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FEMS Microbiology Ecology, 92, 2016, fiw181

doi: 10.1093/femsec/fiw181
Advance Access Publication Date: 24 August 2016
Research Article

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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

Received: 22 April 2016; **Accepted:** 18 August 2016
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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.

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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 classifiied. 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

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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).

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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.

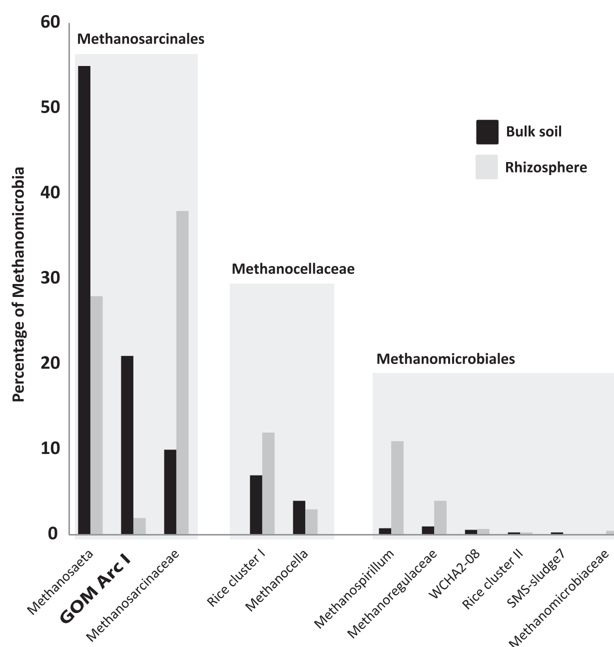

Figure 1. Distribution of 16S rRNA gene reads belonging to the order of Methanomicrobia in the bulk soil and in the rhizosphere of the Vercelli paddy field. Values are expressed as percentage of total Methanomicrobial sequences.

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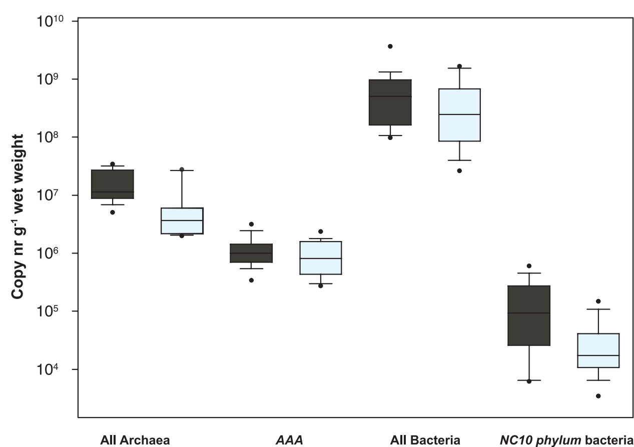

Figure 2. Boxplot of abundance of all archaea, AAA, all bacteria and NC10 phylum bacteria as assessed by quantitative PCR of the 16S rRNA gene in bulk soil (black boxes) and rhizosphere (gray boxes). The horizontal line represents the median and the circles mark the 5th and 95th percentiles. The box 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;

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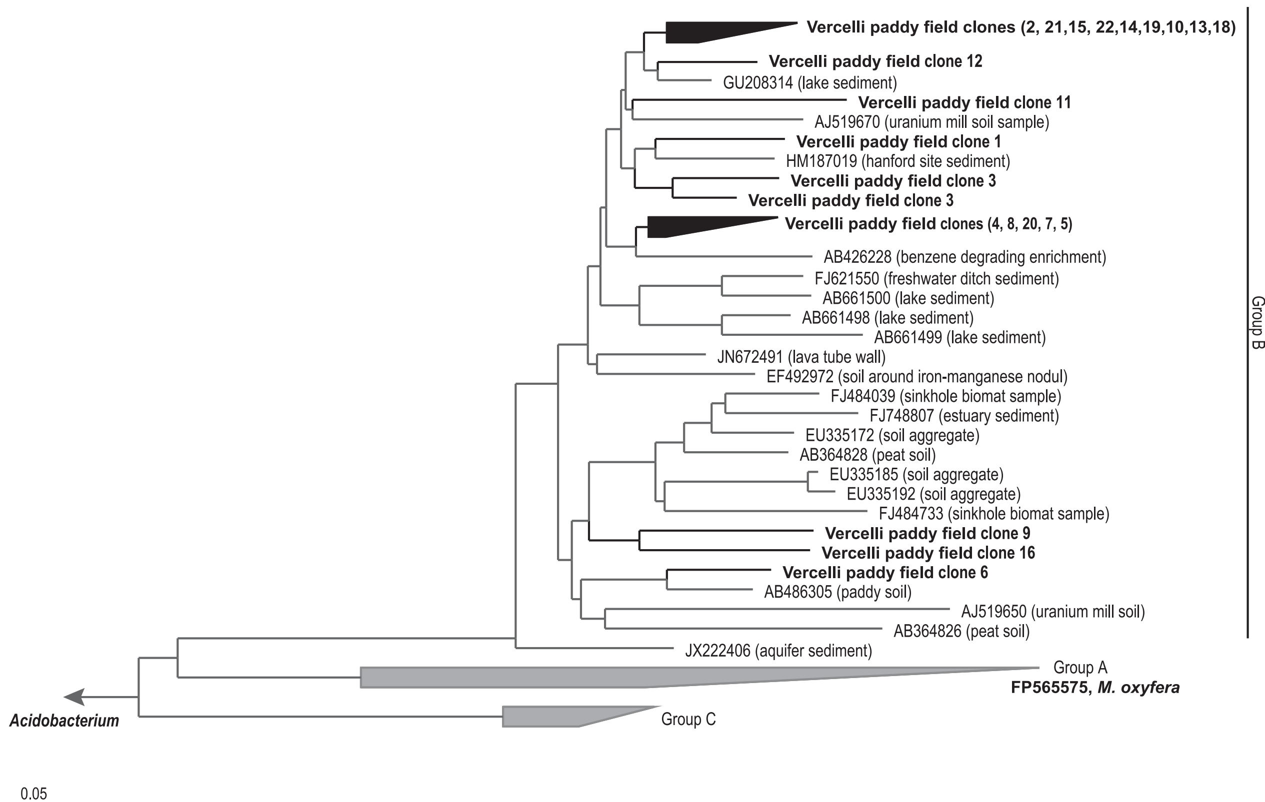

Figure 3. Phylogenetic tree of NC10 phylum bacteria 16S rRNA sequences (841 bp). The tree includes clones that were derived from this study (marked bold) together with a selection of publicly available sequences of environmental samples as well as reference sequence of *M. oxyfera* (acc. no. FP565575, 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.

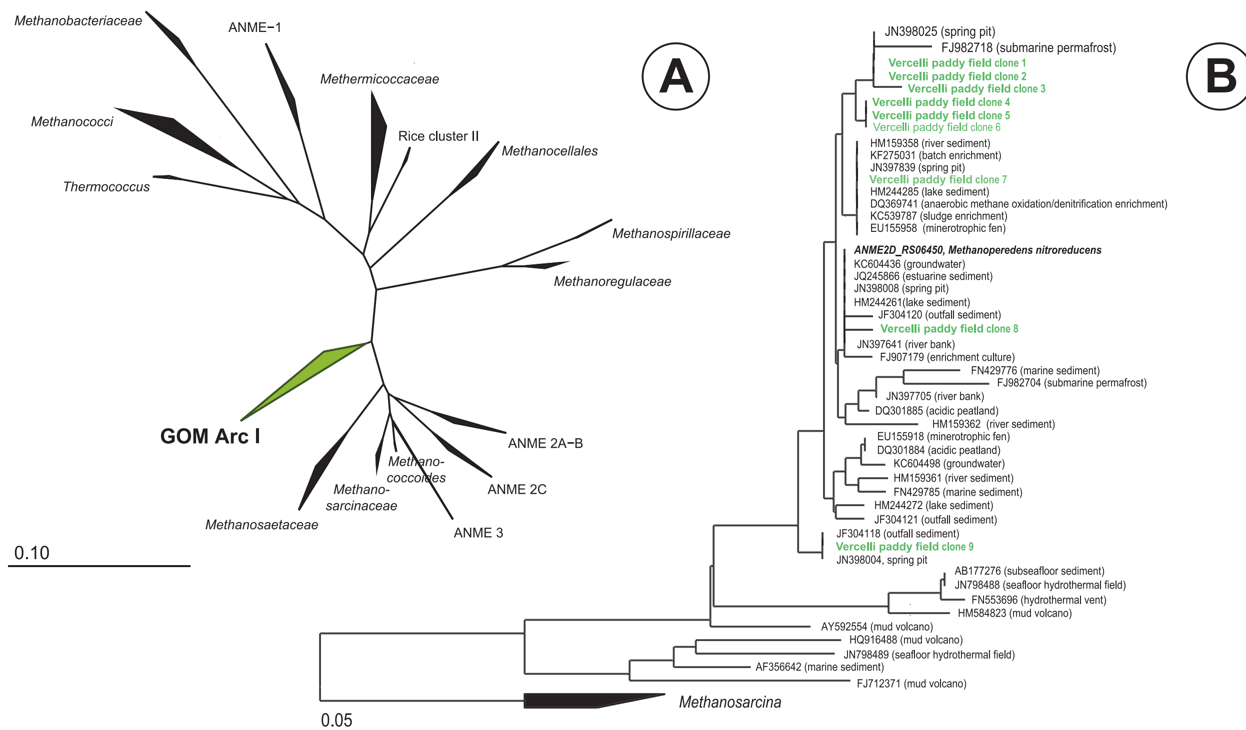

Figure 4. (**A**) Phylogenetic overview of methanogenic and methanotrophic archaea based on 16S rRNA gene sequences. The phylogenetic position of the GOM Arc I archaea is marked in green. (**B**) Detailed presentation of the GOM Arc I clade. Clones (297 and 864 bp) obtained from paddy field are shown 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.

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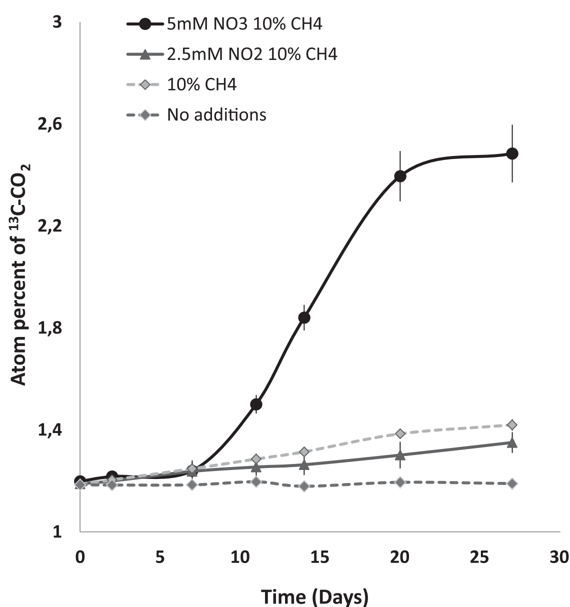

Figure 5. Atom fraction of $^{13}CO_2$ in soil slurries incubated with 10% $^{13}CH_4$ with addition of 5 mM $NaNO_3$ or 2.5 mM $NaNO_2$. As controls soil slurry was incubated with and without 10% $^{13}CH_4$. Measurements were made in triplicate with error bar representing standard deviation.

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.

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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.

ACKNOWLEDGEMENTS

We thank Gabriele Orasen and Marco Canella from the Vercelli Rice Research Unit for help during sampling.

FUNDING

This work was supported by the Dutch organization for Scientific Research [VENI 863.13.007 to KFE], the European Research Council [ERC AG 339880 Eco_MoM to MSMJ], the Dutch government's Gravitation grant [024002002 to MSMJ], and the Spinoza prize to MSMJ. GV was funded by AGER Risinnova [grant n° 2010-2369] and Plant Genetic Resources/(RGV) FAO Treaty [DM 29561].

Conflict of interest. None declared.

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