in Microbiology in 2017.

However it is crucial to assess environmental distribution of AOM microorganisms with culture-independent methods and link it to the methane oxidation activity of environmental samples, the in-depth understanding of the full-potential of '*Candidatus* M. nitroreducens' can only be described by culturing these' microorganism in the laboratory. **Chapter 5** describes the first AOM enrichment, using paddy field soil with significant amounts of '*Candidatus* M. nitroreducens', as inoculum. After two years of enrichment with continuous supply of methane and nitrate as the sole electron donor and acceptor, a stable enrichment dominated by 'Candidatus M. nitroreducens' archaea and NC10 phylum bacteria was achieved. The results of qPCR quantification of 16S rRNA gene copies, analysis of metagenomic 16S rRNA reads,

and fluorescence in situ hybridization (FISH) correlated well and showed that, after two years, '*Candidatus* M. nitroreducens' had the highest ($2.2\pm0.4*10^8$) 16S rRNA copies per mL and constituted approximately 22% of the total microbial community. Whole-reactor anaerobic activity measurements with methane and nitrate revealed an average methane oxidation rate of 0.012 mmol h^{-1} L^{-1}, with cell-specific methane oxidation rates up to 0.57 fmol $cell^{-1}$ day^{-1} for '*Candidatus* M. nitroreducens'. Phylogenetic analysis showed that the 16S rRNA genes of the dominant microorganisms clustered with previously described '*Candidatus* Methanoperedens nitroreducens ANME2D' (96% identity). The diagnostic *mcrA* gene was 96% similar to 'Candidatus M. nitroreducens ANME2D' (WP_048089615.1) at the protein level. The pooled metagenomic sequences resulted in a high-quality draft genome assembly of '*Candidatus* Methanoperedens nitroreducens Vercelli'. This chapter resulted in the article '**Enrichment of anaerobic nitrate-dependent methanotrophic '*Candidatus* Methanoperedens nitroreducens' archaea from an Italian paddy field soil**' published in Applied Microbiology and Biotechnology in 2017.

Together, the chapters of this thesis show that '*Candidatus* Methanoperedens nitroreducens' archaea are very abundant in fertilized water-logged paddy fields soils, and that these archaea could contribute significantly to the anaerobic oxidation of methane in these soils. The established enrichment culture could be used in the future to investigate the metabolic diversity of '*Candidatus* Methanoperedens nitroreducens'.

Samenvatting

Het doel van dit proefschrift was het verkrijgen van een beter inzicht in de diversiteit, de verspreiding en de rol van micro-organismen die anaëroob methaan kunnen oxideren in rijstvelden, met nadruk op de onlangs ontdekte *Methanoperedens* archaea.

Hoofdstuk 1 bestaat uit een algemene inleiding over diverse aspecten van de methaanmicrobiologie in rijstvelden. Het onderzoek naar de mogelijkheid om stikstof-afhankelijke anaërobe methaanoxiderende (N-AOM) micro-organismen in Italiaanse rijstvelden te detecteren, wordt beschreven in **Hoofdstuk 2**. Hierbij wordt ingegaan op de aanwezigheid en verspreiding van twee AOM micro-organismen, *'Candidatus* Methanoperedens nitroreducens' en de NC10 phylum bacterie *'Candidatus* Methylomirabilis oxyfera', in zowel de rhizosfeer en als de bodem van een rijstveld. De microbiële levensgemeenschap in de met water verzadigde bodem en de rhizosfeer, werd geanalyseerd met behulp van 16S rRNA gen amplion sequencing en qPCR. Deze analyse toonde aan dat in waterverzadigde bodem 9% en in de rhizosfeer 1% van alle archaeale reads afkomstig waren van de groep archaea waartoe ook *'Candidatus* M. nitroreducens' behoort. Van de geanalyseerde bacteriële sequenties behoorde slechts minder dan 1% tot het NC10 phylum. Naast het detecteren van de N-AOM micro-organismen, lieten wij met behulp van bodem-incubaties, waaraan nitraat en $^{13}C\text{-}CH_4$ was toegevoegd, zien dat deze rijstveldbodems significante N-AOM activiteit hadden (79,9 nmol g^{-1} dw d^{-1}). De qPCR met nieuw ontwikkelde primers voor het 16S rRNA gen van de N-AOM archaea, gaven aantallen van 0,28 tot 3,9 x 10^6 kopieën g^{-1}_{dw} in de bodem en van 0,27 tot 2,8 x 10^6 kopieën g^{-1}_{dw} in de rhizosfeer weer. Het aantal bacteriële 16S rRNA genen van het NC10 phylum was minstens een grootte van orde lager, dan die van de N-AOM archaea. Hoewel in eerdere studies ook sequenties van *'Candidatus* M. nitroreducens' gedetecteerd werden, werd hun aanwezigheid niet gekoppeld aan N-AOM in rijstvelden. Hoofdstuk 2 werd gepubliceerd als '**Distribution and activity of the anaerobic methanotrophic community in a nitrogen-fertilized Italian paddy soil**' in FEMS Microbial Ecology 2016.

Na het karakteriseren van de eerste twee genomen van *'Candidatus* M. nitroreducens', gepubliceerd in 2013 en 2015, konden specifieke moleculaire detectie methodes ontwikkeld worden voor de kwantitatieve en gevoelige detectie van methyl-coenzym M reductase *(mcrA)* genen van deze belangrijke N-AOM archaea. Hoofdstuk 3 beschrijft twee nieuwe *mcrA* primer combinaties die specifiek gericht zijn op de alfa-sub eenheid van het *mcrA* gen van *'Candidatus* M. nitroreducens'. Het eerste primerpaar resulteert in fragmenten van 186bp die kunnen worden gebruikt om *'Candidatus* M. nitroreducens' cellen te kwantificeren met behulp van qPCR, terwijl het tweede primerpaar een 1191bp lang amplicon oplevert dat geschikt is voor gedetailleerde fylogenetische analyse. Deze nieuwe qPCR primerparen werden getest op zes verschillende bodemmonsters waarbij de aanwezigheid van *'Candidatus* M. nitroreducens'

werd vergeleken met 16S rRNA gen primers. De qPCR resultaten toonde aan dat het grootste aantal kopieën van het *'Candidatus* M. nitroreducens*' mcrA* genen werd gevonden in de bodem van rijstvelden (5,6±0,8*10^6 kopieën g^{-1} nat gewicht), terwijl in monsters van het Indonesische riviersedimenten slechts 4,6±2,7*10^2 kopieën g^{-1} nat gewicht gevonden werden. Naast zoetwateromgevingen werden *mcrA* sequenties ook gedetecteerd in sediment van de Noordzee, dat 2,5±0,7*10^4 kopieën g^{-1} nat gewicht bevatte. De geamplificeerde 1191bp *mcrA* gen sequenties uit de verschillende milieus vielen bij fylogenetische analyse in dezelfde groep als de beschikbare genoomsequenties van bekende *'Candidatus* M. nitroreducens*'* archaea'. Het onderzoek van dit hoofdstuk resulteerde in het artikel '***McrA* primers for detection and quantification of the anaerobic archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens**' en werd gepubliceerd in Applied Microbiology and Biotechnology 2017.

Na het ontwikkelen van een moleculaire methode gebaseerd op een functioneel gen en het aantonen van de aanwezigheid van *'Candidatus* M. nitroreducens' in verschillende ecosystemen waaronder de Italiaanse rijstveldbodems, werd in hetzelfde rijstveld de stratificatie van de microbiële levensgemeenschap en de potentiële activiteit voor AOM onderzocht. **Hoofdstuk 4** had tot doel door middel van qPCR; 16S rRNA gen amplicon sequencing en anoxische ^{13}C-CH_4 activiteittesten met nitraat, nitriet en ijzer als elektronenacceptoren, de microbiële diversiteit en het methaanmetabolisme in de bovenste 60 cm van het rijstveld in kaart te brengen. De op het 16S rRNA gen gebaseerde bacteriële levensgemeenschap bestond voornamelijk uit *Acidobacteria, Chloroflexi, Proteobacteria, Planctomycetes* en *Actinobacteria*. Bij de Archaea domineerden *Euryarchaeota* en *Bathyarchaeota* over *Thaumarchaeota* in de bovenste 30 cm van de bodem. *Bathyarchaeota* reads vormden zelfs 45% van de totale hoeveelheid archaeale reads in de bovenste 5 cm. Bij de methanogene archaea waren de specialistische *Methanosaeta* dominanter dan de meer veelzijdige *Methanosarcina*. De maximale gemeten productiesnelheid van methaan was 444 nmol g_{dw} h^{-1}. De qPCR data bevestigde de resultaten verkregen in hoofdstuk 2: de bodemmonsters bevatten veel meer *'Candidatus* M. nitroreducens*'* cellen dan NC10 phylum bacteriën, behalve op een diepte van 60 cm waar de aanwezigheid van beide groepen zeer laag was. Met een reeks elektronacceptoren werd de potentiële AOM activiteit bepaald. De maximale nitraat-, nitriet- en ijzer-afhankelijke anaërobe methaanoxidatie snelheden waren respectievelijk 57, 55 en 56 nmol $g_{dw}h^{-1}$. Deze resultaten toonden aan dat er een aanzienlijk AOM kan optreden in vruchtbare rijstveldbodems waarbij *'Candidatus* M. nitroreducens' archaea waarschijnlijk de grootste bijdrage levert, mogelijk niet alleen met nitraat maar ook met ijzer als elektronenacceptor. Dit hoofdstuk werd gepubliceerd als '**Stratification and diversity of methane-oxidizing microorganisms in a nitrogen-fertilized Italian paddy soil**' in Frontiers in Microbiology in 2017.

Hoewel het belangrijk is om de verdeling van AOM micro-organismen met cultivatie onafhankelijke methoden te bepalen en deze te koppelen aan de methaanoxidatie

activiteitsmetingen, kan de volledige kennis over *'Candidatus* M. nitroreducens*'* alleen worden verkregen door het ophopen, groeien en karakteriseren van deze micro-organismen in het laboratorium. **Hoofdstuk 5** beschrijft de eerste AOM ophopingscultuur, waarbij natte bodem van een rijstveld met significante hoeveelheden *'Candidatus* M. nitroreducens' werd gebruikt als startmateriaal. Door continue aanvoer van methaan en nitraat als enige elektrondonor en acceptor, werd na twee jaar een stabiele ophopingscultuur verkregen, die gedomineerd werd door *'Candidatus Methanoperedens nitroreducens'* archaea en NC10 bacteriën. Resultaten van de kwantificatie van 16S rRNA gen kopieën middels qPCR, analyse van metagenomische 16S rRNA reads en fluorescente *in situ* hybridisatie (FISH) correleerden goed met elkaar en toonden aan dat, na twee jaar, de *'Candidatus* M. nitroreducens' het hoogste aantal (2,2 ± 0,4 * 10^8) 16S rRNA kopieën had per ml had en dat deze archaea ongeveer 22% van de totale microbiële gemeenschap vormden. Uit de anaërobe activiteitsmetingen van de totale reactor met methaan en nitraat werd duidelijk dat de gemiddelde snelheid van methaanoxidatie 0,012 mmol h^{-1} L^{-1} was, met een celspecifieke methaanoxidatie van ongeveer 0,57 mol $cell^{-1}$ dag^{-1} voor *'Candidatus* M. nitroreducens*'*. Fylogenetische analyse toonde aan dat de 16S rRNA genen van de dominante micro-organismen groepeerden met eerder beschreven *'Candidatus* M. nitroreducens ANME2D' (96% identiteit). Het *mcrA* gen was 96% vergelijkbaar met *'Candidatus* M. nitroreducens ANME2D' (WP_048089615.1) op het eiwitniveau. De samengevoegde metagenomische sequenties resulteerden in een kwalitatief hoogwaardig genoom van *'Candidatus* Methanoperedens nitroreducens Vercelli'. Dit hoofdstuk resulteerde in het artikel '**Enrichment of anaerobic nitrate-dependent methanotrophic '*Candidatus* Methanoperedens nitroreducens' archaea from an Italian paddy field soil**', gepubliceerd in Applied Microbiology and Biotechnology in 2017.

Tesamen laten de hoofdstukken van dit proefschrift zien dat *'Candidatus* Methanoperedens nitroreducens' archaea in behoorlijk hoeveelheden voorkomen in bemeste, natte rijstvelden, en dat deze archaea daar een significant bijdrage kunnen leveren aan de anaërobe oxidatie van methaan. Met de verkregen ophopingscultuur kan in de toekomst de metabole diversiteit van *'Candidatus* Methanoperedens nitroreducens' onderzocht worden.

1
General introduction

Rice is the primary food source for about half of the world's population and the second most widely grown cereal crop. Worldwide, rice provides 27% of dietary energy supply and 20% of dietary protein (Van Nguyen & Ferrero, 2006). Major producers are located in Asia (especially China), where approximately 90% of global rice supply originates. In Europe, Italy is the largest rice producer. Rice is mainly cultivated in flooded paddy fields which are man-made wetlands that contribute substantially to atmospheric methane (CH_4) concentrations and release 25-300 Tg of CH_4 $year^{-1}$ (Bridgham *et al.*, 2013). This represents 10-20% of the global methane emissions (Conrad, 2009, Bodelier, 2011). It is predicted that the land area designated for rice cultivation, which currently is about 160 million hectares, will increase by 60% in the coming decades. As paddy fields are an important source of methane, a large number of studies have focused on; estimation of the methane emission rates from paddy fields; identification of environmental and anthropogenic factors which affect these rates and the microbial communities in paddy fields (Inubushi *et al.*, 2002, Krüger *et al.*, 2002, Chin *et al.*, 2004, Conrad *et al.*, 2008, Bodelier, 2011, Banger *et al.*, 2012, Bao *et al.*, 2016).

In addition to methane being the second most abundant greenhouse gas, it is also 34 times more potent than carbon dioxide (CO_2) in radiative forcing over 100 year span. Since the industrial era, the atmospheric methane concentrations have increased 2.5-fold to above 1800 ppb (Myhre *et al.*, 2013). Both natural and agricultural soils are important contributors to atmospheric methane, despite the large uncertainties in the estimates.

Investigation of the soil microbial community is a complex matter, as soil is not a homogenous environment and there is no 'typical' soil microbiome. The soil environment can vary from micrometers to millimeters in the abiotic characteristics, and the composition of bacterial and archaeal taxa varies on the same scale (O'Brien *et al.*, 2016). The uniqueness of paddy fields environment is due to the cultivation under waterlogged conditions. Unlike many other plants, rice is able to tolerate the submersion much better, due to the formation of gas spaces between the shoot and the root called aerenchyma, which enables the oxygen transport into the submerged parts deeper in soil (Armstrong, 1971, Colmer, 2003, Colmer & Pedersen, 2008). The overlaying water decreases the oxygen penetration depth and functions as a diffusive barrier, which causes anoxia in the majority of soil compartments and favours the process of anaerobic decomposition. Further, paddy fields are commonly fertilized with nitrogen compounds several times over the growing season; such fertilizers maintain soil fertility and increases crop yield (Cassman *et al.*, 1998). Another source of nitrogen in the form of nitrate and nitrite occurs at oxic/anoxic interfaces, where ammonium is oxidized with oxygen released from the roots or from the surface (Brune *et al.*, 2000).

A crucial factor in the understanding of methane emissions is that the majority of methane released to the atmosphere (70-80%) is of biogenic origin (Conrad, 2009). Methane is the end

product of anaerobic digestion of organic matter by the microbial community. Most of the methane is produced in the anoxic layers of soil by methanogenic archaea. These thrive well under the anoxia, where organic matter is fermented and electron acceptors are limited. Yet the extensive root system creates 'hotspots' for microbial diversity. Studies on paddy fields differentiate between the bulk soil and the rhizosphere soil. The bulk soil is often dominated by *Acidobacteria* and *Chloroflexi*, sometimes together with *Proteobacteria* (Ahn *et al.*, 2012, Ahn *et al.*, 2014, Lee *et al.*, 2015). The rhizosphere is often dominated by *Firmicutes*. As roots secrete a considerable amount of fermentable poly- and monomeric sugars, the dominance or high abundance of these fermenting microorganisms in the rhizosphere is to be expected (Aslam *et al.*, 2013, Edwards *et al.*, 2015). In this complex system, many of these fermenting microorganisms provide substrates for the methanogenic archaea, which in the anoxic layers of the soil produce methane either by acetoclastic of hydrogenotrophic methanogenesis.

The abundance of methanogens, community composition and the pathways of methanogenesis have been intensively studied in paddy fields (Chin *et al.*, 1999, Wang *et al.*, 2010, Lee *et al.*, 2014, Lee *et al.*, 2015, Bao *et al.*, 2016). Among many other factors, flooding (Rui *et al.*, 2009), fertilization and straw application (Bao *et al.*, 2016), temperature (Conrad *et al.*, 2009, Noll *et al.*, 2010), rice cultivar and soil type (Conrad et al., 2008) and plant growth stage (Breidenbach & Conrad, 2015) affect the community composition and the amount of methane released.

In the rhizosphere, methanogenic archaea belong mostly to hydrogenotrophic and methylotrophic groups (Daebeler *et al.*, 2013), whereas in the bulk soil strictly acetoclastic *Methanosarcinales* (*Methanosaeta*) seem to dominate. Furthermore, versatile representatives of the *Methanosarcinales*, which may use both acetoclastic and hydrogenotrophic pathways for methane production, have been found to be more abundant in the rhizosphere. Hydrogenotrophic methanogens of the *Methanocellales* or Rice Cluster I (RC I) have been noted as the key players in Italian and other paddy fields (Conrad & Klose, 2006, Erkel *et al.*, 2006, Watanabe *et al.*, 2009).

Surprisingly, strict anaerobic methanogens have also been detected in the rhizosphere and on rice roots (Chin *et al.*, 2004, Xu *et al.*, 2012, Edwards *et al.*, 2015, Lee *et al.*, 2015). For instance, Lee et al. (2015) observed a higher abundance of methanogens in the rhizosphere than in bulk soil. One of the possible explanations for surviving nearby roots and being tolerant to the oxygen released is that methanogens possess mechanisms to counteract the reactive oxygen radical species as has been demonstrated for hydrogenotrophic methanogens (Erkel *et al.*, 2006). The niche in the rhizosphere for strict anaerobes may be either within soil aggregates, which have a local low local oxygen concentration or in non-active roots where no oxygen is released.

The methanogenic process is counteracted by another well studied process: the aerobic oxidation of methane. Despite of overlaying water causing anoxia in the majority of soil compartments, there is a constant oxygen influx into the soil layers through diffusional transport via the aerenchyma and by the radial oxygen loss of the rice roots (Armstrong, 1971, Li & Wang, 2013).

Aerobic methane-oxidizing bacteria (MOB) have been extensively studied since the discovery of "Bacillus methanicus" (Söhngen, 1906). MOB have long been considered the only microbes capable of oxidizing methane. The currently known MOB belong to the phyla *Proteobacteria* and *Verrucomicrobia* (Op den Camp *et al.*, 2009, Semrau *et al.*, 2010). Proteobacterial aerobic methanotrophs inhabit a variety of environments, ranging from tundra soil (Dedysh *et al.*, 2004) and arctic permafrost (Liebner *et al.*, 2009), to sewage treatment sludge (Ho *et al.*, 2013). By phylogenetic analyses of both 16S rRNA and the particulate methane mono-oxygenase subunit A (pmoA) gene, two groups of *Proteobacteria* have been identified as MOB: Type I methanotrophs belonging to the *Gammaproteobacteria* and Type II methanotrophs belonging to the *Alphaproteobacteria* (Trotsenko & Murrell, 2008, Semrau *et al.*, 2010).

Previous research of methane sinks in paddy fields has mainly focused on aerobic *Proteobacterial* methanotrophs, demonstrating that they can oxidize substantial parts of the methane produced in the anoxic parts of the paddy field soil (Krüger *et al.*, 2001, Krause *et al.*, 2010, Ho *et al.*, 2011, Lüke & Frenzel, 2011, Lee *et al.*, 2014). It has been suggested that Type I methanotrophs can likely outcompete Type II methanotrophs for substrates in these nitrogen-loaded environments (Zheng *et al.*, 2014). Studies on selection showed dominance of Type I over Type II, when ammonium or nitrate were supplied as nitrogen source (Pfluger *et al.*, 2011). Compared to the *Proteobacterial* aerobic methanotrophs, the more recently discovered *Verrucomicrobia* often inhabit more extreme environments with low pH values and/or high temperatures (Dunfield *et al.*, 2007, Op den Camp *et al.*, 2009, Sharp *et al.*, 2014, van Teeseling *et al.*, 2014).

In contrast to aerobic methane oxidation, the process of anaerobic oxidation of methane (AOM) is less-well understood and explored in soil systems. Yet in the absence of oxygen, there is a range of electron acceptors available in soils to support methane conversion to carbon dioxide: sulfate, nitrate, nitrite and metal oxides such as manganese and iron oxides.

The process of anaerobic oxidation of methane was first hypothesized to occur based on biogeochemical sediment profiles (Reeburgh, 1976), but the responsible microbes were discovered only much later. Since then, many studies have addressed the importance of sulfate-dependent AOM in marine ecosystems as reviewed in (Knittel & Boetius, 2009) showing that it is mediated by a consortium of anaerobic methanotrophic (ANME) archaea in cooperation

with sulfate-reducing bacteria, or possibly ANME alone (Knittel & Boetius, 2009, Milucka *et al.*, 2012, Scheller *et al.*, 2016). Currently, ANMEs are divided into three main lineages: ANME-1, ANME-2 and ANME-3 (Knittel *et al.*, 2005, Nauhaus *et al.*, 2005, Stadnitskaia *et al.*, 2005). They act as the dominant methane sink in marine environments, accounting for 80% of methane removal in these ecosystems (Durisch-Kaiser *et al.*, 2005, Boetius & Wenzhofer, 2013). In freshwater and terrestrial environments, sulfate concentrations are typically two orders of magnitude lower than in marine systems, making the contribution of microorganisms using other electron acceptors more likely (Strous & Jetten, 2004).

Anaerobic oxidation of methane coupled to nitrate and nitrite reduction (N-AOM) was reported for the first time in 2006 (Raghoebarsing *et al.*, 2006). This enrichment contained archaea distantly related to ANME-2, and bacterium of the candidate division NC10. Over time, as a result of supplying the bioreactor with more nitrite, the enrichment culture became dominated by NC10 phylum bacteria, which was identified as the microorganism capable of catalyzing nitrite-dependent AOM alone and it was named '*Candidatus* Methylomirabilis oxyfera' (Ettwig *et al.*, 2008, Ettwig *et al.*, 2010). '*Candidatus* Methylomirabilis oxyfera' as the name illustrates has 'miraculous' trait of using an intra-aerobic mechanism, whereby two molecules of nitric oxide are probably dismutated into O_2 and N_2. Yet it contains the key enzymes of the aerobic methanotrophic pathway; the particulate methane monooxygenase complex, which can use the produced O_2 for methane oxidation in the same manner as aerobic methanotrophs (Ettwig *et al.*, 2010). The discovery of '*Candidatus* Methylomirabilis oxyfera' identified the first microorganism that couples nitrogen- and methane cycle and since then a number of studies have demonstrated that nitrite-dependent anaerobic oxidation of methane by NC10 phylum bacteria significantly contributes to the methane removal in the sediments of a deep lake (Deutzmann & Schink, 2011, Deutzmann *et al.*, 2014) and in a eutrophic peatland (Zhu *et al.*, 2012). Also in paddy fields, occurrence and significant activity of NC10 phylum bacteria has been demonstrated (Wang *et al.*, 2012, Hu *et al.*, 2014).

Using the 16S rRNA gene as phylogenetic marker, NC10 bacteria have been divided previously into four groups (A, B, C, D) (Ettwig *et al.*, 2009) (Figure 1). All known N-AOM performing '*M. oxyfera*'-like bacteria characterized from enrichment cultures fall into group A (Ettwig *et al.*, 2009, Ettwig *et al.*, 2010, Haroon *et al.*, 2013, He *et al.*, 2015). Very recently, a high-quality draft genome of an NC10 bacterium belonging to group D could be re-constructed from an aquifer sediment metagenome (Hug *et al.*, 2016). Group B and C are to date only represented by environmental sequences and nothing is known about the physiology of these organisms.

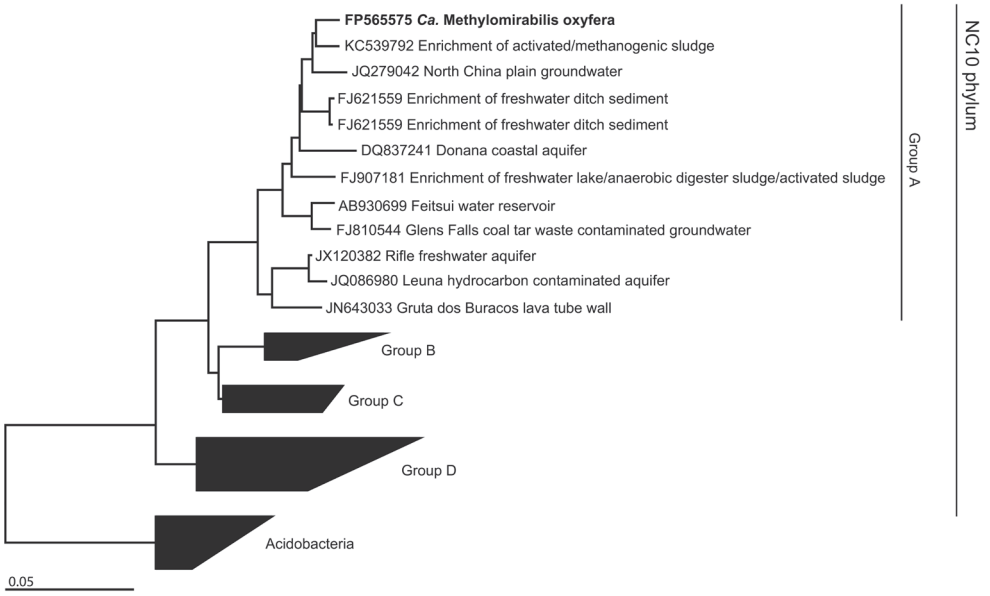

Figure 1: Phylogenetic overview of NC10 bacteria based on 16S rRNA gene sequences. Depicted is the clustering of the NC10 clade into groups A-D. 'Candidatus Methylomirabilis oxyfera' of the group A is marked in bold. The calculation of the tree was carried out by Neighbour-joining algorithm using the Jukes Cantor correction and filter over 1158bp and Acidobacteria as an outgroup (Welte *et al.*, 2016).

The role of the ANME-2 archaea in the first enrichment culture was characterized much later. These *Euryarchaea*, capable of coupling nitrate reduction to anaerobic methane oxidation were identified as '*Candidatus* Methanoperedens nitroreducens' (Haroon *et al.*, 2013). Till 2017, two genomes of ANME-2d archaea, enriched in bioreactors fed with methane, nitrate and ammonium (Haroon *et al.*, 2013) or methane and nitrate, were available (Arshad *et al.*, 2015). '*Candidatus* Methanoperedens nitroreducens' possesses all the genes of the (reverse) methanogenic pathway (Haroon *et al.*, 2013, Arshad *et al.*, 2015). Phylogenetic analysis revealed that '*Candidatus* Methanoperedens nitroreducens' belongs to the order of *Methanosarcinales* and is classified as 'GOM Arc I' in the ribosomal rRNA SILVA database. The group 'GOM Arc I' consists of the ANME-2d group as well as the original GOM Arc I group with sequences from Gulf of Mexico (Mills *et al.*, 2003).

To date the GOM Arc I/ANME-2d group can be split into three defined clusters A, B and C (Figure 2). The 16S rRNA sequences of the two known genomes (Haroon *et al.*, 2013, Arshad *et al.*, 2015) cluster into group A, which is the largest and most uniform group. With few exceptions of sequences found in marine and brackish environments, this group consists of sequences detected in freshwater environments such as aquifers, lakes and rivers (L*i et al.*,

2012, Flynn *et al.*, 2013). Group B and C have no cultured representatives so far and consist exclusively of environmental sequences. The sequences of group B and C have been found in extreme environments such as marine and terrestrial mud volcanoes, marine sediment and hydrothermal vents (Inagaki *et al.*, 2006, Pachiadaki *et al.*, 2011, Yang *et al.*, 2012). Sequences classified as '*Candidatus* Methanoperedens nitroreducens' have been detected previously in paddy fields, including fields in Vercelli, Italy (Lueders *et al.*, 2001, Conrad *et al.*, 2008), Chinese paddy fields (Xu *et al.*, 2012), and Korean paddy fields (Lee *et al.*, 2015) as well as in natural wetlands (Narrowe *et al.*, 2017).

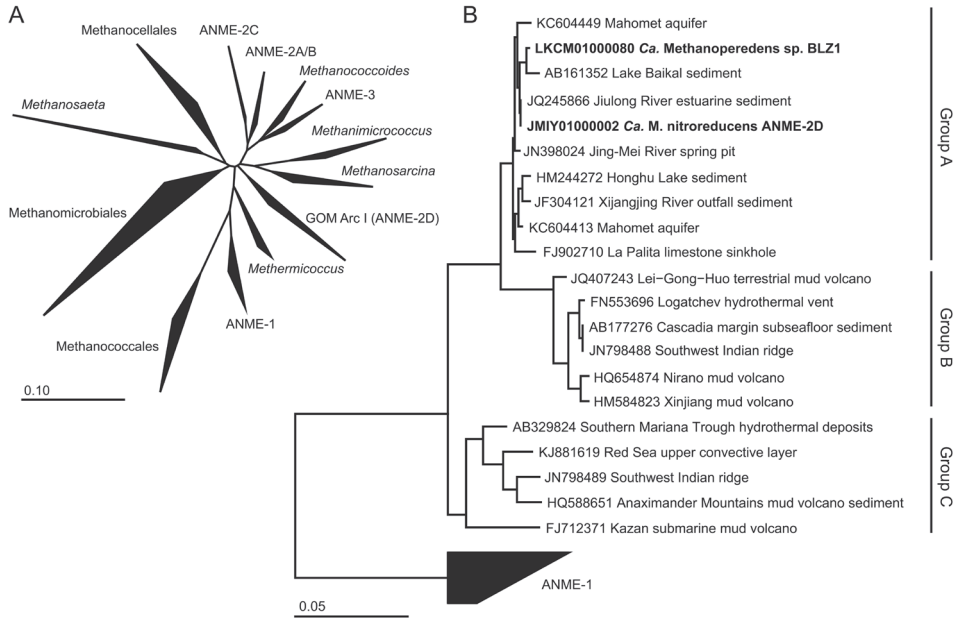

Figure 2: Phylogenetic overview of Methanoperedens-like archaea based on 16S rRNA gene sequences. A. Phylogenetic positioning of GOM Arc I/ANME-2D within other ANME groups and methanogens. B. Clustering of ANME-2D into groups A–C. Cultured representatives are marked in bold. The classification of the groups was performed with all available 16S rRNA gene sequences of ANME-2D and confirmed by Neighbour-joining and maximum likelihood algorithms. The representative Neighbour-joining phylogenetic tree was calculated using the Jukes Cantor correction, filter over 290 bp and ANME 1 as an outgroup. The full 16S rRNA sequences of the two cultured representatives 'M. nitroreducens ANME2D' and 'Methanoperedens sp. BLZ1' are 95.2% identical (Welte *et al.*, 2016).

After the nitrite-and nitrate-dependent AOM microorganisms had been identified, speculations on metal-dependent AOM remained. Metal-dependent AOM seemed to occur in both marine and freshwater environments (Beal *et al.*, 2009, Egger *et al.*, 2015) and possibly have played a role in the early earth, when oxygen was lacking (Beal *et al.*, 2009). Iron is the most abundant

element on earth and in paddy fields iron reduction is considered as a central biochemical process. Anaerobic incubations of paddy field with Fe (III) stimulated methane oxidation (Kumaraswamy *et al.*, 2001). Very recently it was shown that '*Candidatus* Methanoperedens nitroreducens' can use various electron acceptors including iron-citrate and, may thus be able to carry out metal dependent AOM (Ettwig *et al.*, 2016). Metal-dependent AOM has been demonstrated recently also for other ANME enrichments as well (Scheller *et al.*, 2016).

In the light of these new discoveries, it is therefore of primary interest to understand fundamental processes in the paddy soil and the contribution of anaerobic and aerobic microorganisms to the methane balance in the atmosphere. Only then can we re-evaluate the influence of environmental and anthropogenic factors, and start to create better mitigation strategies and minimize the methane emissions from agricultural lands.

In this thesis, we investigated several microbiological aspects of anaerobic oxidation of methane in an Italian paddy field. In chapter 2 and 4 we report the presence, abundance, activity and stratification of both '*Candidatus* Methylomirabilis oxyfera' and of '*Candidatus* Methanoperedens nitroreducens' with an array of complementary methods. In chapter 3 we developed new molecular tools for detection of '*Candidatus* Methanoperedens nitroreducens' based on the key *mcr*A gene of the reverse methanogenesis pathway. Finally in chapter 5, we used Italian paddy field soil to enrich '*Candidatus* Methanoperedens nitroreducens' in a bioreactor fed with methane and nitrate. The culture was analyzed with stable isotope activity tests, metagenomics and microscopy, and all methods showed that a new '*Candidatus* Methanoperedens nitroreducens' strain was obtained.

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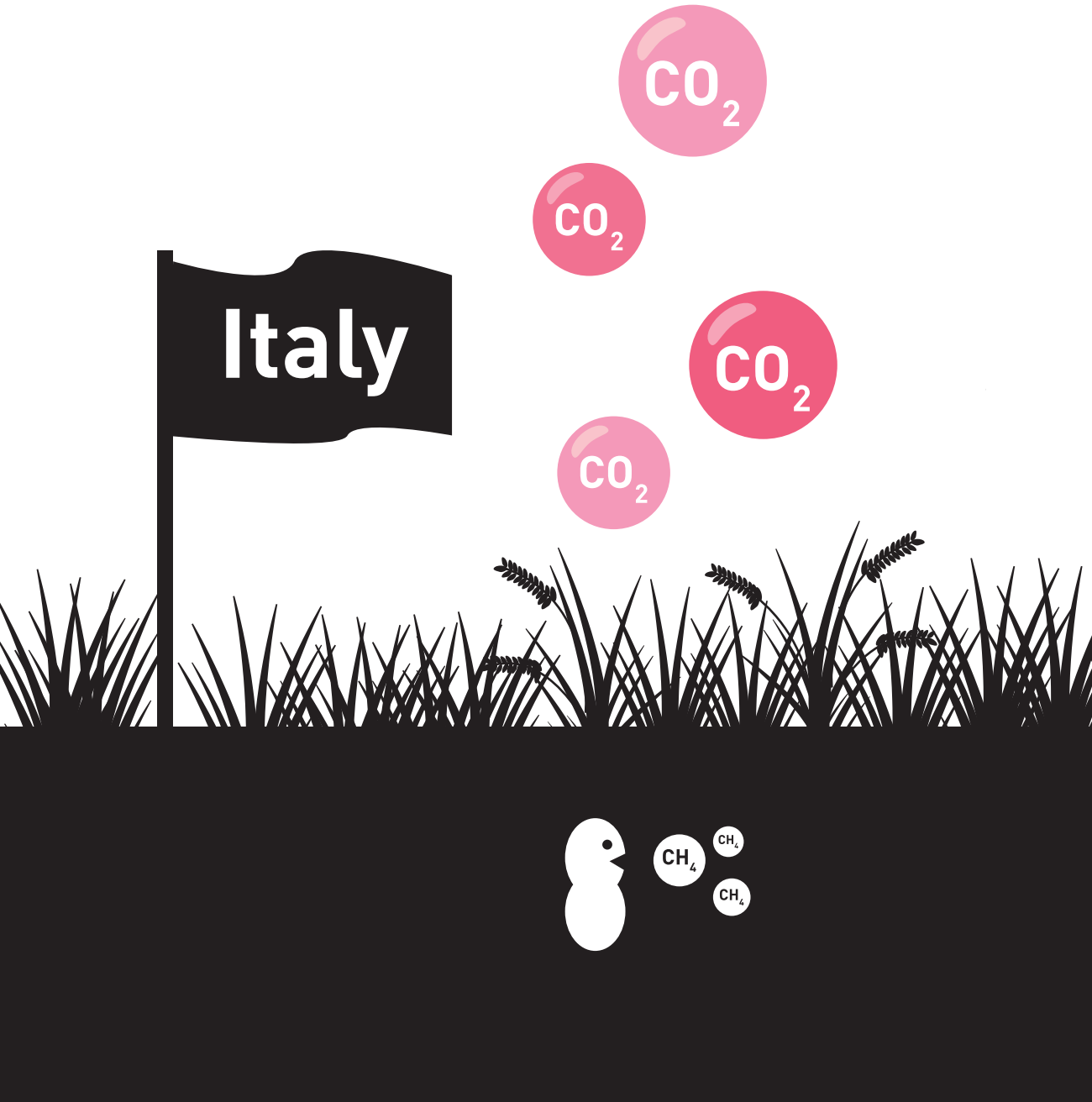
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Distribution and activity of the anaerobic methanotrophic community in a nitrogen-fertilized Italian paddy soil

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RESEARCH ARTICLE

Distribution and activity of the anaerobic methanotrophic community in a nitrogen-fertilized Italian paddy soil

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One sentence summary: Italian paddy soil contains a considerable population of anaerobic methane oxidizing archaea.
Editor: Gary King

ABSTRACT

In order to mitigate methane emissions from paddy fields, it is important to understand the sources and sinks. Most paddy fields are heavily fertilized with nitrite and nitrate, which can be used as electron acceptors by anaerobic methanotrophs. Here we show that slurry incubations of Italian paddy field soil with nitrate and ^{13}C-labelled methane have the potential for nitrate-dependent anaerobic oxidation of methane (79.9 nmol g^{-1}_{dw} d^{-1}). Community analysis based on 16S rRNA amplicon sequencing and qPCR of the water-logged soil and the rhizosphere showed that anaerobic oxidation of methane-associated archaea (AAA), including *Methanoperedens nitroreducens*, comprised 9% (bulk soil) and 1% (rhizosphere) of all archaeal reads. The NC10 phylum bacteria made up less than 1% of all bacterial sequences. The phylogenetic analysis was complemented by qPCR showing that AAA ranged from 0.28×10^6 to 3.9×10^6 16S rRNA gene copies g^{-1}_{dw} in bulk soil and 0.27×10^6 to 2.8×10^6 in the rhizosphere. The abundance of NC10 phylum bacteria was an order of magnitude lower. Revisiting published diversity studies, we found that AAA have been detected, but not linked to methane oxidation, in several paddy fields. Our data suggest an important role of AAA in methane cycling in paddy fields.

Keywords: anaerobic oxidation of methane; microbial community; paddy fields; 16S rRNA gene; amplicon sequencing; rice rhizosphere; *Methanoperedens nitroreducens*; AAA

INTRODUCTION

Methane (CH_4) is an important greenhouse gas that is up to 34 times more potent than carbon dioxide (CO_2) in radiative forcing (Myhre *et al.* 2013). Since the industrial era, the atmospheric methane concentrations have increased 2.5-fold to above 1800 ppb (Myhre *et al.* 2013). Paddy fields cover about 160 million hectares worldwide and have been estimated to contribute 10–20% to the global methane emission (Conrad 2009; Bodelier 2011).

Biogenic methane emission into the atmosphere is the net result of production by methanogens and consumption by methanotrophs. Methanogenic archaea occur mostly in the anoxic layers of soil and sediments, and produce methane mainly from acetate or hydrogen, degradation products of

organic matter (Conrad 2007; Borrel *et al*. 2011). Paddy fields have been studied intensively for their communities of acetoclastic and hydrogenotrophic methanogens (Conrad 2007).

For a long time microbial methane oxidation was considered to be solely dependent on the availability of oxygen as an electron acceptor. Aerobic methanotrophs have been known since 1906, when they were first described (Söhngen 1906). Subsequent studies showed that they belong to at least two bacterial phyla: *Proteobacteria* and *Verrucomicrobia* (Op den Camp *et al*. 2009; Semrau, DiSpirito and Yoon 2010). Representatives of methane-oxidizing *Verrucomicrobia* have so far only been found in acidic environments (Op den Camp *et al*. 2009; Sharp, Stott and Dunfield 2012; Sharp *et al*. 2014; van Teeseling *et al*. 2014). Proteobacterial methanotrophs belong to either the *Alpha-* (type II) or *Gamma-Proteobacteria* (type I) (Trotsenko and Murrell 2008; Semrau, DiSpirito and Yoon 2010), and have been extensively studied in a range of environments, including paddy fields (Ho *et al*. 2011; Lüke and Frenzel 2011; Lee *et al*. 2014).

In contrast to aerobic methanotrophs, anaerobic methanotrophs in non-marine environments are much less explored. The process of anaerobic oxidation of methane (AOM) was first hypothesized to occur based on biogeochemical sediment profiles (Reeburgh 1976), but the responsible microbes were discovered only much later. Since then, many studies have addressed the importance of sulfate- dependent AOM in marine ecosystems (as reviewed in Knittel and Boetius 2009) showing that it is mediated by a consortium of anaerobic methanotrophic (ANME) archaea with sulfate-reducing bacteria, or possibly ANME archaea alone (Knittel and Boetius 2009; Milucka, Widdel and Shima 2012; Scheller *et al*. 2016). This is the dominant methane sink in marine environments, accounting for 80% of methane removal in these ecosystems (Hinrichs and Boetius 2002; Boetius and Wenzhofer 2013). Both the aerobic and anaerobic methanotrophic community in soils and sediments function as biofilters, controlling the amount of methane emitted to the atmosphere. In freshwater and terrestrial environments, sulfate concentrations are typically two orders of magnitude lower than in marine systems, making the contribution of microorganisms using other electron acceptors more likely (Strous and Jetten 2004). In 2006, AOM coupled to nitrate and nitrite reduction was first reported (Raghoebarsing *et al*. 2006). The responsible microorganisms were identified as archaea belonging to the family *Methanosarcinales* but only distantly related to ANME group 2 (ANME-2) archaea, and a bacterium of the candidate division NC10, named '*Candidatus* Methylomirabilis oxyfera'. This NC10 bacterium was found to be capable of catalyzing nitrite-dependent AOM alone (Ettwig *et al*. 2008), using an intra-aerobic mechanism, whereby two molecules of nitric oxide are probably dismutated into O_2 and N_2. The O_2 produced can be used for methane oxidation via the particulate methane monooxygenase complex much like in aerobic methanotrophs (Ettwig *et al*. 2010). The initial co-culture, containing archaea and nitrite-reducing *M. oxyfera*, showed also nitrate-reducing ability, which led to the hypothesis that the archaea may be carrying out nitrate-dependent AOM. Eventually this was confirmed in a mixed culture, where very similar methanotrophic archaea were enriched without the presence of other methanotrophs. These Archaea were shown to oxidize methane anaerobically via a reverse methanogenesis pathway, using nitrate as terminal electron acceptor, and named '*Candidatus* Methanoperedens nitroreducens' (Haroon *et al*. 2013; Arshad *et al*. 2015). These archaea have also been referred to as AOM-associated archaea (AAA) (Knittel and Boetius 2009). They are closely related to the ANME-2d group comprising sequences retrieved from the Gulf of Mexico (Mills *et al*. 2003, 2005) and subsequently renamed GOM Arc I (Lloyd, Lapham and Teske 2006). The current SILVA taxonomy, established based on comprehensive rRNA databases, combines AAA and ANME-2d/GOM Arc I at a higher taxonomic level into one group referred to as GOM Arc I (Quast *et al*. 2013).

Before the discovery of nitrate- and nitrite-dependent AOM, its contribution to biological methane oxidation was not known and could not be taken into account even in well-studied ecosystems. Recent studies demonstrated that anaerobic nitrite-dependent NC10 phylum methanotrophs contributed significantly to methane removal in the sediments of a deep lake (Deutzmann and Schink 2011; Deutzmann *et al*. 2014) and in a eutrophic peatland (Zhu *et al*. 2012). Also in paddy fields, occurrence and significant activity of NC10 phylum bacteria have been demonstrated (Wang *et al*. 2012; Hu *et al*. 2014). To our knowledge, no study has focused yet on nitrate-dependent AOM by AAA in nature, nor estimated their environmental distribution.

In the present study, we therefore investigated the role of anaerobic methanotrophs as a methane sink in a water-logged paddy field soil in Vercelli (Northern Italy). The Vercelli paddy fields have been used as a model system for studying the distribution and activity of aerobic methanotrophs and anaerobic methanogens in a temperate climate. Previous research in these fields has suggested that in the rice field rhizosphere up to 40% of the methane produced is consumed (Krüger, Frenzel and Conrad 2001). This activity was attributed to aerobic methanotrophs, while the contribution of nitrate- or nitrite-dependent AOM has not been addressed so far.

In this study, we (i) characterized the microbial community of the bulk soil and the rhizosphere by 16S rRNA gene amplicon sequencing with a focus on methanotrophic organisms, (iii) quantified the abundance of both bacterial and archaeal anaerobic methanotrophs using qPCR and related their abundance to total numbers of bacteria and archaea, and (iii) demonstrated the potential nitrate-induced AOM activity of the paddy soil.

METHODS

Soil sampling

Sampling of the rhizosphere and collection of bulk soil for molecular analysis was carried out in September 2013 in paddy fields at the Italian Rice Research Unit in Vercelli, Italy (08°22′25.89″E; 45°19′26.98″N). These fields of silt loam soil were flooded with approximately 15 cm of water and regularly tilled. Soil of the experimental field had been fertilized with 147.5 kg ha^{-1} nitrogen and 183 kg ha^{-1} potassium 21 days after flooding. Samples were collected 95 days after the beginning of flooding. At the time of sampling, the porewater nitrate and ammonium concentrations were 0.54 and 0.09 μmol/L, respectively. The rice variety cultivated in the field plots was *Oryza sativa* japonica Onice, a long A Italian rice variety, and samples were collected when rice plants were at the dough maturation stage. All samples were collected from randomly distributed spots on one field with five replicates of both rhizosphere and bulk soil. Bulk soil samples were taken at 10–20 cm depth with a spade. For rhizosphere samples, roots were washed and the majority of the attached soil was removed with gloves. For DNA extractions, samples were stored in 50 ml falcon tubes. Upon arrival at the laboratory in the Netherlands, samples were immediately frozen until the DNA extraction was carried out. For incubation experiments, soil samples were collected and transported in a container and in the container covered with water sampled from the field. After transport to the laboratory, the soil samples for activity assays were stored at 4°C.

DNA extraction

DNA extraction from 0.25–0.75 g of soil or roots was performed in duplicate with the PowerSoil DNA isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. Roots were ground in liquid nitrogen with a mortar and pestle prior to extraction. DNA quantity was assessed by UV-VIS spectroscopy (Nanodrop, ND-1000, Isogen Life Science, The Netherlands) and 1% agarose gel electrophoresis was performed to check the quality of the isolated DNA.

Primer selection and modification for amplicon sequencing

Primers for amplification of 16S rRNA genes for bacteria and archaea were selected based on their coverage for NC10 phylum bacteria and *Methanoperedens nitroreducens* as well as suitability for amplicon sequencing (Klindworth *et al.* 2013). The coverage was assessed using the Testprime Silva online tool (http://www.arb-silva.de/search/testprime). The following primers were selected: forward S-D-Arch-0349-a-S-17 (5′GYGCASCAGKCGMGAAW3′) and reverse S-D-Arch-0519-a-A-16 (5′TTACCGCGGCKGCTG3′) for archaea; and forward S-D-Bact-0341-b-S-17 (5′CCTACGGGNGGCWGCAG3′) and reverse S-D-Bact-0515-a-A-19 (5′TTACCGCGGCTGCTGGCAC3′) for bacteria. For amplicon sequencing, the above-mentioned primers were extended with adapter sequences, specific barcodes and key sequences compatible for Ion Torrent sequencing at the 5′ end, resulting in a total length of 56–60 nucleotides per primer.

Library preparation and Ion Torrent sequencing

DNA extractions from five replicates were pooled in equimolar amounts and used as the template for subsequent amplicon sequencing. Amplicons were obtained by gradient PCR using Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA). The PCR temperature protocol started with an initial denaturation at 98°C for 5 min followed by 55 cycles (a high cycle number due to primer dimer formation) of 15 s at 98°C, primer annealing at 50–60°C for 30 s and elongation at 72°C for 1.5 min. A final elongation step was performed at 72°C for 3 min. The size and the quality of the amplicons were checked using 1% agarose gel electrophoresis. Purification of amplicons with correct size was done using the GeneJET gel extraction kit (Thermo Scientific, Landsmeer, The Netherlands) after extraction of the PCR product from the agarose gel, according to the manufacturer's protocol. Extracted PCR products of the different annealing temperatures were pooled for Ion Torrent library preparation.

For preparation of the library for Ion Torrent sequencing, amplification of the pooled and extracted PCR fragments was performed using 100 μL of Platinum PCR SuperMix High Fidelity (Life Technologies, USA), 5 μL of Library amplification primer mix and 25 μL of unamplified purified PCR product. Amplification was performed with an initial denaturation at 95°C for 5 min followed by five cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 15 s and elongation at 70°C for 1 min. Purification of the library was performed using Agencourt AMPure XP kit (Beckman Coulter, Inc., USA). Concentrations and fragment lengths of the libraries were determined with the Bioanalyzer 2100 and the High Sensitivity DNA kit (Agilent Technologies, USA). The libraries obtained were diluted to a final concentration of 26 pM, and when needed different barcoded libraries were pooled in equimolar amounts before sequencing.

For Ion Torrent sequencing the library fragments were attached to Ion Sphere particles using the Ion One Touch Instrument and Ion PGM Template OT2 400 Kit (Life Technologies, USA) according to the manufacturer's instructions. After enrichment of the Template-Positive Ion Sphere Particles, using the Ion One Touch ES (Life Technologies, USA), they were loaded on either an Ion 314 v2 Chip or an Ion 318 v2 Chip. Subsequently, DNA fragments were sequenced using the Ion PGM Sequencing 400 Kit using 850 nucleotide flows, according to the manufacturer's instructions.

Amplicon sequence data analysis

Initial data analysis was performed using the CLC genomic workbench, including elimination of low quality and short reads (cut-off value 150 nucleotides). The exported reads were further processed by using the automated pipeline of Silva next-generation sequencing (NGS) of the SILVA rRNA gene database project (SILVAngs 1.2) (Quast *et al.* 2013). In this process each read was aligned using the SILVA Incremental Aligner (SINA; SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse, Peplies and Glockner 2012) against the SILVA SSUrRNA SEED and quality controlled (Quast *et al.* 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations, artefacts and reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis. After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered (operational taxonomic units; OTUs), on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering were done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (Li and Godzik 2006) running inaccurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 119; http://www.arb-silva.de) using blastn (version 2.2.28+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho *et al.* 2009). The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. This yields semi-quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the value for (percentage sequence identity + percentage alignment coverage)/2 did not exceed 93, remain unclassified. These reads were assigned to the metagroup 'No Relative' in the SILVAngs fingerprint and Krona charts (Ondov, Bergman and Phillippy 2011). This method was first used in the publications Klindworth *et al.* (2013) and Ionescu *et al.* (2012).

Quantification by qPCR

The quantification of the total bacterial and total archaeal community using the 16S rRNA gene as biomarker was conducted by quantitative polymerase chain reaction (qPCR). The qPCR reactions were run in triplicate on duplicate DNA extractions from each sample, with five samples each obtained from the rhizosphere and the bulk soil. This resulted in a total number of 30 qPCR reactions for each primer pair and compartment. For archaea, the following primers

were used: forward Arch-0349 (5′GYGCASCAGKCGMGAAW3′) and reverse Arch-807 (5′GGACTACVSGGGTATCTAAT3′) (Takai and Horikoshi 2000). For Bacteria: forward S-D-Bact-0341-b-S-17 (5′CCTACGGGNGGCWGCAG3′) (Herlemann *et al.* 2011) and reverse S-D-Bact-0515-a-A-19 (5′TTACCGCGGCTGCTGGCAC3′) (Muyzer, de Waal and Uitterlinden 1993). AAA were targeted by clade-specific primers 641F (5′ ACTGDTAGGCTTGGGACC3′) and 834R (5′ATGCGGTCGCACCGCACCTG3′) (previously reported as specific FISH probes) (Schubert *et al.* 2011). These primers were tested for their specificity and amplification efficiency. They resulted in a 193 bp fragment (single band on 1% agarose gel) with 60°C as optimal annealing temperature, determined by gradient PCR (55–65°C) and agarose gel electrophoresis. Cloning and sequencing resulted in sequences assigned to AAA (data not shown; Vaksmaa *et al.*, in preparation). NC10 phylum bacteria were amplified with 16S rRNA primers p2F_DAMO (5′GGGGAACTGCCAGCGTCAAG3′) and p2R_DAMO (5′CTCAGCGACTTCGAGTACAG3′) (Ettwig *et al.* 2009) resulting in a 277 bp fragment. All qPCR reactions were performed using the PerfCTa Quanta master mix (Quanta Biosciences, USA) and 96-well optical plates (Bio-Rad Laboratories, UK) and reactions were performed using the Bio-Rad IQ 5 cycler (Bio-Rad, USA). Absolute quantification was obtained by comparison with standard curves obtained by 10-fold serial dilutions of pGEM-T Easy plasmid DNA with an insert of the target gene. Standard curve samples were used as an internal control at each qPCR run. Calculations for cell abundances were based on 16S rRNA gene copies targeted.

Cloning and sequencing

Sequences belonging to the NC10 phylum were amplified with 202F and 1043R primers targeting the 16S rRNA gene (Ettwig *et al.* 2009), and AAA sequences were obtained using the specific forward primers 641F in combination with general archaeal reverse primer 915R and general prokaryote primer 1492R (with product lengths of 297 bp and 864 bp). Two different reverse primers were used as no specific reverse primer, resulting in a longer DNA fragment, is available to date. Amplified PCR products (obtained with the same protocol as used for qPCR) were cloned using the pGEM-T Easy cloning vector (Promega, USA) and transformed in *E. coli* XL1 Blue competent cells. Plasmids were isolated from positively screened clones with GeneJET Plasmid Miniprep Kit (Thermo Scientific, The Netherlands) and the insert was sequenced either at the DNA Diagnostic Center of Nijmegen University Medical Center (Nijmegen, The Netherlands) or at BaseClear BV (Leiden, The Netherlands) using the M13F primer (5′TTTCCCAGTCACGACGTTG3′). The quality of sequences was checked with the Chromas lite 2.01 (Technelysium Pty Ltd, Australia) software. All DNA sequences obtained were aligned against the Silva reference database prior to import into the ARB database of SILVA SSU Ref dataset (release 119; http://www.arb-silva.de). ARB version 5.5 was used for phylogenetic comparison. Phylogenetic trees were calculated using a neighbor-joining algorithm with Jukes–Cantor correction. A distance matrix was calculated for all sequences. Representative sequences were deposited at GenBank under the accession numbers KT189180–KT189183 for *M. nitroreducens-like* sequences and KT189184–KT189187 for NC10 sequences.

Soil incubations

Soil samples of bulk soil taken at 10–20 cm depth were used for incubation assays. Soil slurry was prepared by sieving approximately 1 kg of the wet soil through a 0.2 μm sieve to remove roots and stones. Thereafter the sieved soil was mixed with the mineral salt medium as described previously (Ettwig *et al.* 2008). Incubations were done in 120 ml serum bottles with 60 ml of soil slurry, which corresponded to about 14 g dry weight of soil. Bottles were sealed with red butyl rubber stoppers and crimp-caps. The headspace was exchanged with Ar/CO_2 by five cycles of vacuuming and gassing and left with 0.5 bar overpressure. Each treatment was done in triplicate and left untreated as control without any additions, treated by addition of 10% CH_4 v/v, or treated by addition of 10% CH_4 v/v and 5 mM $NaNO_3$ or 2.5 mM $NaNO_2$ (final concentration). Headspace measurements to quantify the CH_4 concentration were carried out by gas chromatography (Hewlett Packard 5890) and the measurements of $^{13}CO_2$ were carried out by gas chromatography–mass spectrometry (Agilent 6890 and 5975C inert MSD, USA) as described previously (Ettwig *et al.* 2009).

RESULTS

Bacterial and archaeal communities in bulk soil and rhizosphere

The total microbial community of the Vercelli paddy field soil and rhizosphere compartment was analyzed by amplicon sequencing of 16S rRNA gene sequences. The sequencing resulted in approximately 18 000–784 000 sequences of high quality per library (see Tables 1 and 2). Archaeal and bacterial community composition was different between bulk soil and rhizosphere (Table 1). In the bulk soil, a very large diversity of bacterial phylogenetic groups was observed, with most of the reads assigned to *Proteobacteria* (32%), followed by *Acidobacteria* (20%). In the rhizosphere the diversity was lower with almost half (46%) of the reads belong to *Firmicutes* followed by *Proteobacteria* (39%).

Differences between the compartments were also evident within *Proteobacteria* at the class level. In the rhizosphere *Gamma-* (28%) and *Alpha-Proteobacteria* (6%) were more represented, while in the bulk soil the dominant classes comprised *Delta-* (15%), *Beta-* (8%) and *Alpha-Proteobacteria* (6%). Sequences related to the methane-oxidizing NC10 phylum made up 0.02% in the rhizosphere and 0.6% in the bulk soil. A detailed look at type I (*Gamma-Proteobacteria*) and type II (*Alpha-Proteobacteria*) aerobic methanotrophs revealed that they made up less than 1% of bacterial reads in both compartments.

Verrucomicrobia could be detected in both compartments; however, sequences related (91% identity) to *Methylacidiphilum*, known to carry out aerobic methane oxidation in acidic environments, were only found in the soil compartment (0.01%).

The archaea showed a similar trend for diversity as the bacteria: the bulk soil had a more diverse community than the rhizosphere. *Euryarchaeota* were more abundant than *Thaumarcheota* in both compartments (86% in rhizosphere and 51% in bulk soil). In the rhizosphere, the *Methanobacteriaceae* (44%) were most abundant, followed by *Methanomicrobia* (41%) and the soil Crenarchaeotic group (SCG; 12%). In bulk soil, *Methanomicrobia* dominated (44%), followed by the miscellaneous Crenarchaeotic group (MCG; 36%), SCG (10%) and *Methanobacteriaceae* (6%).

Methanomicrobia were analyzed further to the level of families, showing a clear differentiation in relative abundance in bulk soil and rhizosphere. The GOM Arc I group, comprising '*Candidatus* M. nitroreducens' made up 21% of *Methanomicrobia* in the bulk soil compared with 2% in the rhizosphere (9 and 0.7% of all archaea respectively) (Fig. 1).

Table 1. Distribution of sequence reads of the bacterial 16S rRNA gene amplification. Only phyla making up ≥1% of reads in at least one of the two compartments are shown. Reads were assigned to phylogenetic groups based on the SILVA NGS pipeline (96 990 reads for the rhizosphere and 783 980 reads for the bulk soil). Phyla comprising potential methanotrophs are highlighted in bold. For the *Proteobacteria*, the individual abundance of known methanotrophic genera is shown.

	Percentage of total reads	
Phylogenetic group	Root compartment (%)	Soil compartment (%)
Acidobacteria	1	20
Actinobacteria	7	9
Bacteroidetes	2	2
Candidate division WS3	0	2
Chloroflexi	3	14
Cyanobacteria	1	0
Firmicutes	46	3
Gemmatimonadetes	0	7
Verrucomicrobia	0.1	2
Nitrospirae	0	3
Planctomycetes	0	3
Proteobacteria	39	32
Alpha-	6	6
Methylosinus	0.2	0.09
Methylocystis	0.04	0.009
Methylocapsa	0.008	0.003
Beta-	2	8
Delta-	2	15
Gamma-	28	2
Methylomicrobium	0.004	0.08
Methylomonas	0.05	0.03
Methylocaldum	0.04	0.01
Methylobacter	0.3	0.05
Methylosarcina	0.02	0.01
Methylococcus	0	0.001
Crenothrix	0	0.0009
Methylosoma	0	0.0004
NC10 phylum	0.02	0.6

Table 2. Distribution of sequence reads of the archaeal 16S rRNA gene amplification, with the known methanotrophic *Methanoperedens nitroreducens* of the GOM Arc I group (marked in bold). Groups making up ≥1% of reads in at least one of the two compartments are shown (18 162 reads for the rhizosphere and 81 078 reads for the bulk soil). Reads were assigned to phylogenetic groups based on the SILVA NGS pipeline.

	Percentage of total reads	
Archaea clade	Rhizosphere compartment (%)	Bulk soil compartment (%)
Euryarcheota	**86**	**51**
Haloarchaea	0	1
Methanobacteria	44	6
Methanomicrobia	41	44
GOM Arc I	**0.7**	**9**
Thermoplasmata	0	1
Thaumarcheota	**14**	**49**
MCG[a]	2	36
SCG[b]	12	10
Group C3	0	1
SAGMCG-1[c]	0	1

[a]Miscellaneous Crenarchaeotic group. [b]Soil Crenarchaeotic group. [c]South African gold mine Crenarchaeotic group 1.

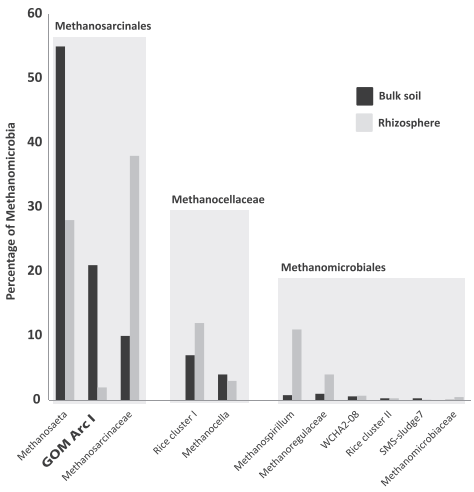

Figure 1. Distribution of 16S rRNA gene reads belonging to the order of Methanomicrobia in the bulk soil and in the rhizosphere of the Vercelli paddy field. Values are expressed as percentage of total Methanomicrobial sequences.

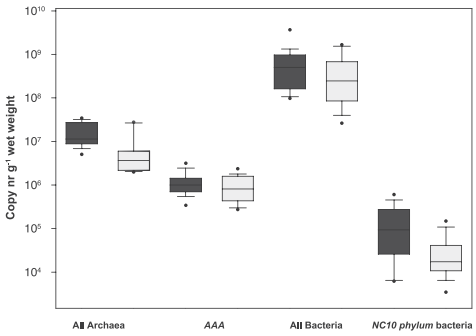

Figure 2. Boxplot of abundance of all archaea, AAA, all bacteria and NC10 phylum bacteria as assessed by quantitative PCR of the 16S rRNA gene in bulk soil (black boxes) and rhizosphere (gray boxes). The horizontal line represents the median and the circles mark the 5th and 95th percentiles. The box upper and lower lines represent the 75th and 25th percentiles respectively. Error bar represents standard deviation.

Quantification of total bacteria, archaea and subgroups of known anaerobic methanotrophs

The qPCR results indicated that the total bacterial abundance was one to two orders of magnitude higher than the archaeal abundance in both bulk soil and rhizosphere. Among the targeted anaerobic methanotrophs, AAA were present in higher gene copy numbers than NC10 phylum bacteria (Fig. 2).

In bulk soil, AAA were approximately seven times more abundant than NC10 phylum bacteria, and in the rhizosphere even 28 times (Fig. 2). This indicates that in both compartments known nitrate-dependent AOM archaea outnumbered nitrite-dependent AOM-mediating bacteria at the 16S rRNA gene level.

Phylogenetic diversity of NC10 phylum bacteria and AAA ('*Candidatus* Methanoperedens nitroreducens'-like archaea)

In addition to the Ion Torrent sequencing, we cloned and Sanger-sequenced longer PCR products of the NC10 phylum 16S rRNA gene for detailed phylogenetic analysis. All obtained sequences (n = 22) clustered into group B (Ettwig *et al.* 2009) (Fig. 3). Sequenced products of the AAA (n = 9) confirmed the identity of the obtained sequences to be more than 99% similar to the AAA from the original Nijmegen enrichment culture (DQ369741, Raghoebarsing *et al.* 2006) and '*Candidatus* M. nitroreducens' (ANME2D_RS06450, Haroon *et al.* 2013) (Fig. 4). Out of all sequences obtained, four representatives of each were submitted to GenBank.

Soil slurry incubations

In order to estimate the rate of nitrate- and nitrite-dependent anaerobic oxidation of methane (AOM), soil slurries were incubated with and without ^{13}C-labelled methane as control and with methane in the presence of either nitrite or nitrate. The concentrations of CH_4 and $^{13}CO_2$ were measured and increase of atom fraction of $^{13}CO_2$ was calculated and used as indication of activity (Fig. 5).

After a lag phase of about a week, linear consumption of CH_4 started in the soil slurry samples supplemented with 5 mM nitrate. This correlated with production of $^{13}CO_2$ and a proportional increase of the atom fraction of $^{13}CO_2$. The average rate of AOM was 79.9 nmol g^{-1}_{dw} d^{-1} in the presence 5 mM nitrate. In the controls without any additions, added $^{13}CH_4$ and $^{13}CH_4$ with 2.5 mM nitrite, methane production prevailed over consumption with 432.3, 357.5 and 185.4 nmol g^{-1}_{dw} d^{-1}, respectively.

DISCUSSION

Paddy fields are important contributors to the emission of the greenhouse gas methane. Biogenic methane emission is the result of a delicate interplay between the presence and activity of methanogenic producers and methanotrophic consumers. Previous research into methane sinks in paddy fields has mainly focused on aerobic Proteobacterial methanotrophs, demonstrating that they can oxidize substantial parts of the methane produced in the anoxic parts of the paddy field soil (Krüger, Frenzel and Conrad 2001; Krause, Luke and Frenzel 2010; Lee *et al.* 2014). In the current study, we investigated the presence and activity of two recently discovered nitrite- and nitrate-dependent anaerobic methanotrophs, NC10 phylum bacteria and archaea of the AAA clade. We showed that AAA are present in significant numbers (up to 10^7 16S rRNA copies g^{-1}_{dw}) in bulk soil and rhizosphere in Vercelli paddy fields, and that they were a few orders of magnitude more abundant than the nitrite-dependent NC10 phylum bacteria.

The paddy field environment provides several distinct niches for microorganisms, for which the proximity of the roots is an important determinant modulating oxygen and organic carbon supply. The rhizosphere has been described as a microbial hotspot, where process rates are much faster compared with the bulk soil (Kuzyakov and Blagodatskaya 2015). For the Vercelli paddy fields, amplicon sequencing indicated a higher diversity of bacteria and archaea in the bulk soil than in the rhizosphere. Abundance, however, was not significantly different between rhizosphere and bulk soil of the paddy field: about 10^8 and 10^7 16S rRNA gene copies g^{-1}_{dw} for bacteria and archaea, respectively. This finding was consistent with previous studies: in soil and rhizosphere in the range 10^8–10^9 bacterial and 10^6–10^7 archaeal 16S rRNA gene copies g^{-1}_{ww} (Lee *et al.* 2015), and in soil 10^{10} bacterial and 10^8–10^9 archaeal 16S rRNA gene copies g^{-1}_{dw} (Ahn *et al.* 2012). The rhizosphere was dominated by only a few phyla, demonstrating the community-shaping power of the roots. The bulk soil microbial community was dominated by *Acidobacteria* and *Chloroflexi*, which were also found in several other studies as dominant phyla, sometimes together with *Proteobacteria* (Ahn *et al.* 2012, 2014; Lee *et al.* 2015). The rhizosphere, in contrast, was clearly dominated by one phylum: almost half of the sequences obtained from the rhizosphere were classified as *Firmicutes*. As roots secrete a considerable amount of fermentable poly- and monomeric sugars, the dominance or high abundance of these fermenting microorganisms in the rhizosphere was to be expected and has been demonstrated also in previous studies (Aslam *et al.* 2013; Edwards *et al.* 2015). Many *Firmicutes* release hydrogen and acetate to the environment, which serve as substrates for methanogens. Consistent with previous findings (Daebeler, Gansen and Frenzel 2013), we found that methanogenic archaea in the rhizosphere belonged mostly to hydrogenotrophic and methylotrophic groups, whereas in the bulk soil strictly acetoclastic *Methanosarcinales* (*Methanosaeta*) dominated. Hydrogenotrophic methanogens of the *Methanocellales* or RC I have been reported to be the key players in various paddy fields (Conrad and Klose 2006; Erkel *et al.* 2006;

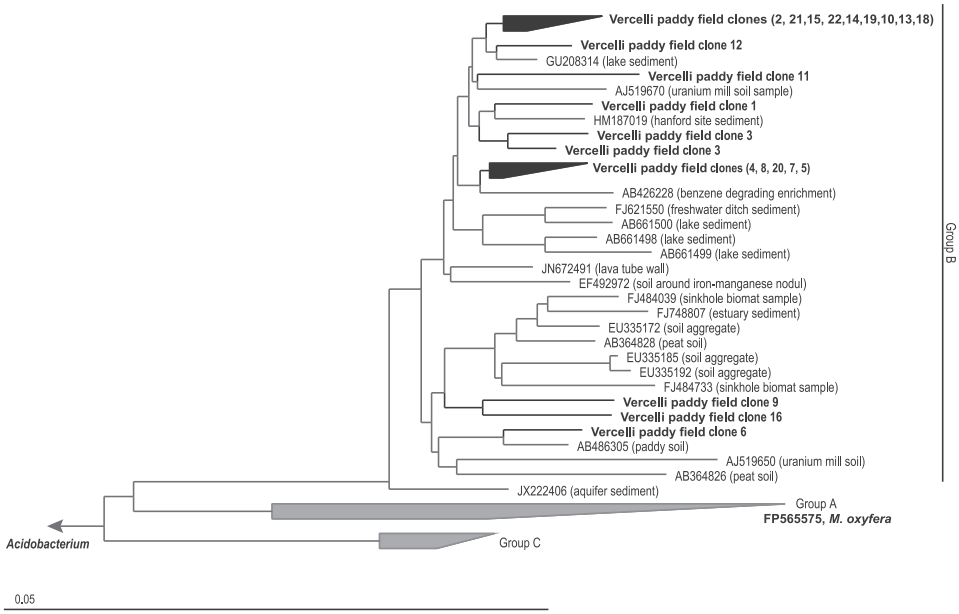

Figure 3. Phylogenetic tree of NC10 phylum bacteria 16S rRNA sequences (841 bp). The tree includes clones that were derived from this study (marked bold) together with a selection of publicly available sequences of environmental samples as well as reference sequence of *M. oxyfera* (acc. no. FP565575, in group A). This covers the whole known diversity of NC10 phylum bacteria. The tree was computed using the neighbor-joining algorithm with Jukes–Cantor correction and a positional variability filter was applied. *Acidobacteria* were used as the outgroup.

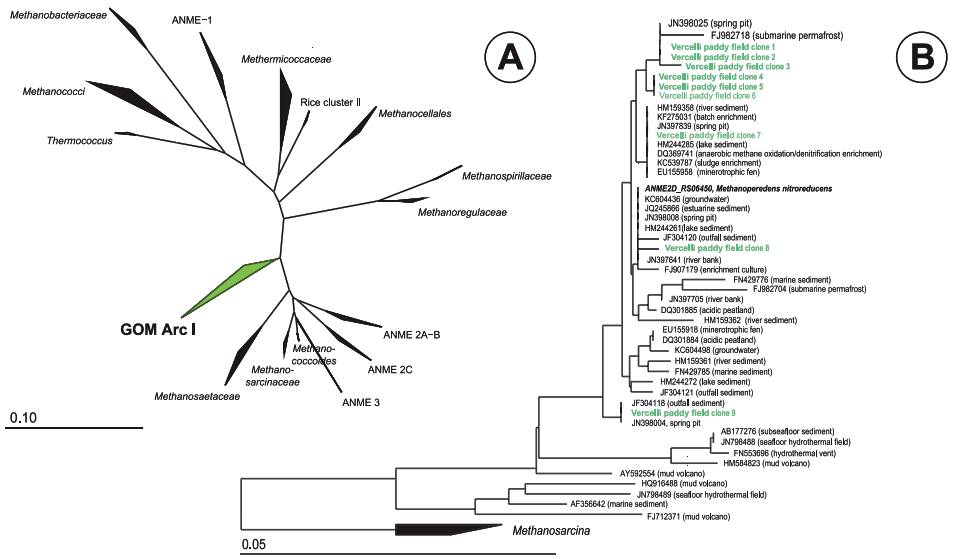

Figure 4. (**A**) Phylogenetic overview of methanogenic and methanotrophic archaea based on 16S rRNA gene sequences. The phylogenetic position of the GOM Arc I archaea is marked in green. (**B**) Detailed presentation of the GOM Arc I clade. Clones (297 and 864 bp) obtained from paddy field are shown in green in relation to other putatively methanotrophic archaea. The neighbor-joining phylogenetic tree was calculated using the Jukes–Cantor correction and *Methanosarcina* as the outgroup.

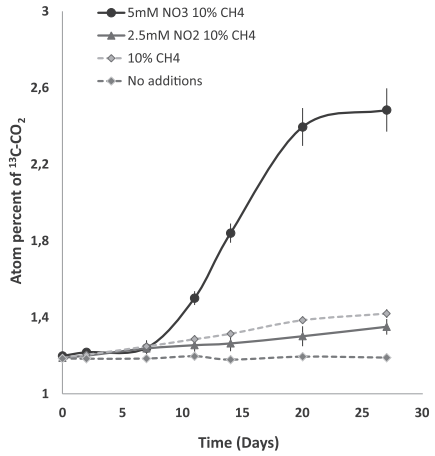

Figure 5. Atom fraction of $^{13}CO_2$ in soil slurries incubated with 10% $^{13}CH_4$ with addition of 5 mM $NaNO_3$ or 2.5 mM $NaNO_2$. As controls soil slurry was incubated with and without 10% $^{13}CH_4$. Measurements were made in triplicate with error bar representing standard deviation.

Watanabe, Kimura and Asakawa 2009). Also versatile representatives of the *Methanosarcinales*, which may use both acetoclastic and hydrogenotrophic pathways for methane production, were four times more abundant in the rhizosphere.

Besides fermentable compounds, roots also secrete O_2, and create microenvironments where oxygen is available simultaneously with methane—favorable conditions for aerobic methanotrophs. Aerobic *Alpha-* and *Gamma-Proteobacteria* methanotrophs were found to make up 0.66% of bacterial reads in rhizosphere and 0.28% in bulk soil, which is comparable with a Korean rice field study where 0.25–3.27% of bacterial reads were assigned to methanotrophs (Lee *et al.* 2015). Besides the Proteobacterial methane oxidizers well-known from paddy fields, we also found, albeit at low numbers, sequences most closely related (91% sequence identity) to the methanotrophic cluster within *Verrucomicrobia* (*Methylacidiphilum*) in the bulk soil. Members of this group have been shown to aerobically oxidize methane; however, they have so far only been found in acidic environments (Op den Camp *et al.* 2009; Sharp *et al.* 2014; van Teeseling *et al.* 2014), whereas the Vercelli paddy field soil has a more neutral pH. Based on 16S rRNA gene sequence comparison alone, it cannot be concluded whether these *Verrucomicrobia* possess the necessary methanotrophic pathways (Op den Camp *et al.* 2009) and contribute to methane oxidation in this paddy field.

In contrast to the aerobic bacterial methane oxidizers, sequences of putative anaerobic bacterial methanotrophs belonging to the NC10 phylum were present only in relatively low copy numbers. Comparably low numbers have been found previously in subtropical Chinese paddy soils (Jiaxing): 6.5×10^3 (60–70 cm) to 7.5×10^4 (30–40 cm) 16S rRNA copies g^{-1} dry soil (Wang *et al.* 2012), whereas copy numbers that were orders of magnitude higher (1.5×10^6 to 4.5×10^6 copies g^{-1} dry soil) have been observed in another Chinese paddy field (Hu *et al.* 2014) albeit at greater depth (60–100 cm). In contrast to the latter study, we only retrieved 16S rRNA sequences belonging to group B of the NC10 phylum, for which to date no methane oxidation activity has been documented. Until now, in all enrichment cultures of NC10 phylum bacteria with demonstrated methane oxidizing activity, only group A was dominant (Ettwig *et al.* 2009; Hu *et al.* 2009; Luesken *et al.* 2011; Zhu *et al.* 2012; Hu *et al.* 2015). It therefore remains speculative whether other groups of the NC10 phylum are involved in anaerobic methane oxidation.

The major finding of our study was the detection of a sizeable population of AAA, archaeal nitrate-reducing methanotrophs including '*Candidatus* M. nitroreducens'. AAA 16S rRNA gene copy numbers were two orders of magnitude higher than those of NC10 phylum bacteria (10^6–10^7 *vs* 10^4–10^5 16S rRNA gene copies g^{-1}_{dw}), as judged by qPCR. To date, only limited genome information is available for these organisms: two genomes of NC10 phylum bacteria are published (Ettwig *et al.* 2010; Hug *et al.* 2016) and two AAA genomes are available (Haroon *et al.* 2013; Arshad *et al.* 2015). All of these genomes possess a single 16S rRNA gene copy indicating a direct link between 16S rRNA copy numbers and cell abundances.

The factors that determine the outcome of competition between NC10 phylum bacteria and AAA are not certain at present, but are likely to include redox potential and oxygen exposure (with NC10 bacteria likely being more tolerant), as well as nitrate, nitrite and methane availability. Low methane concentrations probably rather favor NC10 bacteria with their high methane affinity of about 5–10 μM (Ettwig *et al.* 2008; Winkler *et al.* 2015), compared with the millimolar range of methanotrophic archaea (Nauhaus *et al.* 2002). In planted and submerged Vercelli paddy fields, methane fluxes of 5–36 mmol m^{-2} day^{-1} were measured and the porewater methane concentrations ranged over the planting season between 0.125 and 0.7 mM (measurement for top 60 mm), being highly variable and dependent on several factors (Gilbert 1995).

Several previous studies had already found AAA populations in paddy fields, even in Vercelli soils (Lueders *et al.* 2001; Conrad *et al.* 2008), but classified them as unidentified *mcrA* clusters (Xu *et al.* 2012) or placed them together with methanogens (Lueders *et al.* 2001; Conrad *et al.* 2008; Lee *et al.* 2015). The *mcrA* gene (encoding the α-subunit of methyl-coenzyme M reductase) is used as a marker gene for methanogens, but is also present in archaeal methanotrophs (Knittel and Boetius 2009). Its terminal restriction fragment of 506/507 bp as found in rice paddies studied by Conrad *et al.* (2008) and Xu *et al.* (2012) can be assigned to the *mcrA* of the AAA group, with nucleotide sequence identities >92% to clone A14 of the Dutch enrichment culture (EU495303) (Ettwig *et al.* 2008). In the Chinese paddy field investigated by Xu *et al.* (2012), it was altogether the second most abundant *mcrA* transcript—up to 50% in bulk soil. In a Korean rice field, the GOM Arc I group, comprising the AAA cluster, was found to increase in abundance with depth in both rhizosphere and bulk soil (Lee *et al.* 2015), with up to 60% of all archaeal sequences in bulk soil at 40 cm depth. These findings support an important role of AAA in rice fields. Our study is the first quantitative investigation of these methanotrophs.

To complement the molecular work, we also assessed the methanogenic and nitrite- and nitrate-dependent methane oxidation potential. Our soil slurry incubations demonstrated with and without CH_4 addition high methanogenic activity (432.3 and 357.5 nmol g^{-1}_{dw} d^{-1}, respectively), which is close to previously reported rates of about 547.2–590.4 nmol g^{-1}_{dw} d^{-1} for Vercelli paddy field (Conrad and Klose 2006). Methane production prevailing over methanotrophic activity in nitrite supplemented samples might be due to the low abundance of NC10 phylum bacteria.

The rate observed for nitrate-supplemented samples (79.9 nmol CH_4 g^{-1}_{dw} d^{-1}) was slightly higher than observed for sulphate-dependent methane oxidizing archaea in low-pressure temperate sediments (1–50 nmol CH_4 cm^{-3} d^{-1}; Knittel and Boetius 2009), and in very active marine methane seep sediments (up to 32.9 ± 13.9 nmol g^{-1}_{ww} d^{-1}) (Girguis *et al.* 2003). It clearly exceeded previously measured rates of nitrite-stimulated AOM in a Chinese paddy field (1.68–2.04 nmol g^{-1}_{dw} d^{-1}; Hu *et al.* 2014) by a factor of fifty, indicating that nitrate-dependent AOM may be an even more important methane sink than previously considered.

Assuming that the AAA are responsible for the major part of nitrate-dependent methane oxidation in our study, their estimated methane oxidizing activity would be on average 1.2 fmol d^{-1} per 16S rRNA copy in bulk soil, which also is in the same order of magnitude as quantified for ANME-2 archaea in marine environments (up to 70 fmol d^{-1} per 16S rRNA gene copy, Girguis *et al.* 2003). Compared with the cell-specific AOM rate of NC10 phylum bacteria, ranging from only 0.09 to 0.20 fmol d^{-1} per 16S rRNA gene copy in continuous cultures (Ettwig *et al.* 2009), the archaeal rates are slightly higher, which may also be related to their larger cell size.

To summarize, the methane oxidizing potential of the soil and the high abundance of archaeal methanotrophs observed with qPCR and amplicon sequencing both support a significant role of nitrate-dependent AOM in the investigated paddy field mediated by AAA.

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Conflict of interest. None declared.

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McrA primers for detection and quantification of the anaerobic archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens'

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METHODS AND PROTOCOLS

McrA primers for the detection and quantification of the anaerobic archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens'

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Abstract The nitrogen and methane cycles are important biogeochemical processes. Recently, '*Candidatus* Methanoperedens nitroreducens,' archaea that catalyze nitrate-dependent anaerobic oxidation of methane (AOM), were enriched, and their genomes were analyzed. Diagnostic molecular tools for the sensitive detection of '*Candidatus* M. nitroreducens' are not yet available. Here, we report the design of two novel *mcrA* primer combinations that specifically target the alpha sub-unit of the methyl-coenzyme M reductase (*mcrA*) gene of '*Candidatus* M. nitroreducens'. The first primer pair produces a fragment of 186-bp that can be used to quantify '*Candidatus* M. nitroreducens' cells, whereas the second primer pair yields an 1191-bp amplicon that is with sufficient length and well suited for more detailed phylogenetic analyses. Six different environmental samples were evaluated with the new qPCR primer pair, and the abundances were compared with those determined using primers for the 16S rRNA gene. The qPCR results indicated that the number of copies of the '*Candidatus* M. nitroreducens' *mcrA* gene was highest in rice field soil, with $5.6 \pm 0.8 \times 10^6$ copies g^{-1} wet weight, whereas Indonesian river sediment had only $4.6 \pm 2.7 \times 10^2$ copies g^{-1} wet weight. In addition to freshwater environments, sequences were also detected in marine sediment of the North Sea, which contained approximately $2.5 \pm 0.7 \times 10^4$ copies g^{-1} wet weight. Phylogenetic analysis revealed that the amplified 1191-bp *mcrA* gene sequences from the different environments all clustered together with available genome sequences of *mcrA* from known '*Candidatus* M. nitroreducens' archaea. Taken together, these results demonstrate the validity and utility of the new primers for the quantitative and sensitive detection of the *mcrA* gene sequences of these important nitrate-dependent AOM archaea. Furthermore, the newly obtained *mcrA* sequences will contribute to greater phylogenetic resolution of '*Candidatus* M. nitroreducens' sequences, which have been only poorly captured by general methanogenic *mcrA* primers.

Keywords '*Candidatus* Methanoperedens nitroreducens' · Anaerobic oxidation of methane · ANME · *mcrA*

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Introduction

Methane is an important greenhouse gas (GHG) that contributes approximately 20% to global warming (Myhre et al. 2013). Since the advent of industrialization, atmospheric concentrations of methane have increased by 150%, potentially further exacerbating climate change (Schwietzke et al. 2016). Evaluating the contribution of environmental microorganisms that produce or consume this significant GHG is essential for understanding methane sources and sinks and developing mitigation strategies for methane released into the atmosphere. Most research on microorganisms involved in the methane cycle has focused on aerobic methanotrophic bacteria that inhabit oxic environments or archaea that produce methane in anoxic

zones. However, recent studies have revealed that in the anoxic layers of soils and sediments, methane is consumed by anaerobic methanotrophic bacteria and/or archaea that use alternate electron acceptors such as nitrite, nitrate, or iron (Egger et al. 2015; Ettwig et al. 2010; Raghoebarsing et al. 2006).

Enrichment cultures inoculated with freshwater sediment exhibited coupling of the reduction of nitrite to the anaerobic oxidation of methane (Ettwig et al. 2008; Raghoebarsing et al. 2006). The corresponding nitrite-dependent methanotrophic bacteria were identified as belonging to the bacterial NC10 phylum and named '*Candidatus* Methylomirabilis oxyfera' (Ettwig et al. 2010). This microorganism exhibits an intra-aerobic metabolism in which nitric oxide is hypothesized to be dismutated to oxygen and nitrogen gas. The oxygen could subsequently be used by the canonical particulate methane monooxygenase encoded by *pmoCAB*.

Archaea that oxidize methane anaerobically were initially discovered in marine environments, where they carry out sulfate-dependent anaerobic oxidation of methane (S-AOM). These anaerobic methane-oxidizing archaea (ANME) have been estimated to oxidize up to 90% of released methane before it reaches the atmosphere (Hinrichs and Boetius 2002; Knittel and Boetius 2009). ANMEs are divided into three lineages, ANME-1, ANME-2, and ANME-3 (Knittel et al. 2005; Nauhaus et al. 2005; Stadnitskaia et al. 2005) and are further divided into sub-clades in some cases. All three lineages have been detected in marine and freshwater environments.

Recently, the genomes of ANME-2d archaea enriched in bioreactors fed with methane, nitrate, and ammonium or methane and nitrate were obtained (Arshad et al. 2015; Haroon et al. 2013). These Euryarchaea, which are capable of coupling nitrate reduction to anaerobic methane oxidation, were identified as '*Candidatus* Methanoperedens nitroreducens.' Phylogenetic analysis revealed that these archaea are related to *Methanosarcina* in the Methanosarcinales order (Haroon et al. 2013) and are classified as GOM Arc I in the ribosomal RNA (rRNA) SILVA database. The GOM Arc I consists of the ANME-2d group as well as the original GOM Arc I group with sequences from the Gulf of Mexico (Mills et al. 2003).

'*Candidatus* M. nitroreducens' possesses all genes of the (reverse) methanogenic pathway (Arshad et al. 2015; Haroon et al. 2013). The best-characterized enzyme of methanogenesis and AOM is methyl-coenzyme M reductase (MCR). In methanogenesis, MCR catalyzes the terminal step of the pathway, resulting in the release of methane. In the anaerobic oxidation of methane, MCR functions in a reverse mode (Hallam et al. 2003, 2004; Krüger et al. 2003), catalyzing the activation of methane (Krüger et al. 2003). The genomes of two '*Candidatus* M. nitroreducens' strains have been assembled and analyzed. In both genome assemblies, the complete reverse methanogenesis pathway including the *mcrABCDG* genes was identified (Arshad et al. 2015; Haroon et al. 2013), and the genomes contained only a single copy of the 16S rRNA and the *mcrA* gene. Furthermore, the enzymes for nitrate reduction to nitrite and nitrite reduction to ammonium appeared to be encoded by *narGH*- and *nrf*-type genes, respectively (Arshad et al. 2015).

For '*Candidatus* M. oxyfera' bacteria, specific primers for both the 16S rRNA gene and the *pmoA* gene have been designed (Ettwig et al. 2009; Luesken et al. 2011). Analyses of various environmental samples using these primers have demonstrated that '*Candidatus* M. oxyfera' is present in peat lands, lake sediments, wastewater treatment systems, rice fields, and various other anoxic environments (Deutzmann and Schink 2011; Hu et al. 2014; Zhou et al. 2014; Zhu et al. 2012). As nitrate concentrations in freshwater environments are generally higher than those of nitrite or sulfate, '*Candidatus* M. nitroreducens' may contribute significantly to nitrate-dependent AOM in these environments (Vaksmaa et al. 2016). To detect '*Candidatus* M. nitroreducens' in environmental samples, specific fluorescence in situ hybridization (FISH) probes have been designed (Schubert et al. 2011). The development of quantitative detection methods based on the 16S rRNA gene has also been reported (Ding et al. 2015).

Although the 16S rRNA gene is most commonly used for phylogenetic surveys, the *mcrA* gene is an alternative and more specific biomarker for the detection of methanogens and ANMEs in the environment. Although previously published *mcrA* primers were designed to mainly target all known methanogens and ANMEs, most have a strong bias toward certain methanogens or specific groups of ANMEs (Hales et al. 1996; Juottonen et al. 2006; Luton et al. 2002; Nunoura et al. 2008). Available general *mcrA* primers are not well suited to capturing *mcrA* sequences of '*Candidatus* M. nitroreducens' in the environment, potentially resulting in underrepresentation in molecular surveys. Furthermore, differentiating between phylogenetically closely related methanogens and methanotrophs is crucial to directly link observed diversity with the organisms responsible for either methane oxidation or methane production. In the current study, we developed two novel *mcrA* primer pairs that specifically target '*Candidatus* M. nitroreducens' for use in quantification and more refined phylogenetic analysis. We used these primers to study the distribution and abundance of '*Candidatus* M. nitroreducens' in various ecosystems. For comparison, we validated the use of 16S rRNA gene probes designed for FISH analysis as qPCR primers and compared the results with the diversity and abundance obtained with the novel *mcrA* primers.

Materials and methods

Environmental samples

Environmental samples were obtained from six different locations: rice field soils (Vercelli, Italy), sludge from a brewery

wastewater treatment plant (Lieshout, The Netherlands), North Sea sediment (The Netherlands), polluted Citarum River sediment (Indonesia), Jordan River sediment (UT, USA), and State Channel sediment (UT, USA). In addition to the environmental samples, an enrichment culture (AOM enrichment Vercelli) of '*Candidatus* M. nitroreducens' was used as a sample for primer validation (Vaksmaa et al in preparation). The samples were stored at −20 °C prior to DNA extraction. Detailed information on the geographic locations is presented in Table S1.

Primer design, DNA extraction, and PCR amplification

For primer design, 20,000 high-quality *mcrA* sequences deposited in the NCBI GenBank database (Benson et al. 2013) were downloaded and aligned, and the lengths of these sequences were inspected. From the alignment of 20,000 *mcrA* sequences, 45 available full-length *mcrA* sequences (two belonging to '*Candidatus* Methanoperedens nitroreducens') were used for primer design using the probe design tool implemented in ARB (Ludwig et al. 2004). The designed *mcrA* primer set McrA159F/McrA345R amplifies a 186-bp fragment and has a predicted annealing temperature of 62 °C. The McrA169F/McrA1360R primer pair yields a 1191-bp fragment. Detailed information on the *mcrA* primers and 16S rRNA primers used in this study is provided in Table 1. Commonly used general *mcrA* gene primers were in silico evaluated for their ability to target '*Candidatus* Methanoperedens nitroreducens,' and the number of mismatches is brought out in Table 2. For comparison, the 16S rRNA gene of '*Candidatus* Methanoperedens nitroreducens' was targeted with the clade-specific primers AAA641F and AAA834R (previously reported as FISH probes) (Schubert et al. 2011). These primers amplify a 212-bp fragment with an optimal annealing temperature of 60 °C. DNA was extracted from all samples with the PowerSoil® DNA Isolation Kit. First, 0.1–0.35 g of soil was weighed into the 2-ml tubes provided with the kit, which contained buffer and beads. The following steps were performed according to the manufacturer's protocol (MO BIO Laboratories Inc., Carlsbad, USA). DNA quantity was assessed using a microspectrophotometer (NanoDrop, ND-1000, Isogen Life Science, The Netherlands). All PCR reactions were performed using PerfeCTa Quanta master mix (Quanta Biosciences, Gaithersburg, USA) with the following composition: 1 μl each of 20 μM of the forward and reverse primers, 12.5 μl of PCR master mix and 9.5 μl of Milli-Q water. The PCR temperature gradient program was 96 °C for 5 min, followed by 45 cycles of 96 °C for 30 s, gradient (55–68 °C) for 45 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min.

Cloning, sequencing, and phylogenetic analysis

The sizes of the PCR products obtained with the McrA159F/McrA345R, McrA169F/McrA1360R, or AAA641F/AAA834R primer pairs were evaluated by gel electrophoresis on 1% agarose gels. The fragments were purified using the GeneJET PCR purification kit according to the manufacturer's protocol (Thermo Scientific, Landsmeer, The Netherlands). The amplified PCR products were cloned using the pGEM-T Easy cloning vector (Promega, USA) and used to transform *E. coli* XL1 Blue competent cells. The cells were plated on Luria-Bertani (LB) agar plates containing 20 μl of 100 mg/ml ampicillin, 35 μl of 2% X-Gal, and 35 μl of 100 mM IPTG. The plates were incubated at 37 °C overnight. Colony PCR was performed by direct PCR using the M13F and M13R primers. The PCR program consisted of initialization at 96 °C for 10 min, followed by 40 cycles of amplification at 96 °C for 45 s, 57 °C for 30 s, and 72 °C for 30 s and a final elongation step at 72 °C for 5 min. The colonies resulting in amplification of a fragment of the correct size were grown in 5 ml of LB medium overnight at 37 °C prior to plasmid isolation with a GeneJET Plasmid Miniprep Kit (Thermo Scientific, The Netherlands). The inserts were sequenced at

Table 1 List of PCR and qPCR primers used for mcrA and 16S rRNA gene amplification

Primer name	Sequence 5′–3′	Nr of bases	Primer binding site 5′ to 3′	Tm (°C)	GC (%)	Product size (bp)
McrA159F	AAAGTGCGGAGCAG CAATCACC	22	159–181	66.5	55	186
McrA345R	TCGTCCCATTCCTGCTG CATTGC	23	322–345	71	57	
McrA169F	GCA GCA ATC ACC AAG AAG AGA GG	23	169–192	59.9	52	1191
McrA1360R	TGCCTCTTTGTGGA GGTACATGGA	24	1336–1360	65.6	50	
16S rRNA AAA641F	ACTGDTAGGCTTG GGACC	17	576–593	51.4	59	193
16S rRNA AAA834R	ATGCGGTCGCACCG CACCTG	20	768–788	72.6	70	

Primer binding site refers to the nucleotide position at the *mcrA* gene of '*Candidatus* M. nitroreducens'

BaseClear B.V. (Leiden, Netherlands) or Macrogen (Amsterdam, Netherlands). For short fragments, the MF primer (5′TTTCCCAGTCACGACGTTG′3) was used, and to retrieve longer fragments, sequencing was also performed with the MR primer (5′GGATAACAATTTCACACAGG′3). The quality of the sequences was assessed with the Chromas Lite 2.01 (Technelysium Pty Ltd., Australia) software. All DNA sequences were imported into the *mcrA* ARB database. ARB version 5.5 was used for phylogenetic comparison (Ludwig et al. 2004). Phylogenetic trees based on the DNA sequences were calculated using the neighbor-joining algorithm with the Jukes-Cantor correction. Sequences were further analyzed by BLASTn and BLASTx at NCBI (Altschul et al. 1990).

Quantification by qPCR

The *mcrA* and 16S rRNA gene copy numbers in the environmental samples were quantified with the primer set McrA159F/McrA345R and the 16S rRNA gene primers AAA641F/AAA834R. All qPCR reactions were performed using PerfeCTa Quanta master mix (Quanta Biosciences, Gaithersburg, USA) and 96-well optical plates (Bio-Rad Laboratories, Hercules, England). Each reaction was performed in triplicate on duplicate DNA extractions. All reactions were performed using the Bio-Rad IQ™ 5 cycler (Biorad, USA). Negative controls were added to each plate by replacing the sample volume with autoclaved Milli-Q water. Standard curves were constructed by tenfold serial dilution of a known copy number of the pGEM-T easy plasmid with inserted DNA of the target gene.

In silico evaluation of 16S rRNA primers

The specificity and intra-group coverage of the 16S rRNA gene primers DP397F/DP569R (Ding et al. 2015) and the primers AAA641F/AAA834R, which target '*Candidatus* M. nitroreducens,' were evaluated. The comparison was carried out in ARB (Ludwig et al. 2004) using the GOM Arc I group as a representative group for '*Candidatus* M. nitroreducens' and related sequences. The specificity and intra-group coverage of both primer sets were evaluated using the non-redundant version of the SILVA SSU Ref dataset (release 119; (Quast et al. 2013)), which contains 535,004 high-quality 16S rRNA gene sequences, of which 109 belong to GOM Arc I.

Nucleotide sequence accession numbers

Representative sequences were deposited at GenBank under the accession numbers KX290067–KX290105 for *mcrA* sequences amplified with the McrA159F/McrA345R primers and under the accession numbers KX290017–KX290044 for *mcrA* sequences obtained with the primers McrA169F/McrA1360R. The 16S rRNA gene sequences were deposited under accession numbers KX290045–KX290065.

Results

Specificity of the novel *mcrA* primers for qPCR

To design an *mcrA* primer set specific for '*Candidatus* M. nitroreducens,' available full-length *mcrA* sequences (45 sequences) covering the diversity of known methanotrophs and methanogens were aligned and used for primer design. Representative sequences and the primer-binding positions are depicted in Fig. 1. Two sites at an appropriate distance for qPCR amplification (at nucleotide positions 159–181 and 322–345, respectively) were conserved between the two '*Candidatus* M. nitroreducens' sequences but were different in all other archaeal *mcrA* sequences. The resulting McrA159F/McrA345R primer pair amplifies a fragment of 186 bp, suitable for qPCR. The forward primer McrA159F possesses four mismatches with the *mcrA* sequence of the closest methanogen, *Methanobacterium alcaliphilum.* The reverse primer McrA345R possesses three mismatches with the *mcrA* sequences of the methanogens *Methanothermobacter wolfeii* and *Methanohalophilus halophilus*. The optimal annealing temperature of 62 °C was determined by gradient PCR using DNA extracted from rice field soil. All 40 PCR products amplified from DNA from the environmental samples and the enrichment culture were cloned and sequenced and corresponded to the expected part of the *mcrA* gene. The sequencing resulted in five to seven clone sequences per each environmental sample. All of the sequences had very high similarity to the *mcrA* gene of the two described '*Candidatus* M. nitroreducens' strains (91–100% at the nucleotide level and 97–100% at the amino acid level (Table S2)).

qPCR quantification of '*Candidatus* Methanoperedens nitroreducens' *mcrA* and 16S rRNA gene copies in environmental samples

The newly designed *mcrA* primers McrA159F/McrA345R were used with DNA extracted from six environmental samples. In addition, the results were compared with the copy numbers obtained with the primers AAA641F/AAA834R targeting the 16S rRNA gene of '*Candidatus* M. nitroreducens.'

Two 16S rRNA primer sets, the primer pairs DP397F/DP569R (Ding et al. 2015) and AAA641F/AAA834R, have been proposed to target '*Candidatus* M. nitroreducens' and the GOM Arc I group, respectively. Here, we analyzed the applicability of these primer sets in silico as specific qPCR primers to target the GOM Arc I group. The intra-group coverage and the number of out-

Fig. 1 Excerpt of the full-length sequence alignment of the *mcrA* genes of anaerobic methanotrophs and methanogens. The binding sites and conserved positions of the McrA159F forward and McrA345R reverse primers are indicated in *2*

group targets with one to three allowed mismatches are presented in Table 3. The primer pair AAA641F/AAA834R exhibited higher intra-group coverage (65–84%) than the DP397F/DP569R primers, which covered less than 60% of the GOM Arc I sequences at zero mismatch. Thus, we experimentally tested the AAA641F/AAA834R primers using DNA from the environmental samples and the enrichment culture and sequenced the PCR products. Twenty-one of the resultant clone sequences were highly similar to the 16S rRNA gene sequences of the two described '*Candidatus* M. nitroreducens' strains, whereas two clone sequences did not correspond to '*Candidatus* M. nitroreducens' (Table S3).

In the qPCR analysis, the highest '*Candidatus* M. nitroreducens' copy numbers were obtained in rice field soil, with an average *mcrA* gene copy number of $5.6 \pm 0.8 \times 10^6$ copies g^{-1} wet weight and an average 16S rRNA gene

Table 2 Overview of mcrA primers commonly used to amplify methanogenic and methanotrophic communities

Author	Primer name	Nr of bp	Binding position	Primer sequence 5′–3′	Mismatches
Luton et al. (2002)	ML-F	32	1021–1053	GGTGGTGTMGGATTCACACARTA YGCWACAGC	6
	ML-R	23	1468–1491	TTCATTGCRTAGTTWGGRTAGTT	3
Springer (1995)	McrAF	17	988–1005	TAYGAYCARATHTGGYT	5
	McrAR	17	1477–1491	ACRTTCATNGCRTARTT	4
Hales et al. (1996)	ME1F	20	727–747	GCMATGCARATHGGWATGTC	6
	ME2R	21	1469–1490	TCATKGCRTAGTTDGGRTAGT	4
	ME3F	20	1036–1056	GGTGGHGTMGGWTTCACACA	5
Nunoura et al. (2006)	Type c–d ANME-2	24	984–1008	GCTCTACGACCAGATMTGGCTTGG	3
		25	1058–1083	CCGTAGTACGTGAAGTCATCCAGCA	9
Nunoura et al. (2006)	Type e	25	1220–1245	CHCTGGAAGATCACTTCGGTGGTTC	5
		24	1363–1387	RTATCCGAAGAARCCSAGTCKRCC	5
Nunoura et al. (2006)	Type a-b ANME-1	20	1000–1020	TGGTTCGGAACGTACATGTC	4
		20	1562–1582	TCTYYTCCAGRATGTCCATG	6
Nunoura et al. (2008)	ME3MF	23	1015–1038	ATGTCNGGTGGHGTMGGSTTYAC	5
	ME3MF-3	23	1015–1038	ATGAGCGGTGGTGTCGGTTTCAC	6
Present study	McrA 159F	22	159–181	AAAGTGCGGAGCAGCAATCACC	0
Present study	McrA 345R	23	322–345	TCGTCCCATTCCTGCTGCATTGC	0

The number of mismatches to the '*Candidatus* M. nitroreducens' mcrA sequences is indicated

Table 3 The specificity and fidelity of previously described 16S rRNA gene primers for the total GOM Arc I group

	Intra-group coverage of GOM Arc I (%)				Hits in non-GOM Arc I			
	Mismatches				Mismatches			
	0	1	2	3+	0	1	2	3+
DP397F	23	64	79	85	*3*	*8*	*249*	2221
DP569R	59	*83*	*84*	*85*	*1*	44	343	*1737*
AAA641F	*71*	*89*	*92*	*92*	21	616	3184	9751
AAA834R	*65*	78	80	84	7	*7*	*26*	*76*

The intra-group coverage and the number of non-target hits are shown with up to three mismatches. The highest intra-group coverage and the lowest number of out-group targets per primer are marked in italics

abundance of $1.3 \pm 0.3 \times 10^8$ copies g^{-1} wet weight. Rice field soil was followed by river sediment (State Channel, USA; $4.4 \pm 4.4 \times 10^5$ *mcrA* gene copies g^{-1} wet weight and $1.8 \pm 0.6 \times 10^7$ 16S rRNA gene copies g^{-1} wet weight), wastewater treatment plant sludge ($1.2 \pm 0.8 \times 10^5$ *mcrA* gene copies g^{-1} wet weight and $6.7 \pm 2.2 \times 10^7$ 16S rRNA gene copies g^{-1} wet weight), Indonesian river sediment ($3.0 \pm 0.7 \times 10^4$ *mcrA* gene copies g^{-1} wet weight and $4.2 \pm 2.2 \times 10^6$ 16S rRNA gene copies g^{-1} wet weight), and North Sea sediment ($2.5 \pm 0.7 \times 10^4$ *mcrA* gene copies g^{-1} wet weight and $4.5 \pm 0.3 \times 10^6$ 16S rRNA gene copies g^{-1} wet weight). The lowest abundance was recorded in the sediment of the Jordan River (UT, USA), where qPCR did not result in any 16S rRNA gene amplification and only $4.6 \pm 2.7 \times 10^2$ copies of the *mcrA* gene g^{-1} wet weight were detected (Fig. 2).

Phylogenetic analysis

In addition to the qPCR primers McrA159F/McrA345R, a second primer set was designed to amplify longer *mcrA* fragments. Conserved regions were identified at nucleotide positions 169–192 and 1336–1360. The resulting primer set, McrA169F/McrA1360R, amplifies a fragment of 1191 bp, suitable for detailed phylogenetic analysis. The primers were again tested using DNA extracted from the environmental samples and the enrichment culture as described in the "Materials and methods" section. Amplification resulted in a single band of the expected size, and sequence analysis indicated that all 40 sequences were highly similar to '*Candidatus* M. nitroreducens.' The phylogenetic positions of these clones are depicted in Fig. 3. Clustering of sequences from the same environment was not observed, although all sequences clustered more closely with '*Candidatus* Methanoperedens sp. DS-2015' than '*Candidatus* Methanoperedens nitroreducens ANME-2d.' On average, the sequences exhibited higher identity to '*Candidatus* Methanoperedens sp. DS-2015' (87–99% nucleotide sequence identity) than to '*Candidatus* Methanoperedens nitroreducens ANME-2d' (85–90% nucleotide sequence identity). The sequence identities of all clones to the two described strains are provided in Table S4.

Discussion

In this study, we developed specific and sensitive molecular detection tools to target nitrate-dependent anaerobic methanotrophic '*Candidatus* M. nitroreducens' archaea. We designed two novel PCR primer sets for the *mcrA* gene of '*Candidatus* M. nitroreducens,' thus providing a straightforward detection and quantification method. The primer set McrA159F/McrA345R results in the amplification of a 186-bp fragment and is suitable for quantification of *mcrA* gene copies by qPCR. The other primer set, McrA169F/McrA1360R, results in the amplification of a 1191-bp fragment that can be used in more accurate and detailed phylogenetic analyses.

The genomes of known '*Candidatus* M. nitroreducens' strains possess only a single copy of the 16S rRNA gene and the *mcrA* gene, although copy numbers might differ for non-cultivated species. However, the copy numbers in the environmental samples obtained with the 16S rRNA gene primers were approximately two orders of magnitude higher than the copy numbers obtained with the *mcrA* primers. The newly designed *mcrA* primers are highly specific, whereas the 16S rRNA gene primers used in this study have the potential to amplify sequences from the whole GOM Arc I clade, possibly capturing a larger diversity of sequences that are less related to '*Candidatus* M. nitroreducens'. The target specificity was reflected in the sequence diversity: the sequenced PCR products obtained with the qPCR primer combination McrA159F/McrA345R all corresponded to the '*Candidatus* M. nitroreducens' *mcrA* gene (97–100% identity at the amino acid level), whereas the sequenced PCR products of the 16S rRNA gene also included sequences (9%) that could be identified as closely related methanogens. This difference in specificity further suggests that the results obtained with these 16S rRNA gene PCR primers may overestimate the copy numbers of '*Candidatus* M. nitroreducens' in the environment. Overall, the *mcrA* primers were more specific, and qPCR quantification of *mcrA* copy numbers may more accurately reflect the number of '*Candidatus* M. nitroreducens' cells in a specific environment.

Among the different environments, '*Candidatus* M. nitroreducens' was most abundant in rice field soil, followed

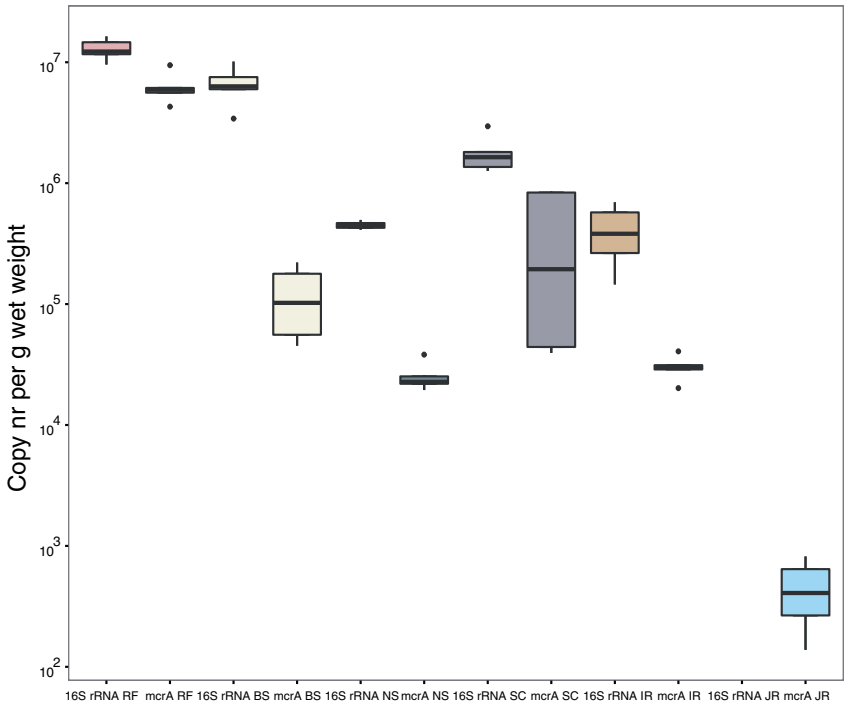

Fig. 2 Boxplot depicting the abundance of '*Candidatus* M. nitroreducens' in environmental samples as assessed by quantitative PCR of the 16S rRNA gene and *mcrA* gene. For each sample, six independent qPCR reactions of two DNA samples were performed. The environmental samples originated from rice field soil (RF), wastewater treatment plant sludge (BS), North Sea sediment (NS), State Channel sediment (SC), Indonesian river sediment (IR), and Jordan River sediment (JR). The *horizontal line* within each *box* represents the median, and the *error bars* represent the standard deviation. The *upper* and *lower* in each *box lines* represent the 75 and 25 percentiles, respectively. For the Jordan River sediment, no amplification was detected with Methanoperedens-specific 16S rRNA gene primers

by wastewater treatment plant sludge. The lowest copy numbers were obtained in the investigated river sediments (Fig. 2). In a previous study (Ding et al. 2015), 16S rRNA gene primers were designed to quantify '*Candidatus* Methanoperedens nitroreducens' in two lake sediments, a river sediment, and a rice field soil sample. In that study, the total abundance of 16S rRNA gene copy numbers in rice field soil was one to two orders of magnitude lower than that obtained in the present study (3.72×10^4 to 2.30×10^5 copies μg^{-1} DNA versus $1.7 \pm 0.4 \times 10^6$ copies μg^{-1} DNA in this study). This variation may be due to differences in the environmental samples used; in addition, the 16S rRNA gene primers used in that study may have been more species-specific. Importantly, the relatively high gene copy numbers obtained in both studies suggest that these anaerobic methanotrophic archaea play a significant role in mediating nitrate-dependent AOM in rice fields and contribute to mitigating methane emissions to the atmosphere.

For accurate phylogenetic analysis, only a few '*Candidatus* M. nitroreducens' *mcrA* gene sequences with lengths greater than 500 bp are available in public databases. These sequences were derived from deep groundwater (Nyyssonen et al. 2012), paddy fields (Bao et al. 2014), river sediments (Jiang et al. 2011), and lake sediments (GenBank accession number JQ080004, unpublished). All of these sequences were retrieved with the general *mcrA* primer pair ME1F/ME2R, which yields a sequence length of 763 bp (Hales et al. 1996). These primers have a high number of mismatches with the two available full-length '*Candidatus* M. nitroreducens' *mcrA* sequences: six mismatches in the forward primer and five in the reverse primer. Thus, the presence of these microorganisms and their diversity in environmental studies may be underestimated because presently used primers simply do not capture them. These archaea have been assumed to be freshwater microorganisms, and thus, it is even more remarkable that we amplified both 16S rRNA and *mcrA* gene sequences

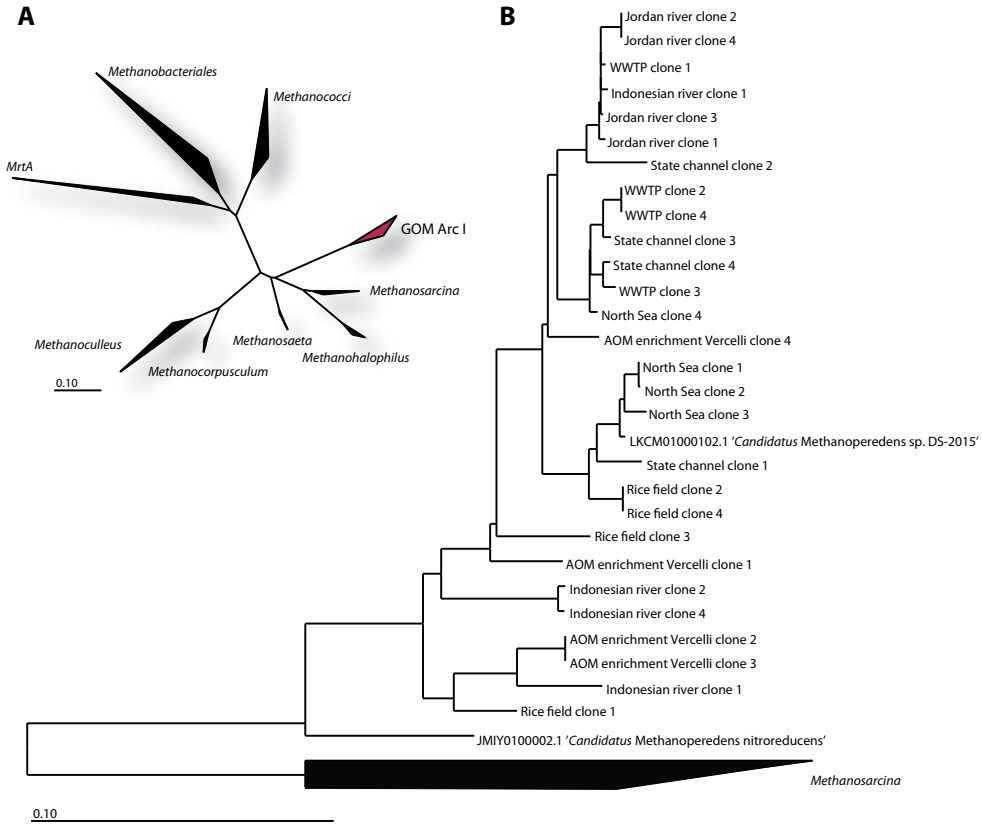

Fig. 3 **a** Phylogenetic overview of methanogenic and anaerobic methanotrophic archaea based on *mcrA* gene sequences. The phylogenetic position of GOM Arc I archaea is marked in *pink*. **b** Phylogenetic tree of '*Candidatus* M. nitroreducens' *mcrA* clone sequences (n = 28, 1191 bp). The tree includes the clones derived from this study as well as reference sequences of '*Candidatus* Methanoperedens nitroreducens ANME-2d' (GenBank accession number JMY01000002.1) and '*Candidatus* Methanoperedens sp. DS-2015' (GenBank accession number LKCM01000080.1). The tree was computed using the neighbor-joining algorithm with the Jukes-Cantor correction (Color figure online)

of '*Candidatus* M. nitroreducens' from marine North Sea sediment. The NCBI database contains only a few sequence entries from marine samples, e.g., accession number HM746653 (unpublished) and accession number GU182109 (Lever et al. 2013), which were detected in the sediment of the Gulf of Mexico and Juan de Fuca Ridge Flank basalt seafloor sediment, respectively. The sequences have 92 and 90% identity at the nucleotide level to the *mcrA* gene of '*Candidatus* M. nitroreducens' (LKCM01000102.1), respectively. For comparison, the nitrite-dependent AOM bacterium '*Candidatus* M. oxyfera' was reported in a recent study of the Eastern South Pacific oxygen minimum zone off Chile (Padilla et al. 2016). '*Candidatus* M. oxyfera' had previously been solely linked to freshwater environments. However, it seems that both nitrite-dependent bacteria and nitrate-dependent archaea also have niches in marine ecosystems, and their roles in these environments remain to be elucidated.

In contrast to universal *mcrA* primers, universal 16S rRNA gene primers have successfully captured '*Candidatus* M. nitroreducens' sequences with high identity to '*Candidatus* M. nitroreducens' in several environments such as minerotrophic fens (Cadillo-Quiroz et al. 2008), river sediments (Li et al. 2012; Rastogi et al. 2009), lake sediments (Kadnikov et al. 2012; Schubert et al. 2011; Stein et al. 2001), contaminated soils (Kasai et al. 2005), groundwater (Flynn et al. 2013), mud volcanoes (Wrede et al. 2012), and

Antarctic cold seeps (Niemann et al. 2009), among other environments. Based on 109 sequences of the GOM Arc I group in ARB, the phylogenetic trees not only show that the sequences of this phylogenetic group form a distinct cluster but also indicate that their diversity can be further divided into sub-branches within the cluster (Welte et al. 2016). This diversity is partially correlated with the environments from which the sequences were retrieved. Due to the lack of suitable primers, there are insufficiently high-quality *mcrA* sequences available to perform a similar analysis. This study added 28 long '*Candidatus* M. nitroreducens' sequences (1191 bp) suitable for high-resolution phylogenetic analysis (Fig. 3). Additional sequences are needed to confirm the splitting of the *mcrA* gene diversity of '*Candidatus* M. nitroreducens' into sub-branches. Furthermore, additional *mcrA* gene sequences will permit an investigation of the possible link between the phylogeny and distribution of '*Candidatus* M. nitroreducens' in nature.

In this study, we designed two novel primer sets targeting the *mcrA* gene of the anaerobic methanotroph '*Candidatus* M. nitroreducens': one set suitable for quantification and the other for detailed phylogeny. These molecular tools will enable the quantification and classification of these recently discovered anaerobic microorganisms in nature and, in turn, facilitate the further elucidation of the role of this important group of archaea in global nitrogen and methane cycling.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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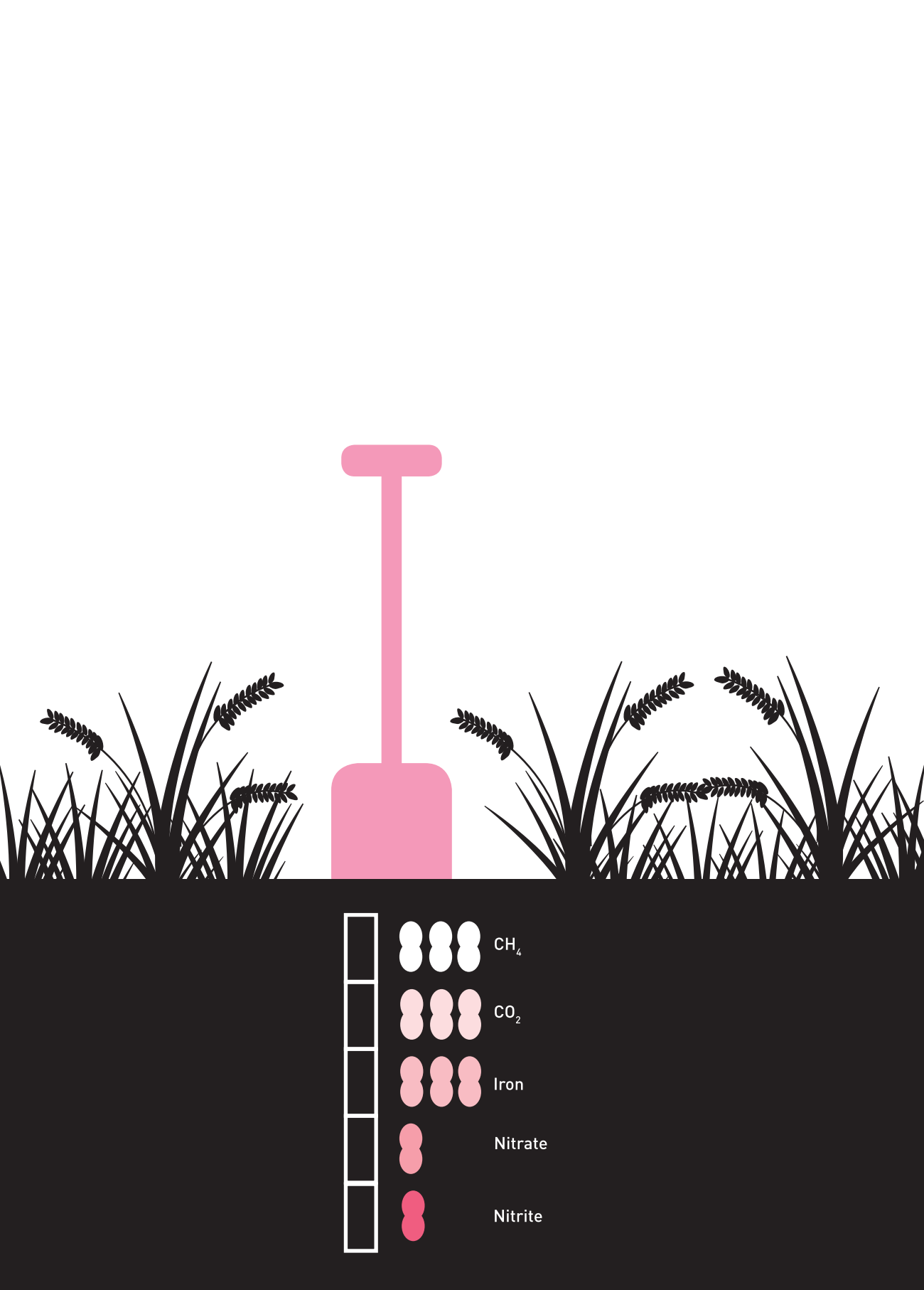
CH_4
CO_2
Iron
Nitrate
Nitrite

4

Stratification and diversity of methane-oxidizing microorganisms in a nitrogen-fertilized Italian paddy soil

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Running Title: *Stratification of methane-oxidizing microorganisms*

Keywords: anaerobic oxidation of methane, paddy fields, 16S rRNA gene amplicon sequencing, *Methanoperedens nitroreducens*, *NC10* phylum bacteria, *Bathyarchaeota*

Abstract

Paddy fields are important ecosystems, as rice is the primary food source for about half of the world's population. Paddy fields are impacted by nitrogen fertilization and are a major anthropogenic source of methane. Microbial diversity and methane metabolism were investigated in the upper 60cm of a paddy soil by qPCR, 16S rRNA gene amplicon sequencing and anoxic ^{13}C-CH4 turnover with a suite of electron acceptors. The bacterial community consisted mainly of *Acidobacteria*, *Chloroflexi*, *Proteobacteria*, *Planctomycetes* and *Actinobacteria*. Among archaea, *Euryarchaeota* and *Bathyarchaeota* dominated over *Thaumarchaeota* in the upper 30cm of the soil. *Bathyarchaeota* constituted up to 45% of the total archaeal reads in the top 5cm. In the methanogenic community, *Methanosaeta* were generally more abundant than the versatile *Methanosarcina*. The measured maximum methane production rate was 444 nmol $g_{dw}h^{-1}$, and the maximum rates of nitrate-, nitrite- and iron-dependent anaerobic oxidation of methane (AOM) were 57 nmol, 55 nmol and 56 nmol $g_{dw}h^{-1}$, respectively, at different depths. qPCR revealed a higher abundance of '*Candidatus* Methanoperedens nitroreducens' than methanotrophic NC10 phylum bacteria at all depths, except at 60cm. These results demonstrate that there is substantial potential for anaerobic oxidation of methane in fertilized paddy fields, with '*Candidatus* Methanoperedens nitroreducens' archaea as a potential important contributor.

Introduction

Methane, a significant greenhouse gas, has up to 34 times the global warming potential over 100 years compared to carbon dioxide (Myhre et al., 2013). Paddy fields contribute substantially to atmospheric methane concentrations and release 25-300 Tg of CH_4 per annum (Bridgham et al., 2013), representing 10-20 % of global methane emissions (Conrad, 2009;Bodelier, 2011). In the next decades, the land area designated for rice cultivation is predicted to increase even further. Without mitigation measures, this will result in elevated methane emission to the atmosphere.

The microbial community structure of paddy fields is influenced by several environmental and anthropogenic factors. Alteration in microbial community composition in paddy fields have been studied wih respect to flooding (Rui et al., 2009), fertilization and straw application (Bao et al., 2016), temperature (Conrad et al., 2009;Noll et al., 2010), rice cultivar and soil type (Conrad et al., 2008) and plant growth stage (Breidenbach and Conrad, 2015). Paddy fields provide a habitat for both aerobic and anaerobic methanotrophs. Aerobic methanotrophs are found in the oxic layers of the soil and in oxic microhabitats of the rhizosphere. Methanogenic archaea, anaerobic methanotrophic archaea and/or bacteria thrive preferentially in the anoxic compartments of the waterlogged soil. The flux of methane to the atmosphere is the net result of production and consumption by methanogenic and methanotrophic microorganisms.

Since the discovery of *"Bacillus methanicus"* (Söhngen, 1906), aerobic methane-oxidizing bacteria (MOB) have been extensively studied. MOB were long considered the only microbes capable of oxidizing methane. Currently, MOB belong to the phyla *Proteobacteria* and *Verrucomicrobia* (Op den Camp et al., 2009;Semrau et al., 2010). Proteobacterial aerobic methanotrophs inhabit a wide variety of environments, ranging from tundra soil (Dedysh et al., 2004) and arctic permafrost (Liebner et al., 2009) to sewage treatment sludge (Ho et al., 2013). Phylogenetic analyses of both 16S rRNA and the particulate methane mono-oxygenase subunit A (*pmoA*) gene have classified *Proteobacteria* into *Gammaproteobacteria* (Type I methanotrophs) and *Alphaproteobacteria* (Type II methanotrophs) (Trotsenko and Murrell, 2008;Semrau et al., 2010). Type I methanotrophs belong to the genera *Methylosarcina, Methylobacter, Methylomonas, Methylomicrobium, Methylosoma, Methylosphera* and *Methylovulum* (Type Ia) and *Methylococcus, Methylocaldum, Methylogaea, Methylohalobius* and *Methylothermus* (Type Ib). Alphaproteobacterial MOB belong to the genera *Methylocystis* and *Methylosinus* (Type IIa) and the genera *Methylocella, Methylocapsa* and *Methyloferula* (Type IIb) (Dumont et al., 2014;Zheng et al., 2014;Knief, 2015). Aerobic methanotrophs have been detected in several paddy field soils (Ho et al., 2011;Lüke and Frenzel, 2011;Lee et al., 2014), and furthermore, it has been suggested that Type I methanotrophs can likely outcompete Type II methanotrophs for substrates in these nitrogen-loaded environments (Zheng et al., 2014). Compared to the

proteobacterial aerobic methanotrophs, the more recently discovered *Verrucomicrobia* often inhabit more extreme environments with low pH values and/or high temperatures (Dunfield et al., 2007; Op den Camp et al., 2009; Sharp et al., 2014; van Teeseling et al., 2014).

Rice cultivation under waterlogged conditions creates anoxia in the majority of soil compartments and, consequently, provides a suitable habitat for methanogenic microorganisms. Rice maturation with the developed and decaying rhizosphere, releases root exudates that, together with dead roots, provide organic matter for an anaerobic food chain. Oxygen influx to soil occurs through diffusional transport via the aerenchyma and radial oxygen loss of the rice roots (Armstrong, 1971;Li and Wang, 2013). Although traditionally considered strict anaerobes, methanogens have been detected in the rhizosphere and on rice roots in several studies (Chin et al., 2004;Xu et al., 2012;Edwards et al., 2015;Lee et al., 2015). Lee et al. (2015) observed a higher abundance of methanogens in the rhizosphere than in bulk soil (Lee et al., 2015). The methanogens in the rhizosphere may live in non-active roots where no oxygen is released or, alternatively, may be oxygen tolerant and have mechanisms to counteract reactive oxygen radical species, as investigated for Rice Cluster I (RC I) (now known as *Methanocella*) methanogens (Erkel et al., 2006). The genomes of RC I harbor genes encoding catalase, three different superoxide anion scavengers, superoxide dismutase and two different super oxide reductase genes for oxygen detoxification (Erkel et al., 2006). The up-regulation of catalase genes in response to oxygen exposure has been observed in both *Methanosarcina* and *Methanocella* (Angel et al., 2011).

Both acetoclastic and hydrogenotrophic methanogens have been identified in paddy fields. Methanogenic archaea of the order *Methanosarcinales* derive methane from the methyl group of compounds such as methanol and methylamine, and until now, only *Methanosarcina* and *Methanosaeta* are known to use acetate for methane production (Jetten et al., 1992;Costa and Leigh, 2014;Welte and Deppenmeier, 2014). Hydrogenotrophic methanogens belonging to the orders *Methanomicrobiales*, *Methanobacteriales* and *Methanocellales* have been commonly found in paddy fields, with the exception of *Methanococcales*, which barely have been detected (Watanabe et al., 2010;Lee et al., 2015). Many of these hydrogenotrophic methanogens can use formate as a substrate but are unable to utilize acetate. Archaea belonging to RC I (*Methanocella*) (Kögel-Knabner et al., 2010), which forms a separate phylogenetic lineage branching between the orders *Methanosarcinales* and *Methanomicrobiales*, are considered key methanogens in rice fields. The reaction stoichiometry of methanogenesis (Conrad and Klose, 1999) indicates that acetoclastic methanogens could contribute approximately two-thirds to methane production, consistent with the dominance of acetoclastic over hydrogenotrophic methanogenesis in paddy fields (Krüger et al., 2001).
Previous theories suggesting a decrease in methane flux as a result of direct stimulation of methanotrophs after amendment with nitrogen fertilizers were unable to link observations

to the activity of the denitrifying anaerobic methanotrophic bacteria and archaea as these microorganisms, were discovered only recently compared to the aerobic methanotrophs. Nitrite- and nitrate-dependent anaerobic oxidation of methane (AOM) were first described in 2006 in an enrichment culture consisting of archaea distantly related to ANME-2d and of bacteria that consume nitrite as an electron acceptor to oxidize methane anaerobically (Raghoebarsing et al., 2006). This novel denitrifying, methanotrophic bacterium of the candidate division NC10 was named *'Candidatus* Methylomirabilis oxyfera*' (Ettwig et al., 2010).* Despite its preference for an anoxic habitat, it is postulated to have an intra-aerobic metabolism. The genome of the bacterium contains all genes of the aerobic methanotrophic pathway and encodes a particulate methane mono-oxygenase complex that can use the O_2 released from nitric oxide for methane oxidation, similar to aerobic methanotrophs (Ettwig *et al.*, 2010).

The genome of ANME-2d archaea was sequenced in 2013 and responsible organism named '*Candidatus* Methanoperedens nitroreducens' (Haroon et al., 2013). This nitrate-reducing archaeon employs a reverse methanogenesis pathway to oxidize methane. The genomes of three different strains of '*Candidatus* Methanoperedens nitroreducens' have been published, and the necessary genes for nitrate reduction and the methanogenic pathway have been identified (Haroon et al., 2013;Arshad et al., 2015; Vaksmaa et al., 2017(AMAB accepted)). Nitrite-and nitrate-dependent AOM microorganisms and/or activity have been detected in several freshwater environments, including paddy fields (Vaksmaa et al., 2016;Welte et al., 2016). Recently it was demonstrated that *'Candidatus* Methanoperedens nitroreducens' can also oxidize methane using iron as electron acceptor (Ettwig et al., 2016).

Besides so far know methanotrophs and methanogens, recent investigations of microbial "dark matter" discovered key genes of the methane pathway to be present in phyla, which previously were not linked to the ability to produce or consume methane. Phylum *Bathyarchaeota,* renamed from *Miscellaneous Crenarchaeotic Group* is a deeply- branching phylum consisting of 17 sub-groups (Kubo et al., 2012). It is abundant in marine environments but is also found in extreme habitats like hot springs, cold sulfur springs, Polar Regions and in mesophilic habitats like sewage waste, fresh water lakes and paddy fields. Though there are no pure isolates, based on culture independent methods, their function was speculated to be important in the global cycle of carbon (Parkes et al., 2005). To date there are eight different genomes annotated, out of which two BA1 and BA2 are hypothesized to be methane metabolizers (Evans et al., 2015;He et al., 2016).

Majority of previous studies of paddy field microbial communities have focused on either a specific group of microorganisms or environmental or anthropogenic effect on methane emissions or sampling had been carried out at a single depth, hindering direct comparison. The aim of the present study was to explore how the microbial communities in a paddy field

are influenced by spatial factors along a depth gradient. The objectives of this study were (i) to characterize the bacterial and archaeal communities in a paddy field soil core by 16S rRNA gene amplicon sequencing with a focus on methane cycle-related organisms; (ii) determine the abundances of total bacteria, total archaea, '*Candidatus* Methanoperedens nitroreducens', NC10 phylum bacteria and *Bathyarchaeota*; and (iii) estimate the anaerobic methane oxidation potential using nitrate, nitrite and iron as electron acceptors at different soil depths.

Materials and methods

Soil sampling

Paddy field soil cores were sampled in August 2015 at the Italian Rice Research Unit in Vercelli, Italy (08°22'25.89''E; 45°19'26.98''N). The sampling fields were cultivated with the rice variety *Oryza sativa* temperate japonica Onice. The paddy fields were flooded for about 90 days, with fertilizer applied in April and twice in June. Soil cores were sampled in triplicate with 80-cm soil augers at approximately 5-m intervals. The porewater nitrate and ammonium concentrations were in average 0.6 µM and 6.8 µM throughout the 80 cm. Amorphous iron oxides over a 50 cm core were in top 25 cm in average 28.5 µmol per gram wet weight (g_{ww}) soil and in lower 25 cm 54.8 µmol per g_{ww} soil, with one maxima at 11 cm 68.6 µmol per g_{ww} soil and at 31 cm 76.0 µmol per g_{ww} soil (data obtained from the previous year), analysis was performed as described in (Egger et al., 2015). For AOM and methanogenic activity incubation assays, the soil was sliced in the field and placed immediately in anaerobic jars. For DNA extraction, the samples were stored in 50-ml conical centrifuge tubes. All samples were stored at 4 °C at the field site laboratory until transport on cool compresses by car. After transport to the lab, samples for DNA extraction were immediately frozen at -20 °C, and samples for activity experiments were stored at 4 °C.

Methane measurements

To measure methane entrapped in the soil, three separate cores with lengths of 51, 58 and 68 cm were sampled. Immediately after sampling, while releasing the core from the auger, samples were taken with a 5-ml open-end syringe. These samples were then transferred to pre-weighed 120-ml bottles filled with saturated NaCl solution. The bottles were sealed with screw-caps with rubber stoppers. The CH_4 concentration was quantified by gas chromatography (Hewlett Packard 5890, USA). Methane concentrations were calculated per gram dry weight (g_{dw}) of the sampled soil at the respective depth.

DNA extraction

For DNA extraction soil cores were divided to 13 different depths. Soil from the same depth of three cores was pooled. DNA was extracted from approximately 0.25 g of soil in duplicate

using a PowerSoil DNA isolation Kit (MO BIO Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol. DNA was extracted from the following depths: 0 cm, 2.5 cm, 5 cm, 7.5 cm, 10 cm, 15 cm, 20 cm, 25 cm, 30 cm, 35 cm, 40 cm, 50 cm and 60 cm. DNA quantity and quality were assessed by UV-VIS spectroscopy (NanoDrop, ND-1000, Isogen Life Science, the Netherlands).

Quantification by qPCR

Quantification of the total bacterial and total archaeal communities using the 16S rRNA gene was performed in triplicate using the duplicate DNA extractions from each depth sample described above. For archaea, the following primers were used: forward Arch-349 (5'GYGCASCAGKCGMGAAW3') (Takai and Horikoshi, 2000) and reverse Arch-807 (5'GGACTACVSGGGTATCTAAT3')(Wang and Qian, 2009). For bacteria, the primers were forward Bact-341 (5'CCTACGGGNGGCWGCAG3') and reverse Bact-785 (5'GACTACHVGGGTATCTAATCC3') (Herlemann et al., 2011). Bathyarchaeota were targeted by primers amplifying 16S rRNA gene: MCG528 forward and MCG732 reverse (Kubo et al., 2012). *'Candidatus* Methanoperedens nitroreducens' was targeted by primers amplifying the mcrA gene: McrA159F forward and McrA345R reverse (Vaksmaa et al., 2017). The 16S rRNA gene of the NC10 phylum was amplified with the primers p2F_DAMO (5'GGGGAACTGCCAGCGTCAAG3') and p2R_DAMO (5'CTCAGCGACTTCGAGTACAG3') (Ettwig et al., 2009). All qPCR reactions were performed using PerfCTa Quanta master mix (Quanta Biosciences, USA) and 96-well optical plates (Bio-Rad, USA) on a Bio-Rad CFX96 Real-Time C1000 Touch Thermal Cycler (Bio-Rad, USA), as described in vaksmaa et al. 2016, 2017 (Vaksmaa et al., 2016;Vaksmaa et al., 2017). Absolute quantification was performed by comparison to standard curves obtained using a 10-fold serial dilution of pGEM-T Easy plasmid DNA (Promega, USA) with an insert of the target gene obtained using the same primers as used for qPCR. Standard curve samples were used as a control for each qPCR run.

Amplicon sequencing

The following primers were used for 16S rRNA gene amplification: forward Arch-0349 and reverse Arch-807 for archaea and forward Bact-0341 and reverse Bact-785 for bacteria. The amplicons were generated in a two-step reaction. DNA was pooled in equimolar amounts per depth to perform PCR under the following conditions: initial denaturation at 96 °C for 3 min; 30-35 cycles of denaturation at 96 °C for 40 s, primer annealing at 60 °C (for archaea) or 61 °C (for bacteria) for 30 s, and elongation at 72 °C for 40 s; and a final elongation at 72 °C for 2 min. Each PCR product was verified by 1 % gel electrophoresis. The obtained PCR products were purified with a GeneJet PCR purification kit (Thermo Scientific, The Netherlands). A second PCR was then performed with the same primers described above, which were extended with adapter sequences, specific barcodes and key sequences compatible with Ion Torrent sequencing at the 5' end. The reaction conditions for this PCR were an initial denaturation

at 96 °C for 10 min; 10 cycles of denaturation at 96 °C for 1 min, primer annealing at 60°C or 61 °C for 1 min and elongation at 72 °C for 2 min; and a final elongation step at 72 °C for 10 min. The products were again pooled per depth and purified as described above. The DNA concentrations of the purified PCR products were then measured and diluted to a range of 0.2 to 0.4 ng/µl. The concentrations and fragment lengths of the libraries were determined with a Bioanalyzer 2100 and a High Sensitivity DNA kit (Agilent Technologies, USA). The obtained libraries were diluted to a final concentration of 100 pM, and the different barcoded libraries were pooled in equimolar amounts before sequencing. For Ion Torrent sequencing, the library fragments were attached to Ion Sphere particles using an Ion One Touch Instrument and Ion PGM Template OT2 400 Kit (Life Technologies, USA) according to the manufacturer's instructions. After enrichment of the template-positive Ion Sphere Particles using the Ion One Touch ES (Life Technologies, USA), the samples were loaded on an Ion 316 v2 Chip. The DNA fragments were then sequenced using the Ion PGM Sequencing 400 Kit and 850 nucleotide flows according to the manufacturer's instructions.

Analysis of 16S rRNA gene amplicon data

The raw sequencing reads were automatically separated into clusters of each depth based on the unique barcodes. After sequencing, all raw reads were imported into CLC Genomics Workbench vs. 9 (QIAGEN Aarhus A/S, Denmark) for initial data analysis, including trimming of low-quality and short reads (cut-off value 200 nucleotides). After trimming, 6661 to 11785 reads were obtained per corresponding depth for archaea; the number of reads obtained per depth for bacteria was 4477 to 7198 reads. The exported reads were further processed using the automated pipeline of Silva NGS (Silva Next Generation Sequencing) of the SILVA rRNA gene database project (SILVAngs 1.2)(Quast et al., 2013). In this process, each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSUrRNA SEED and quality controlled (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2 % ambiguities or 2 % homopolymers were excluded from further processing. Putative contaminants, artifacts and reads with low alignment quality (50 alignment identity, 40 alignment score reported by SINA) were identified and excluded from downstream analysis. After these initial quality control steps, identical reads were identified (dereplication), unique reads were clustered (OTUs) on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering were performed using cd-hit-est (version 3.1.2;http://www.bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. Classification was performed by local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 119;http://www.arb-silva.de) using blastn (version 2.2.28+;http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho et al., 2009). The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. This mapping yielded semi-

quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, and multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, in which the function \(% sequence identity + % alignment coverage)/2" did not exceed a value of 93, remained unclassified. These reads were assigned to the metagroup \No Relative" in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011). This method was first used in the publications (Klindworth et al., 2013) and (Ionescu et al., 2012). The amplicon sequencing data were deposited to the Short Read Archive under Bioproject ID PRJNA378333. Estimated quantities of individual taxa were calculated by multiplication of relative amplicon sequence data with qPCR data.

Soil incubations

Soil samples from the three cores were pooled at depths of 0-5 cm, 5-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm. Soil slurries for each depth were prepared by mixing the soil with mineral salt medium as described by Ettwig et al. (2008). Activity assays were performed in 120-ml serum bottles with 60 ml of soil slurry. The wet and dry soil weight ratio of the slurry was determined in duplicate at each depth. The incubation bottles were sealed with red butyl rubber stoppers and crimp-caps. The headspace was exchanged with Ar/CO_2 by 5 cycles of vacuum and gassing, with a final overpressure of 0.5 bar. Treatments at each of the depths were performed in duplicate and consisted of adding 5 mM $NaNO_3$, 1 mM $NaNO_2$, 20 mM iron nitrilotriacetic acid (FeNTA), or 20 mM ferrihydrite with 10 % ^{13}C-CH_4 v/v (final concentrations) and controls in which either 10 % CH_4 v/v was added or no additions were made to the soil slurry. Each treatment was performed in duplicate with triplicate headspace measurements to quantify the CH_4 concentration by gas chromatography (Hewlett Packard 5890, USA) *as described previously (Ettwig* ***et al.****, 2009)*.. Headspace measurements were carried out over the period of 118 days, with methane concentration measured at day 0, 7, 14, 21, 46, 54, 85, 98 and 118 and the net production or consumption rates were calculated during the linear phase.

Results

Methane measurements in the soil core

The highest methane concentration was measured in the top 15 cm of the soil (Figure 1). The highest peak was measured at 0 cm and 6.5 cm and corresponded to a methane concentration of approximately 165 µmol per g_{dw} in two of the three cores. Below 15 cm, a rapid decrease in the methane concentration was observed; at a depth of 28 cm, methane concentrations were less than 7 µmol per g_{dw}. At depths of 50 cm and below, the methane concentration was at the detection limit of 0.4-2 µmol per g_{dw}.

Quantification of total bacteria, archaea, and subgroups of known anaerobic methanotrophs

The total abundance of bacteria, archaea and nitrate- and nitrite dependent anaerobic methanotrophs was quantified by qPCR. The total bacterial abundance was higher than the archaeal abundance at all depths of the soil core. As depicted in Figure 2, the highest copy number obtained with the archaeal primer combination was observed at a depth of 10 cm ($1.0\pm0.3*10^9$ 16S rRNA gene copies per g_{dw}). Below a depth of 20 cm ($2.6\pm0.4*10^8$ 16S rRNA gene copies per g_{dw}), the archaeal copy numbers decreased gradually until 60 cm, where $4.1\pm2.2*10^5$ 16S rRNA gene copies per g_{dw} was observed. The highest amount of bacterial copies was observed at a depth of 10 cm ($5.6\pm1.4*10^9$ 16S rRNA gene copies per g_{dw}), and the lowest number was observed at a depth of 60 cm ($1.4\pm0.6*10^7$ 16S rRNA gene copies per g_{dw}). The known archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens' exhibited the highest abundance at 20 cm, with $1.8\pm0.3*10^7$ *mcrA* gene copies per g_{dw}, and lowest abundance at 60 cm, with $7.2\pm1.5*10^3$ *mcrA* gene copies per g_{dw}. The anaerobic methanotrophs belonging to NC10 phylum bacteria had two maxima at depths of 10 cm ($2.3\pm0.7*10^5$ 16S rRNA gene copies per g_{dw}) and 35 cm ($2.3\pm0.2*10^5$ 16S rRNA gene copies per g_{dw}). The lowest abundance was observed at a depth of 60 cm ($1.5\pm0.5*10^4$ 16S rRNA gene copies per g_{dw}). Among the targeted anaerobic methanotrophs, '*Candidatus* Methanoperedens nitroreducens' had higher gene copy numbers than NC10 phylum bacteria at all depths except 60 cm, where NC10 phylum bacteria outnumbered '*Candidatus* Methanoperedens nitroreducens'.

Amplicon sequencing of the 16S rRNA gene in the bacterial community

At each depth, the 16S rRNA gene amplicon data were analyzed for both bacteria and archaea. In the bacterial community, a very large diversity was observed (Figure 3, Table S1), with most of the reads assigned to *Acidobacteria*, *Chloroflexi*, *Proteobacteria*, *Planctomycetes* and *Actinobacteria*. Most of the phyla were observed throughout the soil core. However, at depths of 40 cm and below, *Cyanobacteria*, *Bacteroidetes* and *Chlorobi* were hardly or not present at all. The opposite trend was observed for *Latescibacteria*, which increased gradually in relative abundance toward deeper layers. 16S rRNA gene reads assigned to NC10 phylum (phylum *Nitrospirae*) bacteria were recorded at all depths along the gradient of the soil core. The lowest relative abundance was recorded at the top layer of soil (0 cm). Thereafter, the copies increased gradually, with a maximum at a depth of 40 cm, where reads assigned to the NC10 phylum represented 2.4 % of the total bacterial 16S rRNA gene reads. After 40 cm, a rapid decrease was observed in the relative abundance of reads assigned to the NC10 phylum to 50 cm (0.25 %), followed by an increase at 60 cm (1.25 %). The relative abundance of reads assigned to the NC10 phylum at all other depths, except 35 cm, 40 cm and 60 cm, was less than 1 % of the total bacterial reads (Figure 4).

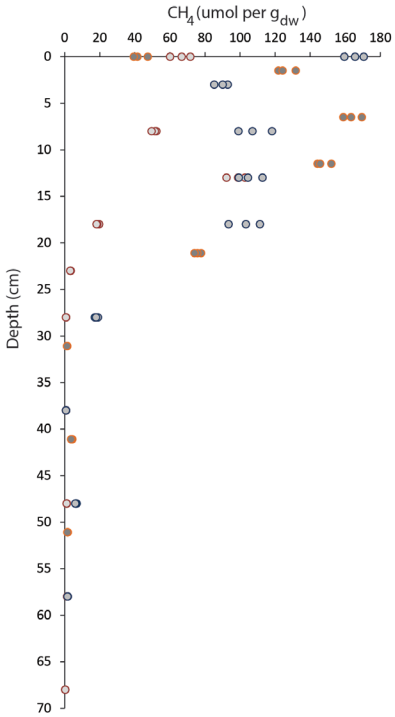

Figure 1: Vertical profile of methane concentrations along the depth gradient of the paddy field soil. The soil depth is depicted vertically, and the methane concentration in µmol per g_{dw} is depicted horizontally. Three separate cores were sampled up to the maximum depth of 68 cm. Measurements of each sample were performed in triplicate by gas chromatography.

Among *Proteobacteria*, the relative abundance of *Alphaproteobacteria* was highest in the top 15 cm of the soil core and gradually decreased in the deeper layers of soil. *Beta-* and *Gammaproteobacteria* showed the lowest relative abundances, but their relative abundances exhibited little variation throughout the soil core. *Deltaproteobacteria* were the second most abundant in the top 15 cm. Their relative abundance peaked at 25 cm, corresponding to 8 % of total bacterial reads, and decreased gradually thereafter. A detailed distribution of the proteobacterial classes is provided in Table S2.

Among sequences assigned to aerobic methanotrophs, most of the reads were assigned to *Methylococcaceae*, except at depths of 0 cm and 15 cm, where more reads were assigned to *Methylocystaceae*. Surprisingly, we observed *Verrucomicrobia* methanotrophs in the paddy soil core, and reads assigned to *Candidatus* Methylacidiphilum were most abundant in the top 5 cm, constituting 20% of the total aerobic methanotrophic community. Overall, the relative

abundance of aerobic methanotrophs was highest at a depth of 10 cm, representing 1.1 % of the total bacterial community. The calculated abundance of aerobic methanotrophs in calculated copy numbers was on the order of 10^7 in the top 20 cm and then gradually decreased to 10^4 at a depth of 60 cm (Figure 4).

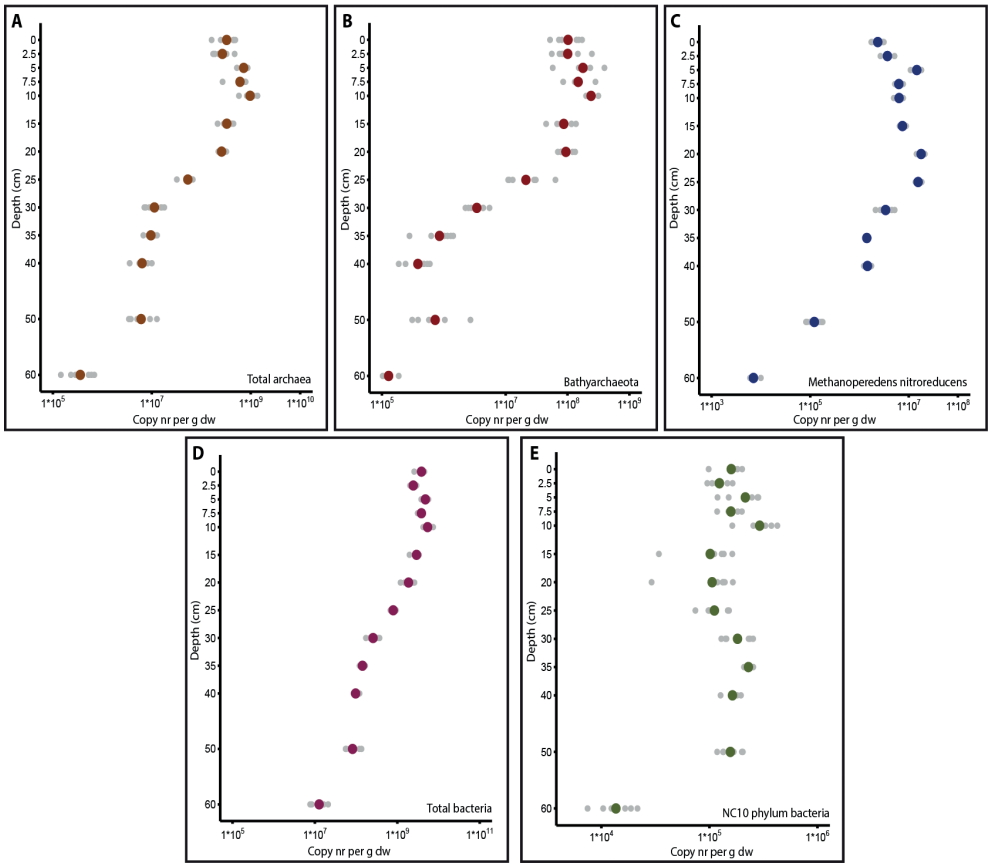

Figure 2: Depth profile of copy numbers of genes of interest obtained by qPCR. In all figures, the soil depth is depicted vertically. Horizontally, the copy numbers obtained by qPCR per gram dry weight are presented in log scale. A. 16S rRNA gene copy numbers of total archaea amplified with Arch349F/Arch807R primers. B. 16S rRNA gene copy numbers of Bathyarchaeota amplified with MCG528F/MCG732R primers C. McrA gene copy numbers of Methanoperedens nitroreducens quantified with McrA159F/McrA345R primers. D. 16S rRNA gene copy numbers of total bacteria quantified with Bac341F/Bac785R primers. E. 16S rRNA gene copy numbers of NC10 phylum bacteria quantified with p2F_DAMO/p2R_DAMO primers.

Phylogenetic diversity of *Verrucomicrobia* and *Candidatus* Methylacidiphilum

Verrucomicrobial methanotrophs have rarely been observed outside acidic volcanic areas. Therefore, we extracted the 16S rRNA gene sequences from amplicon sequencing and analyzed sequences assigned to *Verrucomicrobia* in detail (Figure 5). Sequences clustering with *Candidatus* Methylacidiphilum were found at all depths except 7.5 cm, 50 cm and 60 cm. The extracted sequences clustering with *Candidatus* Methylacidiphilum were 85 % identical at the nucleotide level to cultivated strains of *Candidatus* Methylacidiphilum (van Teeseling et al., 2014).

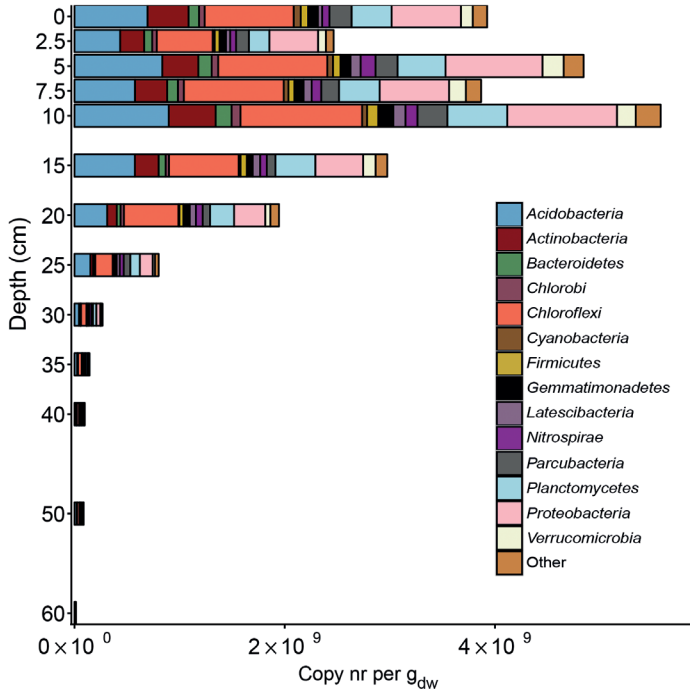

Figure 3: Distribution of 16S rRNA gene reads of major bacterial phyla along the depth profile of the paddy soil core. The soil depth in centimeters is depicted vertically, whereas the total amount of 16S rRNA gene amplicons per gram dry weight is depicted horizontally. The colored bars represent the relative amount of gene copies matching a bacterial phylum present in the soil at a particular depth.

Amplicon sequencing of the 16S rRNA gene of the archaeal community

In the archaeal community reads matching *Euryarchaeota* were more abundant than *Thaumarchaeota* in the top layers until a depth of 30 cm. At deeper depths of 35 cm, 50 cm, and 60 cm (*Euryarchaeota* were dominant at 40 cm), sequences matching *Thaumarcheota* were the most abundant. Sequences matching the 16S rRNA gene of *Bathyarchaeota* (previously known as *Miscellaneous Crenarchaeota Group* (MCG)) were the most abundant in the top layers of

the soil. At depths of 0-5 cm, 43-45 % of the reads were assigned as *Bathyarchaeota*. This proportion decreased gradually throughout the soil core (Figure 6, Table S3).

Analysis of the *Methanomicrobia* and *Methanobacteria* communities in greater detail revealed that sequencing reads assigned to *Methanosaeta* and *Methanosarcina* were most abundant among methanogens throughout the soil core (Figure 7). The top layer of soil had more diverse community than deeper layers. The methanogen community was largest at a depth of 10 cm, 52% of total archaea. The highest relative sequence abundance of the archaeal methanotroph '*Candidatus* Methanoperedens nitroreducens' (GOM Arc I) was found at a depth of 25 cm, comprising 56.4 % of the total archaeal reads. The estimated depth distribution of '*Candidatus* Methanoperedens nitroreducens' calculated based on the sequencing read abundance and on the total archaeal copy numbers is depicted in Figure 4. The abundance based on amplicon data peaked at $1.1*10^8$ copies at a depth of 7.5 cm.

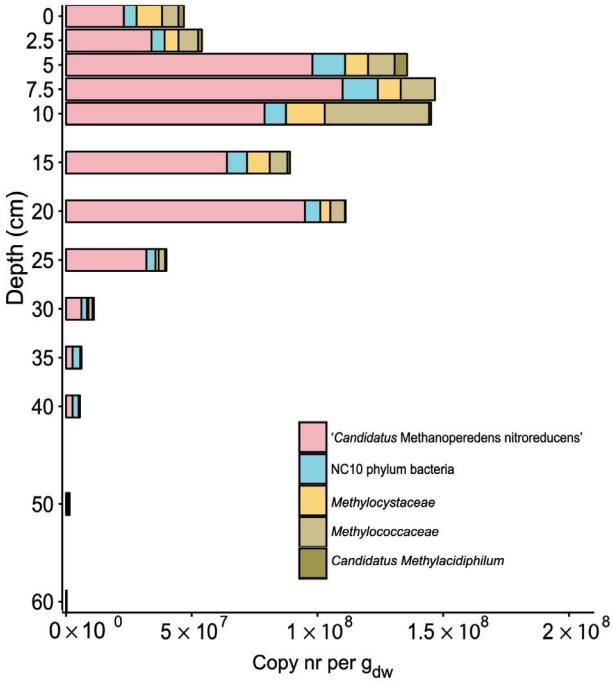

Figure 4: Distribution of sequence reads of proteobacterial (*Methylococcaceae*, *Methylocystaceae*) and verrucomicrobial (*Candidatus* Methylacidiphilum) aerobic methanotrophs together with anaerobic methanotrophs: 'Candidatus Methanoperedens nitroreducens' and NC10 phylum bacteria based on 16S rRNA gene amplification. Reads were assigned to phylogenetic groups based on the SILVA NGS pipeline. The soil depth in centimeters is depicted vertically, and the total amount of 16S rRNA gene amplicons per gram dry weight is depicted horizontally. The colored bars represent the relative amount of gene copies corresponding to aerobic and anaerobic methanotrophs present in the soil at a particular depth.

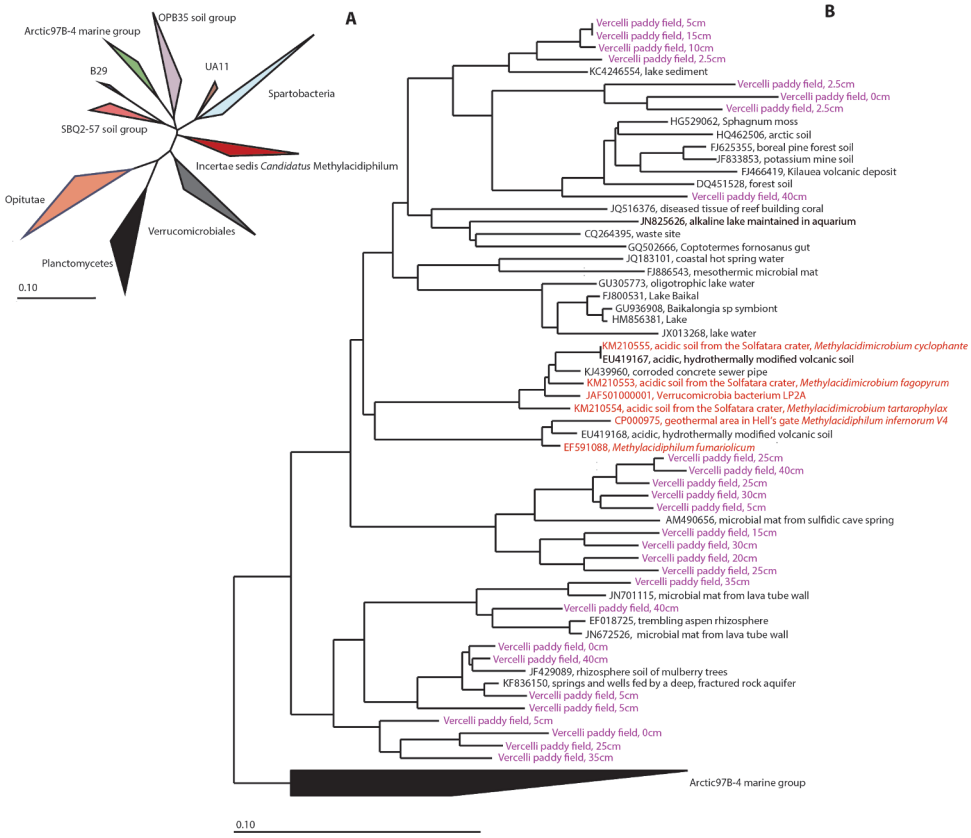

Figure 5: A. Phylogenetic overview of verrucomicrobial 16S rRNA gene sequences. The phylogenetic position of *Candidatus* Methylacidiphilum (*Incertae sedis Candidatus* Methylacidiphilum) is marked in red. B. Detailed presentation of the sequences of *Candidatus* Methylacidiphilum. Sequences of cultivated strains are shown in red, and sequences obtained from paddy field soil are shown in purple. The neighbor-joining phylogenetic tree was calculated using Jukes-Cantor correction and the Arctic 97B-4 marine group as outgroup.

Soil slurry incubations

Soil slurries of different depths were amended with methane and electron acceptors. Controls were prepared with and without addition of methane to detect methanogenic activity. The rates of potential methane oxidation with nitrate, nitrite and two forms of iron, FeNTA and ferrihydrite, were recorded. The potential methane oxidation or production rate was calculated based on methane concentration measurements in the headspace over a time course of 118 days (Figure 8).

The nitrate- and nitrite-dependent methane oxidation rates were highest in the top 20 cm. At depths of (0-5 cm) and (5-10 cm), higher AOM rates were measured in slurries amended with nitrate, 37 nmol g_{dw} h^{-1} and 57 nmol g_{dw} h^{-1}, than in slurries amended with nitrite, 29 nmol g_{dw} h^{-1} and 43 nmol g_{dw} h^{-1}, respectively. At a depth of 10-20 cm, the nitrite-amended samples exhibited the highest methane oxidation rate, 55 nmol g_{dw} h^{-1}, followed by 33 nmol g_{dw} h^{-1} in the nitrate-amended samples. Methane oxidation was measured in the top 20-cm slurries in samples amended with FeNTA, with a peak of 48 nmol g_{dw} h^{-1} at a depth of 5-10 cm.

In the top layers up to 20 cm, addition of ferrihydrite did not stimulate methane oxidation. In the deeper layers, the pattern was the same as that for the addition of FeNTA. The highest methane oxidation rate was observed at a depth of 40-50 cm in slurries amended with FeNTA, 25 nmol/g_{dw}/h^{-1}, followed by ferrihydrite, 20 nmol g_{dw} h^{-1}. At a depth of 50-60 cm, the respective rates for FeNTA and ferrihydrite were 56 nmol g_{dw} h^{-1} and 29 nmol g_{dw} h^{-1}.

In the control samples amended with methane, initial methane oxidation was monitored for a maximum time period of 21 days, after which methane production prevailed, with production of 277 nmol g_{dw} h^{-1}, 369 nmol g_{dw} h^{-1} and 85 nmol g_{dw} h^{-1} at depths of 0-5 cm, 5-10 cm and 10-20cm, respectively. A similar pattern of methanogenesis in soil slurry incubations with no additions was observed. After a lag phase of approximately 21 days, the methane production rate increased. The highest methane production rate, 444 nmol g_{dw} h^{-1}, was observed at a depth of 5-10 cm. At a depth of 10-20 cm, methanogenesis was still observed, with a rate of 69 nmol g_{dw} h^{-1}, which decreased to less than 1 nmol g_{dw} h^{-1} in deeper layers. At depths of 20-50 cm, oxidation prevailed over methane production in slurries amended with methane.

Discussion

Paddy fields are a major source of methane emitted to the atmosphere. The flux of methane is controlled by the microbial community present in the soil, particularly by methanogens and methanotrophs.

The vertical profile of the methane gradient included a higher methane concentration in the top 15 cm of the soil core, followed by a drastic drop. At a depth of approximately 28 cm, methane was nearly undetectable. This depth correlates with the interface of annual plowing and undisturbed soil as well as the rice root penetration depth.

The profile of the total abundance of microorganisms along the depth gradient followed the same trend as methane. The highest copy numbers of both bacteria and archaea were detected at a depth of 10 cm, followed by a decrease to 25 cm, after which the microbial population

size was a few orders of magnitude smaller. The total bacterial and archaeal population sizes correlate well with previous reports. The total bacterial and archaeal 16S rRNA gene copy numbers in a Chinese paddy field ranged from $1.4*10^{10}$ to $2.9*10^{10}$ per g_{dw} and $5.4*10^{8}$-$1.7*10^{9}$ per g_{dw}, respectively (Ahn et al., 2012). In paddy fields in the Philippines, the total bacterial copy numbers and archaeal copy numbers were on the order of 10^{10} and 10^{8} per g_{dw}, respectively (Breidenbach and Conrad, 2015). In paddy fields in India, $9.6*10^{9}$-$1.4*10^{10}$ bacterial 16S rRNA copies per g_{dw} and $7.13*10^{7}$-$3.02*10^{8}$ archaeal 16S rRNA copies per g_{dw} were reported (Singh et al., 2012). We recorded maximum bacterial and archaeal abundances of $5.6±1.4*10^{9}$ and $1.0±0.3*10^{9}$ 16S rRNA gene copies per g_{dw}, respectively.

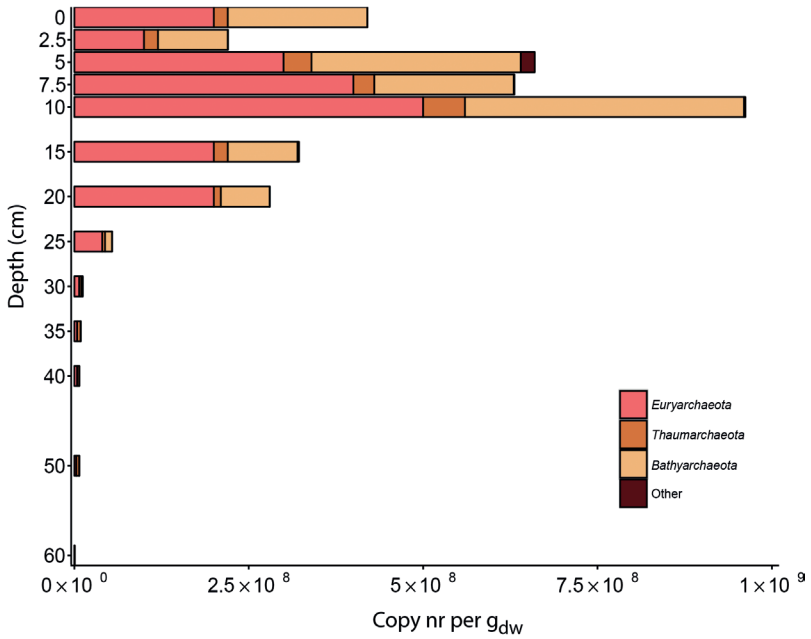

Figure 6: Distribution of 16S rRNA gene reads of major archaeal phyla along the depth profile of the paddy soil core. Reads were assigned to phylogenetic groups based on the SILVA NGS pipeline. The soil depth in centimeters is depicted vertically, and the total amount of 16S rRNA gene amplicons per gram dry weight is depicted horizontally. The colored bars represent the relative amount of gene copies matching an archaeal phylum present in the soil at a particular depth.

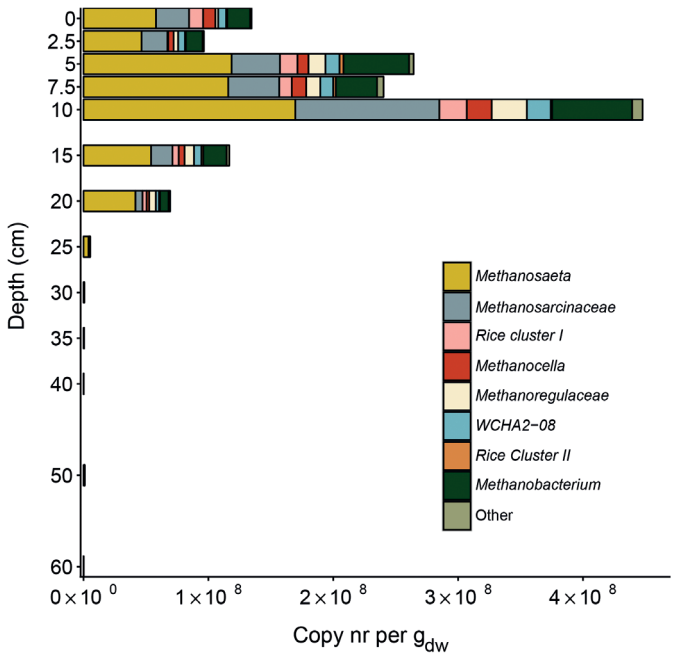

Figure 7: Distribution of 16S rRNA gene reads of methanogens along the depth profile of the paddy soil core. Reads were assigned to phylogenetic groups based on the SILVA NGS pipeline. The soil depth in centimeters is depicted vertically, and the total amount of 16S rRNA gene amplicons per gram dry weight is depicted horizontally. The colored bars represent the relative amount of gene copies matching methanogens present in the soil at a particular depth.

The rice root system has been described as the key determinant in shaping the microbial community via release of root exudates, decaying roots and organic matter as well as oxygen (Kuzyakov & Blagodatskaya, 2015). Diffusion of oxygen to the soil creates micro-oxic niches for oxygen-dependent microorganisms. We detected sequences belonging to aerobic methanotrophs throughout the soil core. The relative abundance of aerobic methanotrophs was highest at a depth of 10 cm and was twice as high as of that in the surface layer. Along the entire depth gradient, MOB were dominated by Type I *Methylococcaceae*, followed by Type II *Methylocystaceae*. *Methylococcaceae* have been detected in several environments with low oxygen concentration, even tolerating periods of hypoxia (Hernandez et al., 2015). The presence of these aerobic methanotrophs in low oxygen environments, such as the investigated paddy field, could possibly be explained by their denitrifying ability as has been demonstrated for *Methylomonas denitrificans*, which during hypoxia carries out nitrate reduction and methane oxidation (Kits et al., 2015). Other *Methylococcaceae*, such as *Methylobacter* contain in their genome besides respiratory nitrate and nitrite reductases as well genes necessary for dinitrogen fixation (Kalyuzhnaya et al., 2015).

In addition to detecting sequences of well-known proteobacterial aerobic methane oxidizers, sequences belonging to methanotrophic *Verrucomicrobia* were identified in this study. Detailed phylogenetic analysis revealed the presence of aerobic methanotrophs distantly related (85 % nucleotide identity of the 16S rRNA gene) to cultured members of *Candidatus* Methylacidiphilum. Only a very small number of verrucomicrobial methanotrophs have been detected in ecosystems other than acidic volcanic areas, including paddy field soil (Genbank JF984005.1), forest soil (Genbank JF420089), lake sediment (Genbank GU305773) arctic soil (Genbank HQ462506) and a few other environments. The reported cultivated strains originate exclusively from extreme hot or acidic environments in Italy, Kamchatka or New Zealand. Further studies are needed to determine if the microbes found in less extreme environments also contain *pmoA* genes in their genome and have the capability to oxidize methane. We hypothesize that there is a niche for these aerobic verrucomicrobial methane oxidizers in less acidic methane-rich environments such as paddy fields.

The translation of 16S rRNA gene sequencing read numbers to copy numbers indicated that the methanogen population abundance was highest at a depth of 10 cm, with 4.5 $*10^8$ copies per g_{dw}, followed by a decline in abundance to 60 cm, with $2.7*10^4$ copies per g_{dw}. The methanogenic population size determined previously in the same Italian paddy field was 10^7-10^8 copies per g_{dw} (Conrad and Klose, 2006). Compared to other sampling sites, our observed abundances are slightly higher than the previously reported methanogen abundances of $1.1*10^7$ or $1.4*10^7$ copies per g_{dw} (Singh et al., 2012) or 10^4-10^5 copies per g_{dw} (Hou et al., 2000). A previous vertical profile study of methanogens identified the highest abundance based on *mcrA* gene copy numbers at a depth of approximately 20 cm in three Japanese paddy fields, peaking at 10^7 (Watanabe et al., 2010). Together, these results suggest that the methanogenic zone is located approximately 10-20 cm below the soil surface and co-occurs with the end of the main root system in soil.

The community analysis of methanogens revealed a diverse composition throughout the soil core. The methanogenic community was dominated by *Methanosaeta, Methanosarcina, Methanobacterium, Methanoregulaceae* and the RC I cluster (*Methanocella*), which have also been found previously in temperate climate paddy fields (Conrad and Klose, 2006;Watanabe et al., 2010). The community throughout the core was dominated by the strictly acetoclastic *Methanosaeta*, followed by more versatile *Methanosarcina spp.* The sampling time of the soil at the end of the growing season, when most root exudates are released (Aulakh et al., 2001) and the ammonia concentration is highest, may explain the methanogen community structure (Singh et al., 2012). *Methanosarcina spp.* have been shown to be present during the rice-growing season, whereas during pre-planting, tilling or post-harvest, *Methanosaeta* were present in lower numbers (Singh et al., 2012), correlating with the lower concentrations of acetate available in the soil (Kruger et al., 2002). In paddy field soil, acetate-dependent

methanogenesis (acetoclastic) generally dominates over hydrogen-dependent methanogenesis (hydrogenotrophic), as demonstrated by ^{13}C-labeling experiments (Conrad, 1999;Conrad et al., 2002;Conrad, 2005;Zhang et al., 2016).

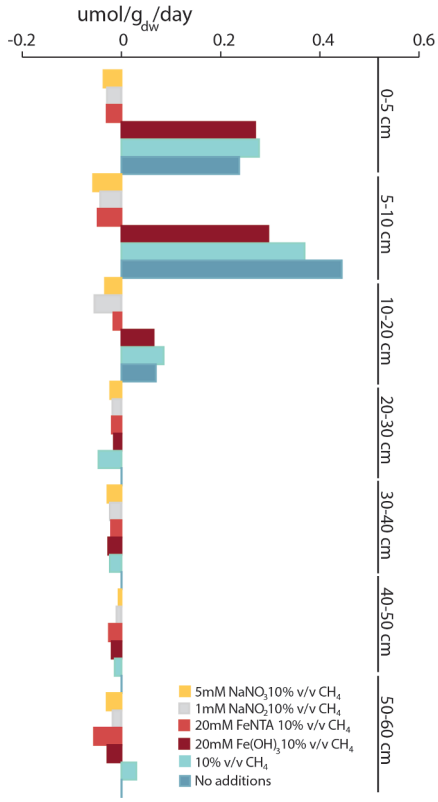

Figure 8: Methane oxidation and methanogenesis rates measured in soil slurries incubated with 10 % v/v 13C-methane with the addition of 5 mM NaNO3, 1 mM NaNO2, 20 mM FeNTA or 20 mM ferrihydrite. As controls, soil slurries were incubated with 10 % v/v 13C-methane and without added methane. GC measurements were performed in triplicate, and rates were calculated in the linear phase. Negative values stand for net oxidation of methane and positive values net production of methane.

The total methane concentration in soil over the course of rice maturation peaks at the flowering and ripening stage (Singh et al., 2012). Previous studies in Italian paddy fields have demonstrated that methane emission rates reach approximately 400 nmol CH_4 per g_{dw} d^{-1} 70-80 days after flooding (Kruger et al., 2005) or even approximately 600 nmol per g_{dw} d^{-1} (Conrad and Klose, 2006). We previously observed methanogenic activity of the same paddy field soil

of 432 nmol and 358 nmol per g_{dw} d^{-1} without and with the addition of methane, respectively, in incubation assays (Vaksmaa et al., 2016). In the current soil core, the highest methanogenic activity was recorded at a depth of 5-10 cm, with rates of 369 and 444 nmol per g_{dw} d^{-1} with and without the addition of methane. In control incubations in which methane was added, methanotrophic activity was initially observed. After three weeks, methanogenesis became the dominant process, with methane oxidation rates identical to those observed in the control treatment without the addition of methane. Furthermore, ferrihydrite added to slurry incubations seemed to stimulate methanogens in the top 20 cm. In those treatments, no initial methanotrophic activity was observed; only stimulation of methanogenic activity was recorded, even when both methane and possible electron donors were supplied. Previous studies in wetlands and paddy fields have demonstrated that the addition of poorly crystalline iron, such as ferrihydrite, has an inhibitory effect on methanogenic activity (Achtnich et al., 1995;Lueders and Friedrich, 2002). In contrast, the addition of highly crystalline iron oxide species of hematite or magnetite stimulates methanogens enriched from paddy field soil via a positive effect on either direct interspecies electron transfer or the availability of diffusive carriers such as hydrogen or formic acid (Kato et al., 2012;Holmes et al., 2017).

Methanotrophic bacteria of the NC10 phylum have been previously detected in paddy field soil based on the 16S rRNA gene or *pmoA* genes and activity assays with nitrite and methane. Conflicting results regarding the vertical distribution of NC10 phylum bacteria in soil have been reported. Zhou et al. (2014) indicated that the highest abundance of $1.0*10^8$ copies per g_{dw} occurred at a depth of 100-120 cm (Zhou et al., 2014). This finding was supported by a study by Hu et al. (2014) of a Chinese paddy field, in which the highest copy number abundance of $1.5±0.2*10^6$ to $4.5±0.3*10^6$ copies per g_{dw} was observed at a depth of 50-60 cm. However, the methane-oxidizing potential of soil slurries amended with nitrite was highest at a depth of 90-100 cm, with values of 1.68±0.03 to 2.04±0.06 nmol of CO_2 per g_{dw} (Hu et al., 2014). By contrast, in a subtropical paddy field soil core sampled to 100 cm, the abundance of NC10 phylum bacteria was highest at the 0-10 cm depth, with $1.0±0.1*10^5$ copies per g_{dw}, followed by $7.5±0.4*10^4$ at 30-40 cm and a subsequent gradual decrease, with no detection at depths of 70 cm and beyond (Wang et al., 2012)

The phylogenetic comparison of the 16S rRNA gene reads obtained from amplicon sequencing revealed that, in the top layers, the 16S rRNA gene reads were assigned exclusively to group B (Ettwig et al., 2009;Welte et al., 2016), with nucleotide identities of 95.6-96.7 % *to 'Candidatus* Methylomirabilis oxyfera'. Sequences belonging to Group A of NC10 phylum bacteria were found only at depths of 40 cm and below. This distribution is consistent with previous reports in which 16S rRNA gene sequencing and relative read abundance indicated that these nitrite-dependent AOM bacteria formed the largest subset of sequencing reads among total bacterial reads at depths of 50 cm and 100 cm (Ding et al., 2015). In our activity assays with nitrite, we

observed the highest methane oxidation potential of 55 nmol per g_{dw} h^{-1} in samples from 10-20 cm, which correlates with the first peak of high abundance of NC10 phylum bacteria. However, all the sequences at 10-20 cm all belonged to group B, for which no methane-oxidizing ability has been demonstrated thus far and needs further investigation.

In addition to detecting nitrite-dependent AOM bacteria of the NC10 phylum, we observed high numbers nitrate-dependent AOM archaea '*Candidatus* Methanoperedens nitroreducens' throughout the soil core. However compared to NC10 phylum bacteria, these archaea were more abundant at all depths, except 60 cm, where NC10 phylum bacteria outnumbered '*Candidatus* Methanoperedens nitroreducens'. Sequences classified as '*Candidatus* Methanoperedens nitroreducens' have been detected previously in paddy fields, including fields in Vercelli, Italy (Lueders et al., 2001;Conrad et al., 2008), Chinese paddy fields (Xu et al., 2012), and Korean paddy fields (Lee et al., 2015) as well as in natural wetlands (Narrowe et al., 2017). We previously quantified and detected '*Candidatus* Methanoperedens nitroreducens' in an Italian paddy field based on 16S rRNA gene (Vaksmaa et al., 2016) and mcrA gene sequences in high abundance (Vaksmaa et al., 2017). High relative sequence abundance has also been observed in other paddy fields based on the 16S rRNA gene, with 60 % of all archaeal reads classified as '*Candidatus* Methanoperedens nitroreducens' (GOM Arc I) at a depth of 60 cm in bulk soil (Lee et al., 2015). In a study by Lee *et al.* (2015), the soil core depth profile exhibited the same trend observed in the current study (Lee et al., 2015). The abundance of '*Candidatus* Methanoperedens nitroreducens' increased with depth, peaking at 20 cm with $1.8\pm0.3*10^7$copies per g_{dw}. The activity assays performed with nitrate and methane indicated that the activity was highest at a depth of 5-10 cm (57 nmol per g_{dw} d^{-1}), followed by a depth of 10-20 cm (33 nmol per g_{dw} d^{-1}). We previously observed a methane-oxidizing potential of 80 nmol methane per g_{dw} d^{-1} in mixed and sieved soil slurry from a depth of 10-20 cm (Vaksmaa et al., 2016). The present study is the first to evaluate potential methane oxidation rates utilizing nitrate as an electron acceptor in different depths of a paddy soil core. Recent research has revealed that '*Candidatus* Methanoperedens nitroreducens' not only can couple nitrate reduction to methane oxidation but is also able to reduce oxidized metals (Ettwig et al., 2016) and may play in important role in both methane and iron cycling in natural and man-made wetlands (Narrowe et al., 2017).

Finally the large number of *Bathyarchaeota* observed in this paddy field and other wetland systems is intriguing, and their potential role in methane cycling needs further investigation (Evans et al., 2015;Narrowe et al., 2017). For the microbial community members, were the function is still unknown, we detected *Bathyarchaeota* to be present throughout soil core with highest abundance at 5cm with $2.1\pm1.1*10^8$ 16S rRNA gene copies per g_{dw}. From the total archaeal community, these account for almost 50%. Albeit their unknown function, their high abundance and wide distribution indicates that though the function is unknown they might be relevant microorganisms. Up to date, the members of phylum *Bathyarcheota* have been

detected in a wide range of habitats from terrestrial to marine, cold and hot temperatures or surface and subsurface environments. Generally they are known to be abundant in marine environments (Teske et al., 2002;Lipp et al., 2008;Kubo et al., 2012). Similarly, other studies showed *Bathyarchaeota* to be present in freshwater environments (Porat et al., 2010;Li et al., 2012). Previous studies have detected *Bathyarchaeota* in paddy fields as well (Lee et al., 2014;Lee et al., 2015) with abundance of *Bathyarchaeota* increasing from 17% to 23% in three different phases of rice cultivation (Breidenbach and Conrad, 2014) and with relative abundance up to 42 % in paddy field sub-soils (Bai et al., 2017).

In summary, we observed high diversity of the archaeal and bacterial microbial communities throughout the soil core and determined the methane-oxidation potential with various electron acceptors at several soil depths. This study highlights the usage of various electron acceptors for the AOM process. Our findings provide support for the significant role of '*Candidatus* Methanoperedens nitroreducens' carrying out nitrate-dependent and/or iron-dependent AOM in paddy fields. NC10 phylum bacteria seem to play a less significant role in AOM in paddy fields. The as-yet unknown functions of members of the *Candidatus* Methylacidiphilum genus and *Bathyarchaeota* in paddy field soil will hopefully be explained in studies in the near future. We acknowledge that the small sample size of our study does have its limitations, and future studies should include more samples in order to more accurately estimate the contribution of AOM in paddy fields on a larger scale.

Conflict of Interest

All authors declare that they have no conflict of interest. All prevailing local, national and international regulations and conventions, and scientific ethical practices, have been respected.

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Author Contributions

AV and CL designed the research and carried out the fieldwork. EL and GV provided the access to the sampling station, and supported the design and fieldwork. AV carried out the experiments in the laboratory. TV and AV collected and interpreted the sequencing data. AV, CL and MJ drafted and finalized the manuscript with input from all authors. The manuscript was checked by a professional peerwith.com editor.

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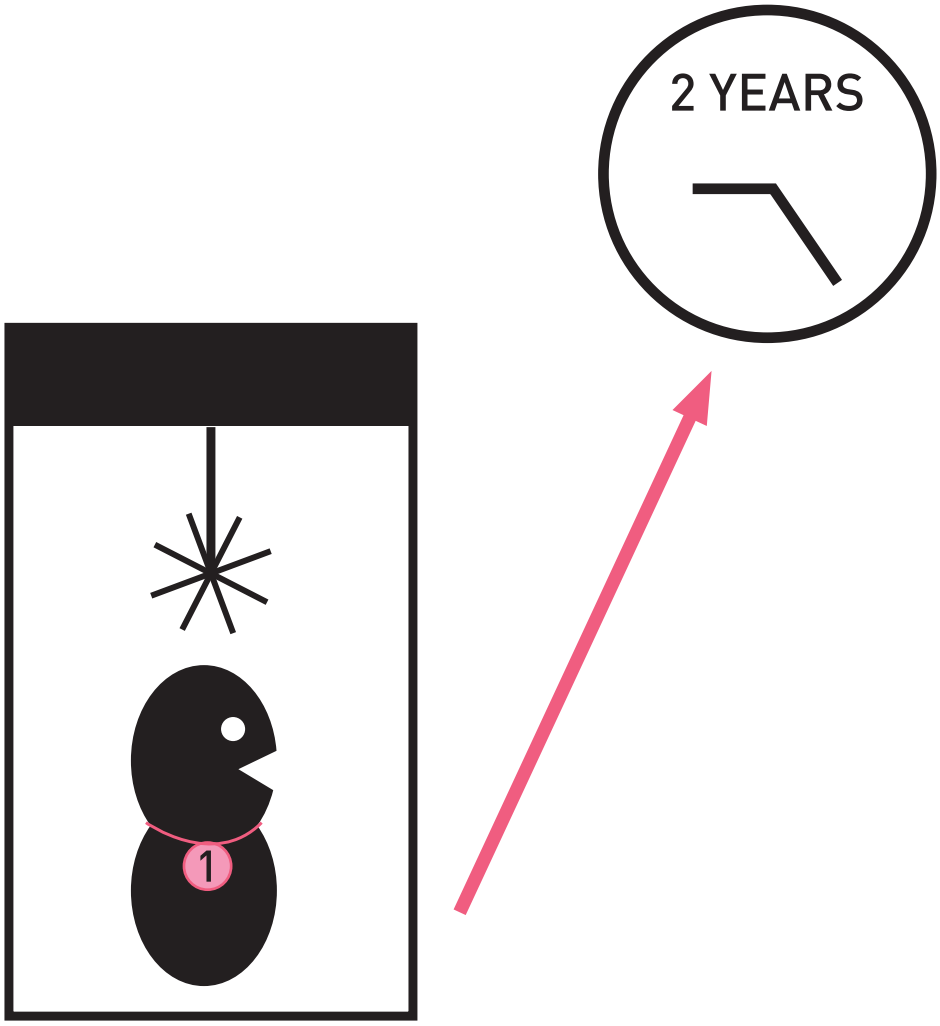
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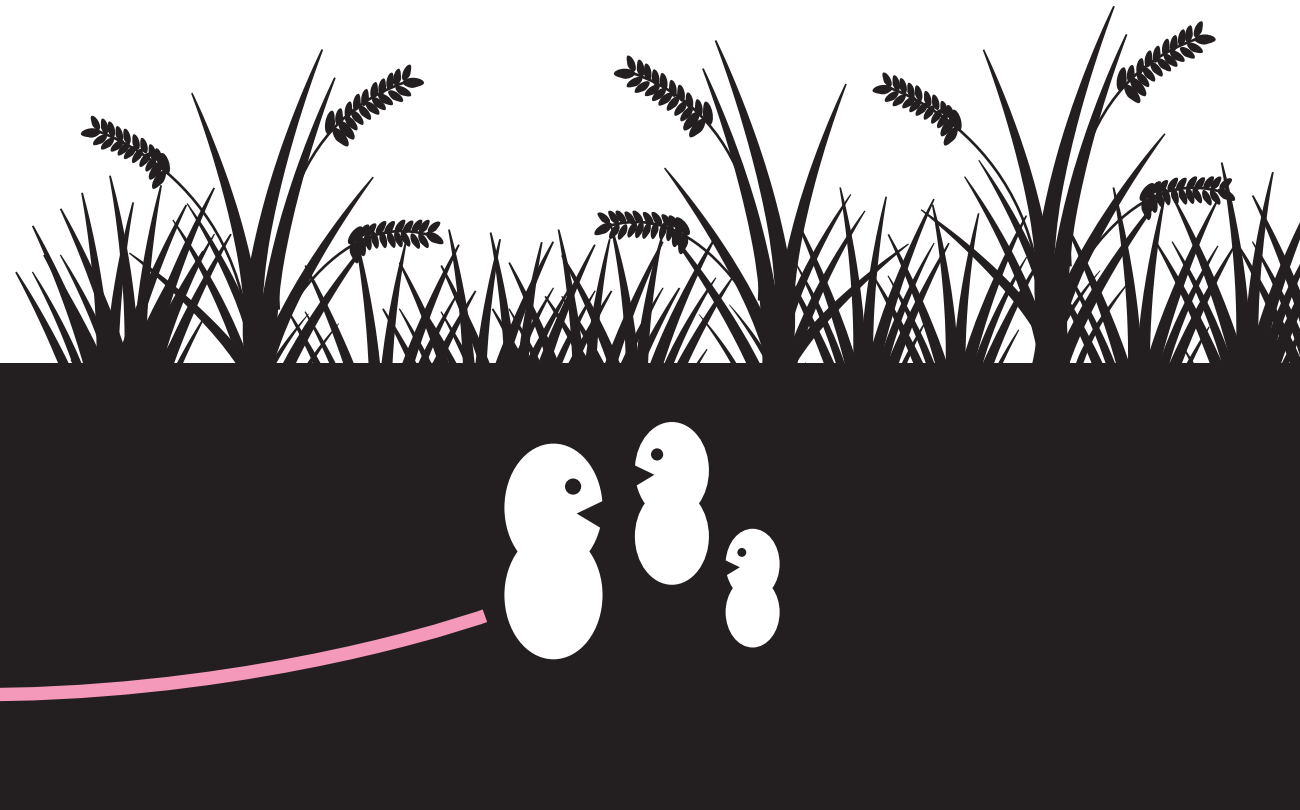
Enrichment of anaerobic nitrate-dependent methanotrophic '*Candidatus* Methanoperedens nitroreducens' archaea from an Italian paddy field soil



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ENVIRONMENTAL BIOTECHNOLOGY

Enrichment of anaerobic nitrate-dependent methanotrophic '*Candidatus* Methanoperedens nitroreducens' archaea from an Italian paddy field soil

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Abstract Paddy fields are a significant source of methane and contribute up to 20% of total methane emissions from wetland ecosystems. These inundated, anoxic soils featuring abundant nitrogen compounds and methane are an ideal niche for nitrate-dependent anaerobic methanotrophs. After 2 years of enrichment with a continuous supply of methane and nitrate as the sole electron donor and acceptor, a stable enrichment dominated by '*Candidatus* Methanoperedens nitroreducens' archaea and '*Candidatus* Methylomirabilis oxyfera' NC10 phylum bacteria was achieved. In this community, the methanotrophic archaea supplied the NC10 phylum bacteria with the necessary nitrite through nitrate reduction coupled to methane oxidation. The results of qPCR quantification of 16S ribosomal RNA (rRNA) gene copies, analysis of metagenomic 16S rRNA reads, and fluorescence in situ hybridization (FISH) correlated well and showed that after 2 years, '*Candidatus* Methanoperedens nitroreducens' had the highest abundance of ($2.2 \pm 0.4 \times 10^8$) 16S rRNA copies per milliliter and constituted approximately 22% of the total microbial community. Phylogenetic analysis showed that the 16S rRNA genes of the dominant microorganisms clustered with previously described '*Candidatus* Methanoperedens nitroreducens ANME2D' (96% identity) and '*Candidatus* Methylomirabilis oxyfera' (99% identity) strains. The pooled metagenomic sequences resulted in a high-quality draft genome assembly of '*Candidatus* Methanoperedens nitroreducens Vercelli' that contained all key functional genes for the reverse methanogenesis pathway and nitrate reduction. The diagnostic *mcrA* gene was 96% similar to '*Candidatus* Methanoperedens nitroreducens ANME2D' (WP_048089615.1) at the protein level. The '*Candidatus* Methylomirabilis oxyfera' draft genome contained the marker genes *pmoCAB*, *mdh*, and *nirS* and putative NO dismutase genes. Whole-reactor anaerobic activity measurements with methane and nitrate revealed an average methane oxidation rate of 0.012 mmol/h/L, with cell-specific methane oxidation rates up to 0.57 fmol/cell/day for '*Candidatus* Methanoperedens nitroreducens'. In summary, this study describes the first enrichment and draft genome of methanotrophic archaea from paddy field soil, where these organisms can contribute significantly to the mitigation of methane emissions.

Keywords '*Candidatus* Methanoperedens nitroreducens' · Anaerobic oxidation of methane · NC10 phylum bacteria

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Introduction

The methane concentration in the atmosphere has increased continuously over the last 150 years. Methane is the second most abundant greenhouse gas and exhibits radiative forcing up to 34 times higher than that of CO_2 (Myhre et al. 2013). Paddy fields are a significant source of methane and contribute 10–20% to global methane emissions (Bodelier 2011; Conrad 2009). The cultivated area dedicated to rice agriculture occupies approximately 160 million ha of land worldwide and

is predicted to increase by 60% in the coming decades. Without changes in cultivation practices, such increases will result in even higher atmospheric methane emissions.

The global biogenic methane budget is directly linked to the activity of methanogenic and methanotrophic microorganisms in the environment. Methanotrophic organisms function as a biofilter, and without their contribution, it is estimated that the atmospheric methane concentration would be 10–60% higher (Conrad 2009). Whereas aerobic methanotrophs are well studied, much less is known about methane removal in oxygen-limited nitrogen-loaded freshwater environments. NC10 phylum ('*Candidatus* Methylomirabilis oxyfera') bacteria and '*Candidatus* Methanoperedens nitroreducens' archaea are the only methanotrophic microorganisms known to directly couple the anaerobic oxidation of methane to the nitrogen cycle. NC10 phylum bacteria use nitrite as an electron acceptor (A), and '*Candidatus* Methanoperedens nitroreducens' archaea perform nitrate reduction (B) with methane as an electron donor according to the following reactions:

$$8NO_2^- + 3CH_4 + 8H^+ \rightarrow 4N_2 + 3CO_2 + 10H_2O$$

$$\left(\mathrm{A}, \Delta G0' = -987\ \mathrm{kJ}/\mathrm{mol}\ CH_4\right)$$

$$2\mathrm{x}\ (4NO_3^- + CH_4 \rightarrow 4NO_2^- + CO_2 + 2H_2O)$$

$$\left(\mathrm{B}, \Delta G0' = -503\ \mathrm{kJ}/\mathrm{mol}\right)$$

$$8HNO_3 + 5CH_4 \rightarrow 5CO_2 + 4N_2 + 14H_2O\ (\mathrm{sum})$$

In 2006, an enrichment culture in which nitrate and nitrite reduction were coupled to the anaerobic oxidation of methane was described for the first time (Raghoebarsing et al. 2006). In that study, an inoculum from the sediment of a freshwater canal was used to start an anaerobic enrichment. After 16 months, the culture was dominated by a consortium consisting of archaea (10–15% of cells) belonging to the *Methanosarcinales* family that were only distantly related to ANME2D (86–87% in 16S ribosomal RNA (rRNA identity)) and a bacterium (approximately 80% of cells) of the candidate division NC10. The enriched co-culture preferred nitrite over nitrate as the substrate, although activity was observed with both substrates (Raghoebarsing et al. 2006). The nitrite-dependent anaerobic oxidation of methane (AOM) was later assigned to phylum NC10 bacteria, which are able to carry out this process in the absence of other microorganisms (Ettwig et al. 2008). The bacterium uses an intra-aerobic mechanism in which oxygen is produced via a putative nitric oxide dismutase and subsequently used for methane oxidation via the particulate methane monooxygenase complex. Assembly of the genome of the NC10 bacterium revealed a complete methane oxidation pathway that included the *pmoCAB* operon and an incomplete denitrification pathway. It was hypothesized that the dismutation of nitric oxide to oxygen and nitrogen supplies O_2 for the methane monooxygenase. These NC10 phylum bacteria were named '*Candidatus* Methylomirabilis oxyfera' (Ettwig et al. 2010). Sequencing of the genome of the AOM archaea and identification of their nitrate reductase indicated that these archaea could couple nitrate reduction to AOM (Arshad et al. 2015; Haroon et al. 2013). The responsible archaea were named '*Candidatus* Methanoperedens nitroreducens.'

Recent microbial ecology studies have indicated sufficient presence and activity of methanotrophic archaea in paddy field soils (Lee et al. 2015; Vaksmaa et al. 2016) to warrant investment in a long-term enrichment procedure to obtain these paddy field AOM archaea and to study their physiology and metabolic potential in more detail.

To achieve this goal, we started a sequencing batch bioreactor continuously fed with nitrate and methane and inoculated with soil from an Italian paddy field soil harboring substantial AOM archaeal cell numbers (Vaksmaa et al. 2016). After establishing nitrate-dependent methane oxidation, the total DNA of this biomass was sequenced using Ion Torrent technology, and the draft genome was annotated and analyzed. The enriched microbial community was further characterized by microscopy, $^{13}CH_4$ and ^{15}N activity assays, and qPCR.

Materials and methods

Source of inoculum

The soil was sampled in September 2013 from paddy fields at the Italian Rice Research Unit in Vercelli, Italy (08° 22′ 25.89″ E; 45° 19′ 26.98″ N). These fields of silt loam soil were flooded with approximately 15 cm of water and regularly tilled. The soil of the experimental field was fertilized with 147.5 kg/ha nitrogen and 183 kg/ha potassium 21 days after flooding. Soil was collected 95 days after flooding. The rice variety cultivated in the field plots was *Oryza sativa japonica* Onice. The soil was sampled down to 20 cm and transported to the laboratory in a container flooded with water sampled from the field. After storage at 4 °C for 6 months, the reactor was started with 200 g of soil (wet weight).

Enrichment culture

A 2-L bioreactor (Applikon, The Netherlands) was operated at 27 °C as a sequencing batch reactor. The sequence consisted of 12 h cycles of 10 h of constant medium supply, 1 h of biomass settling, and 1 h of pumping out of excess liquid. The medium contained $CaCl_2{\cdot}2H_2O$ (0.15 g/L) and KH_2PO_4 (0.01 g/L) and was autoclaved before the addition of $MgSO_4{\cdot}7H_2O$ (0.1 g/L); 5 mL of a trace element stock solution composed of $ZnSO_4{\cdot}7H_2O$

(0.2875 g/L), $CoCl_2 \cdot 6H_2O$ (0.12 g/L), $CuSO_4$ (0.8 g/L), $NiCl_2 \cdot 6H_2O$ (0.19 g/L), H_3BO_3 (0.014 g/L), $MnCl_2 \cdot 4H_2O$ (0.2 g/L), $Na_2WO_4 \cdot 2H_2O$ (0.02 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.0968 g/L), SeO_2 (0.027 g/L), and $CeCl_2$ (0.023 g/L); 3 mL of an iron stock solution composed of $FeSO_4 \cdot 7H_2O$ (5 g/L) and NTA (10.31 g/L); and 1 mL of vitamin solution (DSMZ 141). The medium was constantly sparged with Ar/CO_2 (95:5%) to maintain anaerobic conditions prior to being supplied by peristaltic pump to the bioreactor at a flow rate of 18.75 mL/h. The $NaNO_3$ concentration in the medium was increased from 1.25 to 5 mM after a year of operation due to an increased consumption rate. The bioreactor was operated at pH 7, maintained with automatic supply of $KHCO_3$, stirred at 150 rpm, and sparged with CH_4-CO_2 (95% vol/vol; purity 99.995%; flow rate 4.26 mL/min).

Activity measurements

Activity measurements were performed in the whole reactor in batch mode after cutoff of the supply of medium and methane. The nitrate in the bioreactor was depleted, and the headspace was flushed with Ar-CO_2 (95:5). Once residual methane was no longer detected by gas chromatography, 5 mM ^{15}N-$NaNO_3$ and 20% ^{13}C-CH_4 (vol/vol) were added to the reactor and headspace, respectively. Gas samples of 100 µL were taken at various time points over 3–7 days; the production of ^{13}C-CO_2 was monitored by gas chromatography-mass spectrometry (GC-MS) (Agilent 5975 inert MSD, Agilent, USA), and the consumption of CH_4 was measured by GC (Hewlett Packard 5890, USA). Liquid culture samples of 1 mL (duplicate) were collected for the determination of NO_3 (measured by a Sievers 280i NO analyzer, GE Analytical Instruments, USA) and NO_2 and NH_4 (measured by colorimetric assays as described by Kartal et al. 2006). Liquid samples were centrifuged for 1 min at 14,000*g*, and the supernatant was removed for storage at −20 °C until analysis.

Further activity assays were performed in 60-mL serum bottles with 15 mL of biomass from the reactor. The reactor was stirred at 500 rpm for 5 min before sampling to ensure appropriate mixing of all settled biomass. After transfer of 15 mL of slurry, fresh medium (composition described above) and ^{15}N-$NaNO_3$ (final concentration in bottles, 5 mM) or ^{15}N-$NaNO_2$ (final concentration in bottles, 1 mM) were added. The bottles were made anaerobic by 5 cycles of vacuum and purging with argon-CO_2 (95–5%). An overpressure of 0.5 bar was introduced to the bottles, and 10% ^{13}C-CH_4 was added. Measurements of ^{13}C-CO_2 and CH_4 in a 50-µL headspace sample were obtained by GC-MS and GC, respectively. Calibration was performed with standard gas consisting of QS:1.06%:0.82%:1.32%:459 ppm $He/CO_2/N_2/O_2/N_2O$ (Air Liquide BV, The Netherlands). Analysis of nitrogen compounds was performed as described above for the whole reactor as batch.

DNA extraction

DNA was extracted from 10 mL of reactor biomass in duplicate using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol with addition of 3 min beadbeating step. DNA quantity and quality were assessed by UV-vis spectroscopy (NanoDrop, ND-1000, Isogen Life Science, The Netherlands).

Quantification by qPCR

The abundances of '*Candidatus* Methanoperedens nitroreducens,' NC10 phylum bacteria, total bacteria, and total archaea were quantified based on 16S rRNA gene amplification by qPCR. The qPCR reactions were performed in triplicate on all DNA extracts. '*Candidatus* Methanoperedens nitroreducens' were targeted with the clade-specific primers 641F (5′ ACTGDTAGGCTTGGGACC3′) and 834R (5′ ATGCGGTCGCACCGCACCTG3′) (previously reported as FISH probes) (Schubert et al. 2011). NC10 phylum bacteria were amplified with the 16S rRNA primers p2F_DAMO (5′ GGGGAACTGCCAGCGTCAAG3′) and p2R_DAMO (5′ CTCAGCGACTTCGAGTACAG3′) (Ettwig et al. 2009b). The total number of archaea was quantified using the following primers: Arch-349F (5′ GYGCASCAGKCGMGAAW3′) and Arch-807R (5′ GGACTACVSGGGTATCTAAT3′). For bacteria, the primers Bac-341F (5′ CCTACGGG NGGCWGCAG3′) and Bac-515R (5′ TTACCGCG GCTGCTGGCAC3′) (Klindworth et al. 2013) were used. All qPCR reactions were performed using PerfeCTa Quanta master mix (Quanta Biosciences, Gaithersburg, MD, USA) and 96-well optical plates on a Bio-Rad IQ™ 5 cycler (Bio-Rad, USA). Absolute quantification was performed by comparison to standard curves obtained using a tenfold serial dilution of pGEM-T Easy plasmid DNA (Promega, USA) carrying an insert of the target gene obtained using the same primers used for qPCR. Standard curve samples were used as a control for each qPCR run.

Fluorescence in situ hybridization

'*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria were detected using 2 mL of reactor biomass sample. The sample was pelleted, washed twice with 1 mL of 1× PBS, and fixed with paraformaldehyde on ice for 3 h. Fluorescence in situ hybridization (FISH) was performed as described by Ettwig et al. (2008).

Metagenome sequencing

Ion Torrent sequencing was performed on DNA samples obtained from the bioreactor after 1 and 2 years of operation. DNA was isolated as described above. In total, 185 ng of isolated genomic DNA was sheared for 9 min using a Bioruptor® UCD-200 (Thermo Fisher Scientific Inc., USA). Libraries were prepared using an Ion Plus Fragment library kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. For size selection of the adapter ligated fragments, an E-Gel® electrophoresis system was used with a 2% E-Gel® SizeSelect™ agarose gel (Life Technologies, Bleiswijk, The Netherlands). Eight cycles of amplification of the size-selected fragments were performed as suggested in the protocol. The concentrations and fragment lengths of the libraries were determined with a Bioanalyzer® 2100 and High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA.) The library was diluted to a final concentration of 26 pM for emulsion PCR. Emulsion PCR was performed using an Ion OneTouch™ 2 Instrument and Ion PGM™ Template OT2 400 Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. The template-positive Ion Sphere™ Particles (ISPs) were enriched using the Ion One Touch™ ES (Thermo Fisher Scientific Inc., USA), loaded on an Ion 318™ v2 Chip and sequenced using an Ion PGM™ Sequencing 400 Kit with 850 nucleotide flows according to the manufacturer's instructions. After sequencing, all raw reads were imported into CLC Genomics Workbench v. 9 (QIAGEN Aarhus A/S, Denmark) for initial data analysis, including trimming of low-quality and short reads (cutoff value of 100 nucleotides), followed by assembly of the reads obtained from both sequencing runs (word size 30, bubble size 5000). The raw reads of metagenome sequencing after 1 and 2 years have been deposited to the European Nucleotide Archive, with study accession number PRJEB20370. To extract the contigs of '*Candidatus* Methanoperedens nitroreducens Vercelli,' the contigs were binned based on GC content and coverage using RStudio (RStudio Team 2015) with the GC script. The contigs of '*Candidatus* Methanoperedens nitroreducens Vercelli' were extracted from all assemblies, and reads mapping to contigs were reassembled in CLC (word size 30, bubble size 5000). The completeness of the draft genome and contamination were assessed by CheckM (Parks et al. 2015). MaGe, online full annotation and integration automated pipeline (Vallenet et al. 2009, 2006, 2017), was used to annotate the genome of '*Candidatus* Methanoperedens nitroreducens Vercelli,' and this subsequently was visualized in Artemis (Rutherford et al. 2000). The annotated genome of '*Candidatus* Methanoperedens nitroreducens Vercelli' has been deposited at GenBank under the accession ERS1800110. BLAST was used to search for key genes in '*Candidatus* Methylomirabilis oxyfera.' The contigs from *'Candidatus* Methylomirabilis oxyfera' were extracted after differential mapping. The reads from each year were mapped to the assembly from the combined years (0.5 length fraction and 0.95 similarity), and both values for each read were plotted against one another in RStudio. The contigs containing the *nod*, *pmo*, *nirS*, and 16S genes were manually curated to extend them over the ends of the genes. The annotated contigs were checked using the visualization and annotation tool Artemis.

Results

Enrichment procedure for anaerobic oxidation of methane organisms in the bioreactor

The microbial cells in the inoculum and enrichment culture were quantified by qPCR based on the 16S rRNA gene. The abundance of '*Candidatus* Methanoperedens nitroreducens' was one to two orders of magnitude higher than that of NC10 phylum bacteria in the inoculum slurry (Table 1). After 2 years of enrichment, '*Candidatus* Methanoperedens nitroreducens' constituted approximately 22% of the total microbial community based on the qPCR results and was one order of magnitude more abundant than NC10 phylum bacteria. The growth of '*Candidatus* Methanoperedens nitroreducens' started after 10 months of enrichment. The lag phase of NC10 phylum bacteria appeared to be longer, but after a year, their 16S rRNA gene copy numbers had already increased from 10^3 to 10^7 per milliliter (Fig. 1).

Fluorescence in situ hybridization

Biomass samples from the enrichment culture were analyzed with specific probes for '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria after 2 years of enrichment. Both microorganisms were present in the reactor (Fig. 2), although the cell numbers of the NC10 bacteria appeared to be higher than determined by qPCR.

Activity of the nitrate-dependent anaerobic oxidation of methane co-culture

The culture in the bioreactor oxidized CH_4 to CO_2 using nitrate as an electron acceptor. The oxidation rates increased over the time span of 2 years. The initial potential to oxidize methane at the expense of nitrate in the soil slurries was 16.8 nmol/g dry weight/day with 2 mM $NaNO_3$ versus 3.7 nmol/g/day in the controls, which were incubated without any external electron acceptor and 10% methane in the headspace (Vaksmaa et al. 2016). During the 2 years of bioreactor operation, neither nitrite (<80 μmol/L) nor ammonia was detected (below the detection level) in significant quantities.

Table 1 16S rRNA gene copies of total archaea, '*Candidatus* Methanoperedens nitroreducens,' total bacteria, and NC10 phylum bacteria in the enrichment at the start of the reactor and after 0.5, 1, and 2 years of operation (mean ± SE; $n = 6$), calculated per 1 mL of reactor sample

	$T = 0$	0.5 years	1 year	1.5 years	2 years
Total archaea	$2.6 \pm 0.2 \times 10^6$	$6.7 \pm 0.3 \times 10^6$	$1.3 \pm 0.8 \times 10^8$	$2.4 \pm 0.4 \times 10^8$	$6.6 \pm 0.9 \times 10^8$
M. nitroreducens	$1.9 \pm 0.1 \times 10^5$	$2.7 \pm 0.4 \times 10^6$	$3.2 \pm 0.1 \times 10^7$	$1.7 \pm 0.0 \times 10^8$	$2.2 \pm 0.4 \times 10^8$
Total bacteria	$1.6 \pm 0.1 \times 10^8$	$3.0 \pm 1.2 \times 10^7$	$2.3 \pm 0.0 \times 10^7$	$1.7 \pm 0.0 \times 10^7$	$3.2 \pm 0.3 \times 10^8$
NC10 phylum bacteria	$1.9 \pm 0.9 \times 10^3$	$8.8 \pm 4.8 \times 10^3$	$2.2 \pm 0.3 \times 10^6$	$2.0 \pm 0.1 \times 10^6$	$7.9 \pm 0.3 \times 10^7$

After 2 years, the nitrate consumption and methane consumption were 0.055 and 0.012 mmol/h/L, respectively (Fig. 3). Activity measurements with ^{13}C-CH_4 with 1 mM nitrite and 5 mM nitrate in serum bottles after 2 years of enrichment indicated that only the conversion of methane to CO_2 (0.19 mmol/h/L) occurred in bottles amended with nitrate. Surprisingly, the methane conversion rates in serum bottles amended with nitrite were similar to those in the control sample, where no activity was seen (Supplementary Fig. S1).

Metagenomic analysis and classification based on the 16S ribosomal RNA gene

After 1 and 2 years of enrichment, DNA was extracted from the culture and sequenced by Ion Torrent technology (Supplementary Table S1) to first analyze the 16S rRNA gene composition and then to assemble draft genomes.

From the two metagenomes obtained after 1 and 2 years of enrichment, 1014 and 1423, 16S rRNA reads were extracted, respectively. The phylogenetic classifications for groups with an abundance of greater than 1.5% of the total number of 16S rRNA gene reads are shown in Fig. 4. Of the 1014 16S rRNA reads from the first year, 19.1% were assigned to GOM Arc I (the group to which '*Candidatus* Methanoperedens' is classified in the ARB SILVA database (Ludwig et al. 2004)), followed by 8.5% assigned to *Chloroflexi*, 7% OC31, 6.9% *Candidatus* Methylomirabilis (classified as *Nitrospirae* in the ARB SILVA database), and 4.4% *Phycisphaeraceae* of *Planctomycetes*. Of the 1423 reads obtained after 2 years of enrichment, 22% were assigned as GOM Arc I, followed by 15% *Candidatus* Methylomirabilis, 6.1% *Rhodocyclaceae* of *Betaproteobacteria*, 5.6% *Comamonadaceae* of *Betaproteobacteria*, and 3.5% *Anaerolineaceae* of *Chloroflexi*. Draft genomes were binned based on GC content-coverage (Supplementary Fig. S2) and assembled and annotated in MaGe (17). The '*Candidatus* Methanoperedens nitroreducens Vercelli' draft genome contained 250 contigs, with a total size of 3.5 Mb. The completeness as assessed by CheckM was 97.7%. The genome contained all key enzymes for the reverse methane oxidation pathway and nitrate reductase (Supplementary Table S2). Phylogenetic analysis of the 16S rRNA gene from the assembled genome revealed that the '*Candidatus* Methanoperedens nitroreducens' 16S rRNA gene had 96% identity to '*Candidatus* Methanoperedens nitroreducens ANME2D'

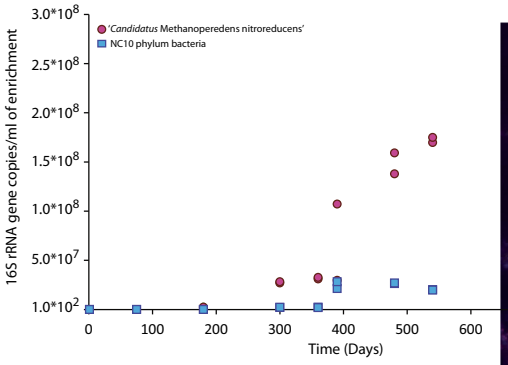

Fig. 1 qPCR quantification of the 16S rRNA gene copy numbers of '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria over the period of 2 years (all time points were analyzed using duplicate DNA extractions and triplicate qPCR reactions). The time in days is depicted horizontally, whereas the 16S rRNA copies per milliliter of enrichment are depicted vertically

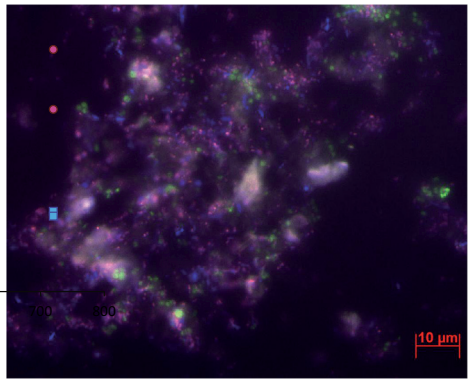

Fig. 2 Fluorescent micrograph of biomass sample from the enrichment culture. *Blue* corresponds to Cy5-EUBMIX, total bacteria, *pink* to NC10 phylum bacteria (Cy5-EUBMIX, DAMO193), and *green* to '*Candidatus* Methanoperedens nitroreducens' (FLUOS 641) (Color figure online)

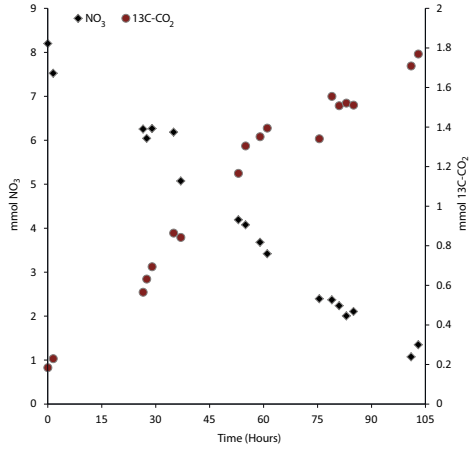

Fig. 3 Nitrate consumption and ^{13}C-CO_2 production in batch assays of the total reactor. The time in hours is depicted horizontally, whereas the total amounts in millimole of nitrate (*left axis*) and ^{13}C-CO_2 (*right axis*) are depicted vertically

(JMIY01000002.1) (Haroon et al. 2013) and 97% identity to '*Candidatus* Methanoperedens BLZ1' (LKCM01000080.1) (Arshad et al. 2015) (Fig. 5). The diagnostic methyl-coenzyme M reductase *mcrA* gene showed 96% identity at the protein level to (WP_048089615.1) and 89% identity to (KPQ44219.1) (Fig. 6). The 16S rRNA gene of the NC10 phylum bacteria had 99% nucleotide identity to '*Candidatus* Methylomirabilis oxyfera' (locus tag DAMO__16s_rRNA_1) (FP565575) and clustered within group A of the NC10 phylum (Fig. 7). The '*Candidatus* Methylomirabilis oxyfera' draft genome contained the diagnostic *pmoCAB*, *mxaF* methanol dehydrogenase, *nirS cd1* nitrite reductase, and putative NO dismutase genes. The analyzed *pmoA* gene had 95% identity at the protein level to '*Candidatus* Methylomirabilis oxyfera' (CBE69519). We also identified the methanol dehydrogenase large subunit (*mxaF*), with 85% identity (CBE67248) at the protein level, and two copies of the putative nitric oxide dismutase (*nod*) with 98% identity to CBE69502 and 92% identity to CBE69496. The identified nitrite reductase (*nirS*) had 93% identity to '*Candidatus* Methylomirabilis oxyfera' (CBE69462).

Discussion

Nitrate-dependent anaerobic oxidation of methane (N-AOM) was discovered a decade ago, but the characterization of the metabolism has been hindered by the slow growth of the responsible organisms. The N-AOM microorganisms '*Candidatus* Methanoperedens nitroreducens' archaea and NC10 phylum bacteria have been detected in various fresh water sediments (Welte et al. 2016). In this study, we started an enrichment culture fed solely with methane and nitrate using a paddy field soil harboring significant amounts of '*Candidatus* Methanoperedens nitroreducens' (Vaksmaa et al. 2016) as the inoculum. Based on qPCR, FISH, and metagenome analyses, the enrichment was dominated by '*Candidatus* Methanoperedens nitroreducens' after 2 years of enrichment.

Many previous enrichments were fed with nitrite or a mixture of nitrite and nitrate instead of nitrate only; such conditions are presumably advantageous to NC10 phylum bacteria. We intentionally omitted ammonium from the medium as other studies showed that such cultures would yield a mixed culture of '*Candidatus* Methanoperedens nitroreducens' and anammox bacteria, which could outcompete NC10 phylum bacteria for nitrite (Shi et al. 2013). Our previous field work demonstrated a high abundance of '*Candidatus* Methanoperedens nitroreducens' in the paddy field soil (Vaksmaa et al. 2016),

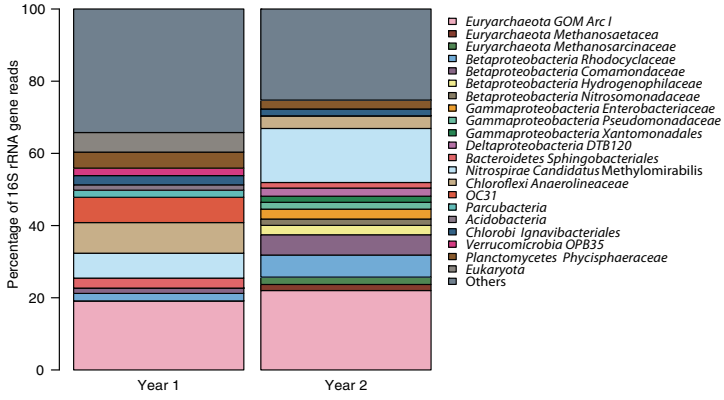

Fig. 4 Phylogenetic classification based on 16S rRNA gene reads obtained from the metagenome after enrichment for 1 and 2 years

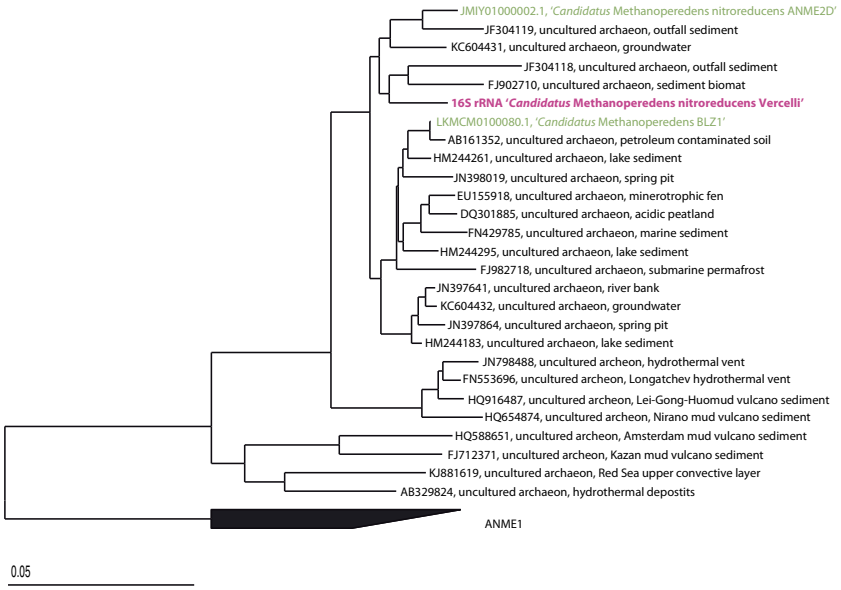

Fig. 5 Phylogenetic tree illustrating the relationships between the assembled 16S rRNA contig of '*Candidatus* Methanoperedens nitroreducens' and closely related sequences. The phylogenetic tree was constructed in ARB using the neighbor-joining method. The tree was rooted to the ANME1 cluster. The *scale bar* represents a difference of 0.05 substitutions per site

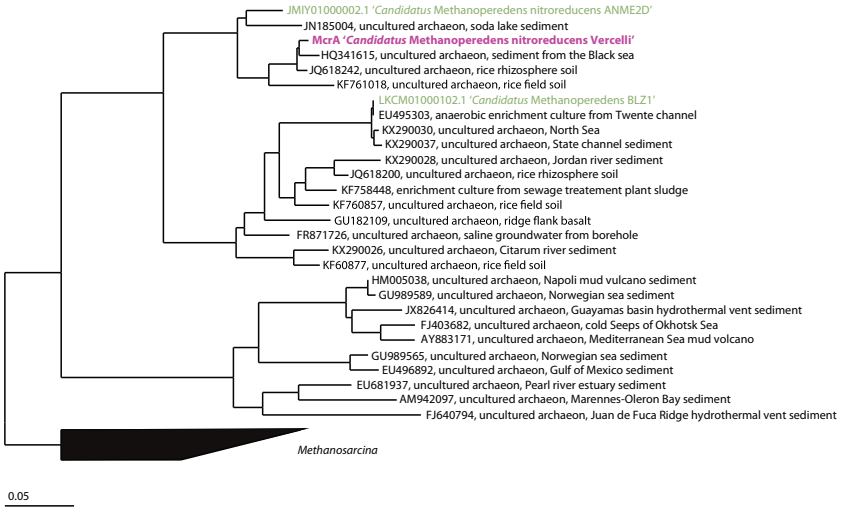

Fig. 6 Phylogenetic tree illustrating the relationships between the *mcrA* contig of '*Candidatus* Methanoperedens nitroreducens' and closely related sequences. The phylogenetic tree was constructed in ARB using the neighbor-joining method. The tree was rooted to the *Methanosarcina* cluster, including *Methanosarcina mazei*. The *scale bar* represents a difference of 0.05 substitutions per site

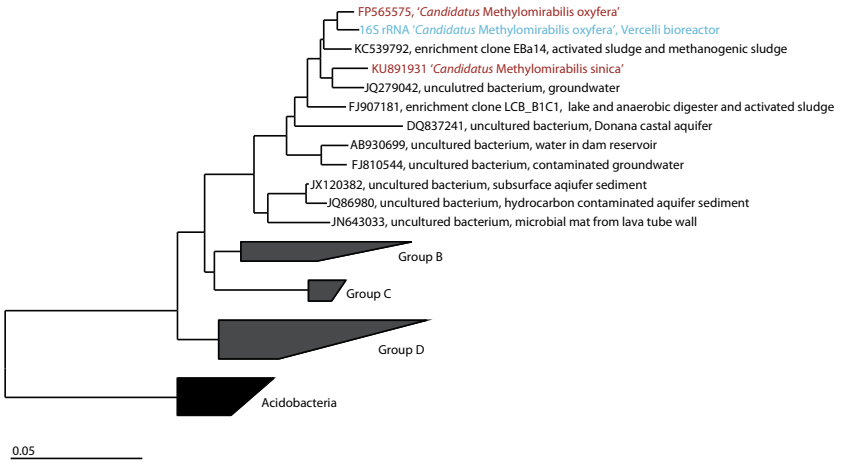

Fig. 7 Phylogenetic tree illustrating the relationships between the 16S rRNA contig of Methylomirabilis bacteria from the metagenome and closely related sequences. Depicted is the clustering of the NC10 clade into groups A–D. '*Candidatus* Methylomirabilis oxyfera' and '*Candidatus* Methylomirabilis sinica' of group A are marked in *red*. The *scale bar* represents a difference of 0.05 substitutions per site. The tree was constructed in ARB using the neighbor-joining algorithm with Jukes-Cantor correction. The tree was rooted to *Acidobacteria* (Color figure online)

which was confirmed by qPCR of the inoculum slurry. The inoculum slurry contained approximately $1.9 \pm 0.1 \times 10^5$ copies per milliliter of the 16S rRNA gene of '*Candidatus* Methanoperedens nitroreducens' and $1.9 \pm 0.3 \times 10^3$ copies per milliliter of NC10 phylum bacteria. After 2 years of enrichment, these numbers had increased to $2.2 \pm 0.4 \times 10^8$ 16S rRNA copies per milliliter of '*Candidatus* Methanoperedens nitroreducens', corresponding to 22% of the total detected 16S rRNA copies (bacteria plus archaea). These numbers indicate a doubling time of 1 to 2 months. The abundance of 16S rRNA gene copies of NC10 phylum bacteria was $7.9 \pm 0.3 \times 10^7$, corresponding to approximately 4% of the total copies.

The qPCR copy numbers correlated well with the metagenome sequencing results for '*Candidatus* Methanoperedens nitroreducens', with an abundance of 16S rRNA reads of 22% after 2 years. The percentage of reads assigned to NC10 phylum bacteria was 15%, possibly indicating underestimation by qPCR. The results based on the two methods presented here provided insight into the growth dynamics of both methane oxidizers. The growth of '*Candidatus* Methanoperedens nitroreducens' was observed after approximately 10 months of acclimatization, whereas for NC10 phylum bacteria, more than a year was necessary before a substantial increase in cell numbers was observed. The initial growth of NC10 phylum bacteria was presumably nitrite-limited. Similar lag phases of the growth of NC10 phylum bacteria in enrichment cultures have been reported previously. Zhu et al. showed that '*Candidatus* Methanoperedens nitroreducens' only started to increase in an enrichment obtained from minerotrophic peatland after 9 months, when significant methane oxidation rates (9 nmol/day/g in serum bottles, based on CO_2 production) indicated microbial growth (Zhu et al. 2012). In addition to substrate preference and availability, temperature has been implicated as a decisive factor in the outcome of AOM enrichments. In enrichments started from wastewater treatment plant sludge and lake sediments, a co-enrichment of NC10 phylum bacteria and '*Candidatus* Methanoperedens nitroreducens' was obtained at 35 °C, whereas at 22 °C, only NC10 phylum bacteria were enriched (Hu et al. 2009).

In our enrichment culture, the methane oxidation potential increased in accordance with the 16S rRNA copy number. The batch incubations performed with the whole bioreactor revealed average methane oxidation and nitrate reduction after 2 years of 0.055 and 0.012 mmol/h/L, respectively. Based on ^{13}C-CO_2 production, the cell-specific methane oxidation rates after 2 years were 0.57 fmol/cell/day for '*Candidatus* Methanoperedens nitroreducens'. This is in the same range as a previously reported nitrate-dependent AOM rates that we measured in paddy field soil in which the estimated cell-specific rates were 1.2 fmol/cell/day of CH_4 (Vaksmaa et al. 2016) as well as NC10 phylum bacteria enrichment in which the cell-specific rates were about 0.2 fmol/cell/day of CH_4 (Ettwig et al. 2009b) and is also comparable to rates reported for sulfate-dependent AOM by ANMEs (0.7 fmol CH_4/cell/day) (Nauhaus et al. 2005). Unfortunately, we did not observe nitrite-dependent methane oxidation in the batch incubation, suggesting that either 1 mM nitrite was greater than the

inhibitory concentration for the organism, regardless of their capacity to metabolize nitrite or the biomass requires a longer adaptation time to overcome the previous nitrite limitation. Based on the stoichiometry of the reactions for nitrate- and nitrite-dependent anaerobic oxidation of methane, AOM organisms accounted for approximately 46% of nitrate consumption, whereas presumably other nitrate reducers in the reactor, such as denitrifiers, were responsible for the remaining 54% of nitrate loss.

Metagenome analysis revealed that only a few phyla other than '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria were represented in greater than 5% abundance. *Anaerolineales* (8.5% abundance) belonging to *Chloroflexi* are obligate anaerobes that have previously been observed in anaerobic methanotrophic (Ettwig et al. 2009a; Siniscalchi et al. 2015) and methanogenic enrichment cultures (Gray et al. 2011; Liang et al. 2015; Yamada et al. 2005). *Anaerolineales* may be responsible for the degradation of *n*-alkanes and release formate, acetate, hydrogen, and carbon dioxide. Hug et al. indicated that *Anaerolineales* may provide organic acids to other microorganisms such as acetoclastic methanogens (DeSantis et al. 2006). The physiology of Candidate division OC31 (7% abundance), which was discovered more recently, remains unknown. *Phycisphaerae*, a class of *Planctomycetes* (4.4% abundance), has also been shown to degrade heteropolysaccharides (Wang et al. 2015) and was previously found to be highly abundant in AOM and other anaerobic enrichment cultures. After 2 years of enrichment, *Rhodocyclaceae* accounted for 6.1% and *Comamonadaceae* for 5.6%. Both of these belong to *Betaproteobacteria*. Members of *Comamonadaceae* can perform denitrification, which may explain the observed nitrate reduction rate, which was higher than expected based on the methane oxidation rate alone.

The 3.5-Mb size of the draft genome of '*Candidatus* Methanoperedens nitroreducens Vercelli' is comparable to those of the publicly available genomes of '*Candidatus* Methanoperedens BLZ1' (3.7 Mb) and '*Candidatus* Methanoperedens nitroreducens ANME2D' (3.2 Mb). The GC content of the '*Candidatus* Methanoperedens nitroreducens Vercelli' genome is 44.1% and is more similar to that of '*Candidatus* Methanoperedens nitroreducens ANME2D' (GC content 43.2%) than '*Candidatus* Methanoperedens BLZ1' (40.8%). Functional gene analysis revealed that the *mcrA* gene has 96% identity to '*Candidatus* Methanoperedens nitroreducens ANME2D' and 89% identity to '*Candidatus* Methanoperedens BLZ1' at the protein level. A similar trend was observed for the majority of enzymes in the reverse methanogenesis pathway. Analysis of the denitrification pathway revealed the presence of nitrate reductases as well as nitric and nitrous oxide reductases in the draft genome, whereas no nitrite reductase could be identified.

In summary, this is the first enrichment culture from paddy field soil supplied solely with nitrate and methane to enrich '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria. The newly enriched co-culture will be used in future studies to unravel the ecophysiological properties of AOM microbes and investigate their role in mitigating methane emissions from paddy fields.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interest The authors declare that they have no conflict of interest.

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6

Integration and outlook

In this thesis, several environmental and eco-physiological aspects of anaerobic oxidation of methane (AOM) were investigated experimentally. In the environmental studies on Italian paddy fields (Chapters 2 and 4) we characterized the presence, abundance and stratification of NC10 phylum bacteria, carrying out nitrite-dependent AOM, and of '*Candidatus* Methanoperedens nitroreducens', an archaeon responsible for coupling nitrate reduction to AOM. The get more insight into the environmental distribution of these recently described archaea we designed new molecular tools (Chapter 3) for detection of '*Candidatus* Methanoperedens nitroreducens' based on the key enzyme of the reverse methanogenesis pathway; the methyl-coenzyme M reductase (mcrA). In order to describe '*Candidatus* Methanoperedens nitroreducens' in more detail, an enrichment culture fed with methane and nitrate was established by using Italian paddy field soil as inoculum. After 2 years of operation the microbial community was investigated with isotope activity tests, metagenomics and microscopy, and all methods showed that a new '*Candidatus* Methanoperedens nitroreducens' strain (Chapter 5) dominated the culture.

Microbial communities in paddy fields and the role of anaerobic methanotrophs

Bacterial and archaeal communities in paddy field soil are very diverse. Paddy fields provide a wide range of environmental conditions creating a variety of environmental niches; under flooding conditions, oxygen is rapidly consumed in the top soil layers, creating anoxia in deeper layers of soil, where radial oxygen loss through roots creates oxic pockets. The roots also provide organic matter to the rhizosphere microorganisms (Liesack *et al.*, 2000). During the cultivation stage of rice, nitrogen fertilizers are applied to the field and provide external input of electron acceptors derived from the oxidation of fertilizer ammonium to nitrate and nitrite. After the harvest, during the resting and drainage stage, fields are dry and the oxygen penetration depth to the soil increases. The microbial communities in paddy fields are able to adapt to these fluctuating conditions and seem to be rather stable in composition. It is more the relative abundance and activity of specific groups that changes (Kögel-Knabner *et al.*, 2010, Noll *et al.*, 2010, Watanabe *et al.*, 2010). The activity of the microbial communities in paddy fields soil shows both a high synergy and dependency on these fluctuating conditions and in return assist rice growth by nutrient cycling.

Although microbial communities have been studied in the paddy fields before, anaerobic oxidation of methane in these system was generally not addressed, thus we were able to expand our microbial eco-physiology knowledge of this important ecosystem. In addition, our 16S rRNA gene amplicon surveys showed the presence of *Bathyarchaeota* and *Verrucomicrobia* in the paddy fields

Aside from commonly found phyla, we observed to our surprise that, in the paddy field microbial community, the for a long time neglected phylum of *Bathyarchaeota* constituted up to 50% of the total archaeal reads at the depth of 10 cm of soil. Although this phylum is detected in a wide range of habitats, from permafrost (Shcherbakova *et al.*, 2016), marine methane hydrate sediments (Inagaki *et al.*, 2006) to petroleum contaminated soil (Kasai *et al.*, 2005) and eight genome sequences are available, their role in the environment can only be speculated upon now. So far their carbon and energy source is known (Kubo *et al.*, 2012). Due to recent advances in sequencing technology, two genomes of the *Bathyarchaeota* were assembled from coal-bed methane wells in Australia, which contained genes encoding for the MCR complex (mcrABGCD). This allowed to hypothesize that they may be methylotrophic methanogens (Evans *et al.*, 2015). As these microorganisms are highly abundant in a variety of ecosystems, it is very relevant to investigate their role in such environments. Therefore, there is a high need to perform genome assisted enrichments and isolation.

Another fascinating finding, based on the 16S rRNA gene diversity analysis, was the detection of potential aerobic *Verrucomicrobial* methanotrophs in several layers of the paddy field soil. These microbes of the *Methylacidiphilum* cluster were until now only observed in extreme environments, such as mud volcanoes, acidic hot springs and geothermal areas (Dunfield *et al.*, 2007, Islam *et al.*, 2008, Op den Camp *et al.*, 2009, van Teeseling *et al.*, 2014). Both mesophilic and thermophilic *Verrucomicrobial* methanotrophs are able to use oxygen as electron acceptor to oxidize methane and their genome contains multiple gene clusters encoding for the *pmoCAB*; the key enzyme for aerobic methanotrophy. The observed 16S rRNA gene sequences in our study were up to 96% similar to the described strains of *Methylacidiphilum*. Future metagenomic and eco-physiological studies in paddy fields should be directed to enrich and isolate these less-extreme relatives to reveal if they are aerobic methanotrophs. If so, we may reevaluate the contribution of these particular microorganisms in many other environments as well.

As mentioned above, the main focus of this thesis was the AOM process and microorganism carrying out nitrate-and nitrite-dependent anaerobic oxidation of methane. As '*Candidatus* Methanoperedens nitroreducens' and its genome was only recently described (Haroon *et al.*, 2013, Arshad *et al.*, 2015), we were for the first time able to assess the importance of this intriguing microorganism in Italian paddy fields.

Several wetlands, including paddy fields, have been studied for the activity and abundance of nitrite-dependent AOM NC10 phylum bacteria (Deutzmann & Schink, 2011, Wang *et al.*, 2012, Zhu *et al.*, 2012, Shen *et al.*, 2014, Shen *et al.*, 2015). As these were recognized as AOM microorganisms earlier (Raghoebarsing *et al.*, 2006, Ettwig *et al.*, 2008) than '*Candidatus* Methanoperedens nitroreducens', much of the observed nitrate-and nitrite-dependent

AOM activity has been assigned to these bacteria. AOM research including '*Candidatus* Methanoperedens nitroreducens' has only emerged recently.
To date only few studies have investigated the co-occurrence of '*Candidatus* Methanoperedens nitroreducens' and NC10 phylum bacteria in paddy fields (Lee *et al.*, 2015, Ding *et al.*, 2016, Vaksmaa *et al.*, 2016) (Chapter 2 and 4). Their potential contribution to mitigate methane needs to be further investigated. In accordance with Lee et al., 2015, we also found that '*Candidatus* Methanoperedens nitroreducens' was more abundant than NC10 phylum bacteria. In our study the abundance was even a magnitude higher. We demonstrated that the activity measured by ^{13}C-CH_4 incubation assays was higher with nitrate than with nitrite, to further support the important role and contribution of these archaea.

However, what determines the higher abundance of one AOM mediating microorganism over another in environments with high methanogenic activity and supply of nitrite and nitrate, remains enigmatic. One plausible explanation may lay in the structure of rice rhizosphere, which has a deep penetration depth in comparison with many other wetland plants, as well as the flooding regime and exposure to oxygen. The sensitivity to high oxygen concentrations of NC10 phylum bacteria has been demonstrated by a decrease in methane oxidation rate and by decreased transcription of key enzymes (Luesken *et al.*, 2012). The effect of low oxygen concentrations (in µM range) on NC10 phylum bacteria is unknown, as it is difficult to investigate this in a mixed culture. Methanogens have been detected in oxic soils and demonstrated to have enzymatic machinery to counteract the effects of oxygen. The genome of RCI cluster methanogens harbors genes encoding catalase, three different superoxide anion scavengers, superoxide dismutase and two different super oxide reductase genes for oxygen detoxification (Erkel *et al.*, 2006). The up-regulation of catalase genes in response to oxygen exposure has been observed in both *Methanosarcina* and *Methanocella* (Angel *et al.*, 2011). Could it be that NC10 phylum bacteria are more sensitive to oxygen than '*Candidatus* Methanoperedens nitroreducens'? Does '*Candidatus* Methanoperedens nitroreducens' harbor similar defense systems as methanogens? Studies on this aspect of AOM should be carried out and this may partially explain the high abundance of Methanoperedens in the paddy field environment.

Although paddy fields seem to be an ideal spot to mediate nitrite- and nitrate-dependent AOM, these are not the sole possible electron acceptors available in this environment. Metal-dependent AOM and characterization of the responsible organism has been an important topic during the past decade. The process itself has been demonstrated in marine and freshwater environments (Beal *et al.*, 2009, Egger *et al.*, 2015). Recently it was shown that in a sediment dominated by ANME-2a/c clusters, which commonly form syntrophic consortia with sulfate-reducing *Deltaproteobacteria*, methane oxidation also occurs when no sulfate was present and when the incubations were fed with ferric citrate and Fe-EDTA (Scheller *et al.*, 2016). Furthermore, it was demonstrated that '*Candidatus* Methanoperedens nitroreducens' could

mediate metal-dependent AOM. Freshwater ANME-2d ('*Candidatus* Methanoperedens nitroreducens') enrichment culture containing also NC10 phylum bacteria was positively tested for AOM activity with Fe (III) and Mn (IV). Knowing this activity cannot be assigned to NC10 phylum bacteria, as shown by the appropriate controls, the observed iron-dependent methane oxidation in the enrichment was attributed to the ANME-2d (Ettwig *et al.*, 2016). Our paddy field slurry incubation assays revealed that addition of nitrate, nitrite, Fe-NTA and Ferrihydrite stimulated the anaerobic methane oxidation in the top 20 cm of the soil. In the deeper layers (40-60cm) of the soil, highest activity was observed with both forms of iron: Fe-NTA and Ferrihydrate. In environmental studies observations have been made that link the biogeochemical iron profiles of wetland systems to high abundance of *Methanoperedens*-like archaea (Narrowe *et al.*, 2017, Weber *et al.*, 2017), this further supports the possible role of '*Candidatus* Methanoperedens nitroreducens' in metal-dependent AOM.

McrA as a biomarker for *'Candidatus* Methanoperedens nitroreducens'

Several studies are based on either amplicon sequencing of the 16S rRNA gene or PCR based quantification of the 16S rRNA gene. The first specific molecular detection tool for *'Candidatus* Methanoperedens nitroreducens*'* was based on PCR primers derived from oligonucleotides used as FISH probes (Schubert *et al.*, 2011). These oligonucleotides were a good starting point for qPCR quantification, however the methyl-coenzyme M reductase gene *mcrA* may provide a much more specific molecular marker to detect *'Candidatus* Methanoperedens nitroreducens*'*. *McrA* has been commonly used as a marker to characterize methanogenic communities and even primers to specifically try to capture ANME were developed (Zhou *et al.*, 2014).

The analysis of the *mcrA* gene of *'Candidatus* Methanoperedens nitroreducens*'* revealed that the general *mcrA* primers designed for methanogens do not capture their *mcrA* sequence, as there are up to six mismatches in the primer sequence. In chapter 3, the necessary and specific molecular tools to capture the *mcrA* sequences of *'Candidatus* Methanoperedens nitroreducens*'* are described. When comparing *mcrA* and 16S rRNA gene primers for their suitability to quantify the abundance of *'Candidatus* Methanoperedens nitroreducens*'*, mcrA primers yielded in most cases lower abundances. This is due to the fact that the 16S rRNA gene is universal to all microorganisms and will nearly always also capture out-groups. The novel qPCR mcrA primers were tested in a range of environments and *'Candidatus* Methanoperedens nitroreducens*'* was found to be abundant in a variety of different ecosystems. We detected these AOM archaea in rice field soils (Vercelli, Italy), sludge from a brewery wastewater treatment plant (Lieshout, Netherlands), North Sea sediment (Netherlands), polluted Citarum River sediment (Indonesia), Jordan River sediment (Utah, United States) and State Canal sediment (Utah, United States). The most surprising was the detection of *'Candidatus* Methanoperedens

nitroreducens' in the marine sediment of the North Sea. To date only two *mcrA* fragment sequences (about 500bp) have been reported from marine environments. These originate from two studies, where Twing and co-workers (HM746653) (unpublished) and Lever and colleagues (GU182109) detected a *mcrA* fragment in the sediment of Gulf of Mexico and Juan de Fuca Ridge Flank basalt seafloor sediment, respectively. The anaerobic methanotrophs coupling sulfate reduction to methane oxidation are widespread in marine environments and are estimated to consume up to 80-90% of methane produced. To date, the role and contribution of *'Candidatus* Methanoperedens nitroreducens' in marine sediments are unknown. In costal sites, the terrestrial runoff could feed the nitrate-dependent AOM, though in deep marine sediments, nitrate may not be the most abundant electron acceptor. Could it be that there is strain diversity and that there is a niche for iron reduction linked to AOM in marine sediments mediated by *'Candidatus* Methanoperedens nitroreducens'?

Enrichment of *'Candidatus* Methanoperedens nitroreducens'

In 2006, an enrichment culture, in which nitrate and nitrite reduction were coupled to the anaerobic oxidation of methane, was described for the first time (Raghoebarsing *et al.*, 2006). In that study, an inoculum from the sediment of a freshwater canal was used to start an anaerobic enrichment. After 16 months, the culture was dominated by a consortium consisting of archaea (10-15% of cells) belonging to the *Methanosarcinale*s family that were only distantly related to known ANMEs (86-87% in 16S rRNA gene identity) and a bacterium (approximately 80% of cells) of the candidate division NC10. The enriched co-culture preferred nitrite over nitrate as the substrate, although activity was observed with both substrates (Raghoebarsing *et al.*, 2006).

To date there are a number of enrichment cultures containing NC10 phylum bacteria (Ettwig *et al.*, 2009, Hu *et al.*, 2009, Hu *et al.*, 2014, Ding *et al.*, 2016) that were started with a variety of inoculum sources including paddy field soil and even costal mudflat sediment (He *et al.*, 2015). Until now, the number of reported enrichments dominated by *'Candidatus* Methanoperedens nitroreducens' is limited (Haroon *et al.*, 2013, Hu *et al.*, 2015, Ettwig *et al.*, 2016). In chapter 5 we described the enrichment of these AOM microorganisms, using paddy field soil as an inoculum. The bioreactor was fed with methane and nitrate as the only electron donor and acceptor. In our enrichment the relative abundance of *'Candidatus* Methanoperedens nitroreducens' was 15% after one year and 22% after two years of enrichment, based on 16S rRNA gene sequences detected in metagenomic reads, while the abundance of NC10 phylum bacteria was 7% and 15%, respectively. In this setting, nitrite produced due to nitrate reduction by *'Candidatus* Methanoperedens nitroreducens', was scavenged by the NC10 phylum bacteria. As part of this research we assembled the genome of *'Candidatus* Methanoperedens nitroreducens Vercelli',

which is the third publically available genome of this AOM archaeon. Out of the three genomes available, two are of high-quality, though none of those genomes are complete. In order to investigate differences between strains, originating from different environments, more high-quality genome sequences need to become available to carry out comparative genomics.

Many questions remain still unanswered regarding the physiology of *'Candidatus* Methanoperedens nitroreducens'; Does, and if so under which conditions production of ammonia by this archaea occurs, which could feed the anammox process in nature? In the genome of *'Candidatus* Methanoperedens nitroreducens' genes necessary for dissimilatory nitrate reduction to ammonium (DNRA) (Haroon *et al.*, 2013) have been identified. In an enrichment of *'Candidatus* Methanoperedens nitroreducens' production of ammonia has been measured as well (Ettwig *et al.*, 2016), yet to answer definitely if it really is an attribute of *'Candidatus* Methanoperedens nitroreducens', pure cultures are needed. However, so far pure cultures have not been obtained due to slow growth and isolation difficulties of these ´impossible´ microbes.

In order to unravel the mystery of *'Candidatus* Methanoperedens nitroreducens' strain diversity and environmental distribution in fresh and marine environments, high enrichments or pure cultures are needed to address the question of salt tolerance of different strains. For ANME-1 the occurrence in hypersaline environments has been demonstrated (Lloyd *et al.*, 2006, Yakimov *et al.*, 2007), as well as adapted cell membranes with glycerol dialkyl glycerol tetraethers (Niemann & Elvert, 2008). Would marine strains of *'Candidatus* Methanoperedens nitroreducens' also possess such specific adaptive mechanisms and differentiate from freshwater strains?

In the current state of knowledge, several electron acceptors are known to serve as substrates for ANMEs. Methane oxidation potential has been shown with iron and manganese for *'Candidatus* Methanoperedens nitroreducens'. Yet all the evidence is based on activity measured in sediment or in an enrichment culture. We do not know if the ANMEs can use both electron acceptors at the same time or switch from one to the other upon availability. Could they still grow? What is the importance of nitrate- versus iron-mediated AOM in paddy fields? To answer these questions, future research should address the enrichment of *'Candidatus* Methanoperedens nitroreducens' by excluding nitrate and adding oxidized metals as sole electron acceptor in long term enrichments; only then the growth rates could be revealed. Comparative activity assays with all possible electron acceptors in ideally pure culture would give insight into the potential methane oxidation contribution, which could be extrapolated to the environment. Not only shall the substrate preference be understood, but also underlying mechanism of the process; how does *'Candidatus* Methanoperedens nitroreducens' carry out metal-dependent AOM?

How exactly does this process take place, what is the electron shuttling mechanism and what is the necessary proximity of organism and poorly bioavailable solid iron particles in nature? Studies on ANME activity and the dependency of that on the proximity of the syntrophic sulfate reducing bacteria (SRB), revealed that the activity is highest when there is least distance. That allows the transferal of electrons directly from one organism to another. Having such syntrophic interface means greater syntrophic benefit for both partners the ANME-2 and associated *Deltaproteobacteria* via direct interspecies electron transfer (McGlynn *et al.*, 2015). The same mechanism is proposed for ANME-1 and their SRB HotSeep-1 (Wegener *et al.*, 2015). SRB have been studied for the genomic potential for interspecies electron transfer, and genes necessary were present in contrast to other SBR which do not live in syntrophic consortia with ANMEs (Skennerton *et al.*, 2017).

An interesting study for the future in this regard is to investigate if *'Candidatus* Methanoperedens nitroreducens*'* carry out interspecies electron transfer or as they are not dependent on other microbes, how do they interact with iron particles to support methane oxidation. As iron is solid and *'Candidatus* Methanoperedens nitroreducens*'* prefers to attach to surfaces, could these archaea move in order to reach a preferred site of substrate? For several methanogens it has been demonstrated that either in conditions of stress, they are able to synthesize an archaellum. In the genome of *'Candidatus* Methanoperedens nitroreducens*'* we identified several of the necessary Fla genes. Would *'Candidatus* Methanoperedens nitroreducens*'* make use of this strategy in order to find substrate or avoid stress, is another question to answer in the future.

In science, as soon as a few questions are answered, many new ones will arise. The current research has shed light into environmental distribution and substrate utilization of *'Candidatus* Methanoperedens nitroreducens*'*, which has indicated the need to establish specific and high enrichments in order to unravel their full physiological potential.

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About the Author

Annika Vaksmaa was born on the 22nd of April 1985 in Tallinn, Estonia. There she obtained the degree of Bachelor of Science in Applied Chemistry and Biotechnology at the Tallinn University of Technology. During her Bachelor studies she took part in an exchange program and spent six months at Aarhus University, Denmark, focusing on Biochemistry and Molecular Biology. Her final Bachelor thesis was a result of an internship at Philips research, Eindhoven, the Netherlands, on the topic of 'Detection cell-free RNA in human blood'. In 2007 she started her Master studies in Roskilde University, Denmark, and in 2010 she obtained the degree of Master of Science in Molecular and Environmental biology. Her final thesis focused on 'Effect of temperature on gene expression in corals'. Aside of her Master studies she took on a project to investigate the biofouling on novel ship coatings and performed field, and laboratory tests in Sultan Qaboos University in Muscat, Oman. This project was continued after obtaining her Master degree, and she returned to Oman. In 2013 she started her PhD in Radboud University of Nijmegen in the Microbial Ecology Department.

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