Anaerobic oxidization of methane in a minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria

Baoli Zhu[1], Gijs van Dijk[2,3], Christian Fritz[2], Alfons JP Smolders[2,3], Arjan Pol[1], Mike S.M. Jetten[1], Katharina F. Ettwig[1*]

[1]Department of Microbiology, Institute for Water and Wetland Research, Radboud University Nijmegen, The Netherlands

[2]Department of Aquatic Ecology and Environmental Biology Ecology, Institute for Water and Wetland Research, Radboud University Nijmegen, The Netherlands

[3]B-WARE Research Centre, Radboud University Nijmegen, The Netherlands

* For correspondence: Dr. Katharina F. Ettwig, Department of Microbiology, Institute for Water and Wetland Research, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. Email: k.ettwig@science.ru.nl; phone: +31-24-36 52 557

Abstract

The importance of anaerobic oxidation of methane (AOM) as methane sink in freshwater systems is largely unexplored, particularly in peat ecosystems. Nitrite-dependent anaerobic methane oxidation (n-damo) was recently discovered and reported to be catalyzed by the bacterium '*Candidatus* Methylomirabilis oxyfera' that is affiliated with the NC10 phylum. So far, several *M. oxyfera* enrichment cultures have been obtained using a limited number of fresh water sediments or wastewater treatment sludge as inoculum. In this study, using stable isotope measurements and pore water profiles, we investigated the potential of n-damo in a minerotrophic peat land in the south of the Netherlands that is infiltrated by nitrate-rich ground water. Methane and nitrate profiles suggested that all methane produced was oxidized before reaching the oxic layer, and NC10 bacteria could be active in the transition zone where counter-gradients of methane and nitrate occur. Quantitative PCR showed high NC10 bacterial cell numbers at this methane-nitrate transition zone. This soil section was used to enrich the prevalent NC10 bacteria in a continuous culture supplied with methane and nitrite at an *in situ* pH of 6.2. An enrichment of nitrite-reducing methanotrophic NC10 bacteria was successfully obtained. Phylogenetic analysis of retrieved 16S rRNA and *pmoA* genes showed that the enriched bacteria were very similar to the ones found *in situ*, and constituted a new branch of NC10 bacteria, with an identity percentage of less than 96% and 90% to the 16S rRNA and *pmoA* genes of *M. oxyfera*, respectively. The results of this study expand our knowledge of the diversity and distribution of NC10 bacteria in the environment, and highlight their potential contribution to nitrogen and methane cycles.

Introduction

Wetlands are the largest single source of methane with estimated emissions of 103 Tg per year, which account for about 20% to 40% of the global annual atmospheric methane flux (1, 8, 19). It is estimated that about 50% of the methane produced in wetlands is consumed before it reaches the atmosphere; this significant microbial methane sink is usually considered to consist exclusively of aerobic methanotrophic bacteria, which degrade methane using oxygen as electron acceptor (2, 5, 19, 39). In ecosystems where oxygen is depleted but sufficient alternative electron acceptors, e.g. sulfate or nitrate are present, methane can also be converted anaerobically (25, 38). Anaerobic oxidation of methane (AOM) coupled to sulfate reduction is performed by a consortium of anaerobic methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB) (25, 47). Its significance is well established for marine ecosystems, where it may consume more than 90% of the produced methane (39). In freshwater wetlands, and especially peatlands, electron acceptors are scarcer, with concentrations typically in the low µM range (37). Due to this reason, redox processes are mostly limited by electron acceptor supply, very dynamic and highly susceptible to alterations e.g. by influx of polluted groundwater and atmospheric deposition of nitrogen and sulfur species (18, 46). The influence of nitrogen pollution on methane oxidation is complex, and not all feedback loops are well understood (2, 3, 16, 29). In principal the role of the alternative electron acceptors nitrate and sulfate for diverting carbon fluxes away from methane production is better established, given that sulfate and nitrate reduction are thermodynamically more favorable than methanogenesis (17, 30, 31, 51). However, these alternative electron acceptors can in principle also enable methane oxidation (47, 54), but this topic has received only little attention with respect to methane cycling in peatlands (43).

In the meantime, for other freshwater ecosystems, more and more evidence about the occurrence of AOM coupled to sulfate (11, 40), iron(III) (42) and nitrate reduction (9, 38, 44, 50) has become available. Whereas nothing is known about the microorganisms mediating ferric iron reduction with methane, for sulfate reduction a very similar consortium of methanotrophic Archaea and SRB as in marine ecosystems is hypothesized to be responsible (11, 40). Nitrate- or nitrite-dependent AOM (n-damo), when linked to organisms, was so far always found to be performed by one bacterial species affiliated to the NC10 phylum (9, 13). Genome sequencing, expression studies and physiological experiments indicated that this bacterium, then named *Candidatus* Methylomirabilis oxyfera, is an "intra-aerobic" methanotroph that produces its own oxygen from the dismutation of nitric oxide into dinitrogen gas and oxygen. The produced oxygen is then used for canonical aerobic methane oxidation starting with the methane monooxygenase enzyme complex (12). Although 16S rRNA sequences similar to *M. oxyfera*'s were found in various environments (14), so far n-damo enrichment cultures have only been obtained from two types of ecosystems: eutrophic freshwater sediments and wastewater treatment sludge. The dominant bacteria in all described cultures were closely related (≥97% identity of the 16S rRNA gene sequence) to *M. oxyfera* (13, 14, 20, 33). Currently it is unclear, however, if *M. oxyfera*-related species are the only nitrite-dependent methane oxidizing bacteria; if methane oxidation is a general feature of NC10 phylum bacteria or limited to (close relatives of) *M. oxyfera*, and how important these bacteria are for methane cycling in various ecosystems.

In this paper, we studied a minerotrophic peatland infiltrated by nitrate-containing groundwater. At the sampling site, no methane emission was detectable. Porewater profiling revealed a nitrate-methane transition zone below the oxic layer that could provide an ecological niche for n-damo microorganisms. NC10 bacteria abundance in soil cores was analyzed using quantitative PCR, and the section with highest cell numbers of *M. oxyfera*,

coinciding with the methane-nitrate transition zone, was used as inoculum for the enrichment of n-damo bacteria. Mimicking field conditions as much as possible by using nitrite-amended peatland water in continuous cultivation, a new cluster of *M. oxyfera*-like bacteria was enriched.

Materials and methods

Site description. The Brunssummerheide peatland (50°55'39.63"N/5°59'50.73"E) is a small (15ha) spring fen located in an oligothrophic sandy valley fed by locally upwelling, weakly buffered nitrate-polluted groundwater. The peat layer is relatively thin (maximum 2.5 m) and vegetation is dominated by *Sphagnum* spec., *Narthecium ossifragum* and *Molinia caerulea*. At the sampling site, nitrate-enriched groundwater overflows the peatland surface and infiltrates into the peat layer.

Porewater profile determination and soil sampling. Nitrate and methane profiles were determined by measuring the concentrations in porewater samples collected using 5 cm ceramic cups (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands) connected to Teflon tubes. Porewater samples were obtained at least in duplicate from the depth of 20 cm to 220 cm at 5 or 10 cm intervals in December 2009 and June 2010. Porewater for methane analyses was collected in vacuumed anaerobic glass bottles (40 ml) prefilled with 5 g sodium chloride and sealed with butyl rubber stoppers. For chemical analyses, porewater was collected in 60 ml syringes. Samples were transported to the laboratory within two hours in a cooling box, and stored at 4 ^{o}C for maximum 14 days before analysis. Methane in the bottle headspace was measured after pressure equilibration with argon using gas chromatography as described previously (14). Nitrate was analyzed colorimetrically on a Traacs 800+ auto-analyzer as described previously (48). Redox potential measurements were performed by

111 gently pushing platinum electrodes into pre-drilled holes and allowing them to equilibrate.
112 Stable readings were obtained after 30 min (15). Soil samples were obtained from 50 cm to
113 130 cm depth with a Russian peat corer, sliced into 5-20 cm intervals in the field, immediately
114 put into self-sealing plastic bags, and stored in air-tight bins with oxygen scavenger
115 (Anaerogen, Oxoid, USA), then transported to the laboratory and stored anaerobically at 4 ^{o}C
116 until further analysis.

117 **Incubation.** Initially, 200 ml soil slurry of the depth layers of 80-100 cm, 100-120 cm and
118 120-135 cm (sampled in July 2009) were incubated in separate bottles (500 ml). Surface water
119 from the peatland was collected and used for medium preparation after removal of particles
120 by filtering through a hemo-filter (Hemoflow HF80S, Fresenius Medical Care, USA). The
121 medium contained: 2 mM $KHCO_3$, 0.2 mM $Na^{15}NO_2$ (99.6% ^{15}N; Isotec, USA) and 0.5 mM
122 $NaNO_3$. The bottles were made anaerobic by 6 cycles of vacuuming and gassing with Ar/CO_2
123 (75:25), followed by 5 min of flushing with Ar/CO_2. Then 10 ml $^{13}CH_4$ was injected into the
124 headspace (final concentration ca. 20%). The pH in the bottles was around 6.0 and the bottles
125 were incubated at 25 ºC, with gentle shaking at 100 rpm. The production of $^{13}CO_2$ was
126 measured by GC-MS in the headspace (see below).

127 After three months incubation, the bottle with strongest $^{13}CO_2$ production was used as
128 inoculum for continuous culturing in a 3 liter glass bioreactor (working volume 1.5 l;
129 Applikon, Schiedam, The Netherlands) that was operated in sequencing batch mode to
130 prevent biomass loss. One cycle constituted of 23 h of continuous supply of medium, 0.5 h
131 settling, finally followed by 0.5 h discharging with a level-controlled pump. To keep the
132 culture anaerobic, the reactor was continuously flushed with 20 ml min^{-1} Ar/CO_2 (95:5) and 5
133 ml min^{-1} methane. The temperature was controlled at 25 ^{o}C and the pH at 6.0 to 6.2.
134 Dissolved oxygen, temperature and pH in the reactor were monitored by respective

electrodes. Medium was prepared as described above, except using unlabeled nitrite. The nitrite concentration in the reactor was estimated daily with Merckoquant test strips (0-80 mg l^{-1}; Merck, Germany), and the concentration in the medium was slowly increased from 0.2 mM to 2.5 mM dependent on the activity of the continuous culture. Nitrite concentrations in the reactor were kept below 20 mg l^{-1} (0.44 mM). The medium loading to the reactor was between 200 to 500 ml per day.

Activity analysis. Methane oxidizing activity in bottles was measured by determining the amount of $^{13}CO_2$ produced from $^{13}CH_4$ oxidation with GC-MS (Agilent 5975C inert MSD; Agilent, United States) as previously described (14). Activity in the reactor was tested in batch experiments with the whole culture. First medium supply was stopped and unlabeled nitrite was allowed to be depleted. The reactor was flushed with Ar-CO_2 (95:5) for 1 h while stirring, and checked for residual methane in the headspace. When undetectable, 0.2 mM $^{15}NO_2^-$ and 50ml $^{13}CH_4$ were added. 20 µl gas samples were taken every hour for $^{13}CO_2$, $^{15,15}N_2$, $^{15,14}N_2$ analysis. At the same time, 1 ml culture liquid was taken and centrifuged; the supernatant was kept at 4 ^oC for nitrite analysis. Nitrite concentrations were determined with colorimetric methods as described elsewhere (23). The influence of pH on activity was determined in batch incubations of 10 ml biomass in 40 ml serum bottles, buffered with MES (2-(N-morpholino) ethanesulfonate, 20 mM) to pH values between 5.9 and 6.7, and with MOPS (3-(N-morpholino) propanesulfonate, 20 mM) to pH values between 6.75 and 7.4 (measured at the end of incubation).

DNA isolation. Total DNA from soil samples was isolated with the PowerSoil$^{\circ,R}$ DNA isolation kit (MO BIO Laboratories Inc., USA) according to the manufacturer's manual. Approximately 0.3 g homogenized soil was used for DNA isolation, and two independent isolations were carried out for each depth interval. DNA was eluted three times with pre-

159 warmed Milli-Q water from the column to ensure that the entire DNA had been collected.
160 DNA in the third elution was undetectable by agarose gel electrophoresis (<0.2 ng μl^{-1}). DNA
161 obtained from the same depth interval was pooled for qPCR analysis to minimize the
162 influences from soil inhomogeneities. DNA from enrichment cultures was isolated with a
163 method based on bead-beating and SDS lysis, as described previously (14). DNA quality was
164 checked on agarose gel, and concentrations were measured in triplicate with NanoDrop (ND-
165 1000, ISOGEN Life Science, The Netherlands).

166 **Quantitative PCR.** In order to quantify n-damo bacteria and all bacteria in different depths of
167 the soil cores, quantitative PCR (qPCR) targeting the 16S rRNA gene was performed. To
168 account for imperfect primer matching and known variability of results (14), two different
169 primer pairs were used for each group. For NC10 phylum bacteria, primer pairs p1F & p1R
170 and p2F & p2R, and for all bacteria, primer pairs 1100F & 1492R and 533F & 805R (Table 1)
171 were applied. All q-PCR assays were performed according to the MIQE guidelines (Minimum
172 Information for Publication of Quantitative Real-Time PCR Experiments) (4). qPCR
173 experiments were carried out with the Bio-Rad IQTM 5 cycler real-time detection system
174 using IQTM SYBR green Supermix (Bio-Rad, United States) in 25µl reaction volume as
175 previously described (14), except using 65 ^{o}C for n-damo specific primer pairs and 58 ^{o}C for
176 universal primer pairs as annealing temperature, which had been determined as most suitable
177 for the present samples by gradient PCR. The qPCR reactions were carried out in 96 well
178 plastic plates (Bio-Rad, United States) sealed with Opti-Seal Optical disposable adhesive
179 (BIOplastics, The Netherlands). Fluorescence signals were obtained at 72 ^{o}C at the end of the
180 elongation step of each cycle. PCR products obtained with n-damo specific and universal
181 bacteria primer pairs were cloned and sequenced using the vector pGEM-T Easy (Promega,
182 United states). The sequences retrieved were of the correct length (201 bp for p1F & p1R; 292
183 bp for p2F & p2R; 291 bp for 515F & 805R and 410 bp for 1100F & 1492R), and the

obtained n-damo sequences were similar (>97.2% identity) to the sequence of *M. oxyfera* (accession no. FP565575). Standard curves for n-damo bacteria and general bacteria were constructed with plasmids containing corresponding inserts, taking into account the molecular mass of the plasmid including the insert, and the plasmid concentration. Plasmids copy numbers used as standard were between 30.7 to 3.07×10^{8} μl^{-1} for NC10 bacteria, 86.9 to 8.69×10^{8} μl^{-1} for all bacteria. Two soil cores with partial overlap were analyzed. Both cores were sliced in sections between 5 and 10 cm in the field (see soil sampling and DNA isolation). In Fig. 1, each depth interval is represented by its average depth. DNA isolated from soil of 85-90 cm depth was used to test dilution effect; 10 times and 100 times had a maximum difference of 8.7%, compared with non-diluted ones. For NC10 bacteria non-diluted DNA was used as templates; but for primers targeting all bacteria, 100 times diluted DNA was used. PCR efficiencies calculated based on standards were between 90.6% and 99.2%. Both standards and samples were run in triplicates. The copy numbers in samples were calculated based on comparison with the threshold cycle values of the standard curve, taking into account the dilution and the amount of total DNA obtained per gram soil.

Phylogenetic analysis. PCR was performed with DNA isolated from the soil layer used as an inoculum (80-100 cm depth), the enrichment culture after 3 months of incubation in bottles, and the continuous culture after 1 and 17 months of enrichment in the reactor. 16S rRNA sequences of n-damo bacteria were obtained with universal bacteria primer 8F or n-damo specific primer 193F in combination with n-damo specific primer 1043R (Table 1). PCR products of the correct size were ligated into the pGEM-T Easy cloning vector (Promega, United States) and amplified in *Escherichia coli* DH5α. Plasmids were isolated from 10 to 15 randomly selected white colonies per library using the GeneJet miniprep kit (Fermentas, Lithuania), and were sequenced at the DNA Diagnostics Center of Nijmegen University Medical Center. The sequences were aligned to reference sequences with the MUSCLE

algorithm. Phylogenetic trees were constructed with MEGA5 using the neighbor-joining method and the robustness of tree topology was tested by bootstrap analysis (1000 replicates).

With the same DNA samples also functional gene (particulate methane monooxygenase subunit A, *pmoA*) clone libraries were constructed. The particulate methane monooxygenase catalyzes the first step of methane oxidation and is well conserved in methane oxidizing bacteria, therefore *pmoA* is widely accepted as a marker gene for assessing diversity of aerobic and *M. oxyfera*-like anaerobic methanotrophs in the environment (34, 36). Two different forward primers targeting either most methanotrophs (A189b) or only close relatives of *M. oxyfera* (cmo182) were combined with a specific reverse primer (cmo682) (Table 1). A *pmoA* phylogenetic tree based on nucleotide sequences was constructed as described above.

Fluorescence in situ hybridization. On a monthly basis, 1.5 ml biomass was harvested from the reactor and forced through a 0.5 mm needle to break big cell aggregates. Then the sample was centrifuged and the pellet was washed twice with 1 ml 1×PBS, and fixed with paraformaldehyde on ice for three hours. Fluorescence in situ hybridization (FISH) was performed as previously described (13), using 40% formamide concentration. The following oligonucleotide probes were used: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 specific for n-damo bacteria (38) and a mixture of EUB I-III and V for most Bacteria (7). Images were acquired with a Zeiss Axioplan 2 epifluorescence microscope equipped with a CCD camera, together with the Axiovision software package (Zeiss, Germany).

Nucleotide sequences accession numbers. Representative 16S rRNA and *pmoA* gene sequences were deposited at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) with the accession numbers JX262153- JX262155 (*pmoA*) and JX262156-JX262161 (16S rRNA).

Results

Porewater profiles. Porewater depth profiles of the Brunssummerheide sampling location were determined on five occasions between July 2009 and May 2011, with an overall very similar pattern. Representative winter (December 2009) and summer (June 2010) profiles are shown in Fig. 1. Nitrate concentration decreased with depth, and became undetectable below 100 cm. No methane was detected in the upper 80 cm, but methane gradually increased below the depth of 80 cm and reached the maximum concentration at around 120 centimeters (Fig.1 A, B). Redox data indicated that the soil was completely anoxic below 50 cm depth, and living roots of vascular plants were not found below 60 cm depth. The maximum concentration of nitrate (0.6 mM) in June 2010 (Fig.1 B) was about 0.2 mM higher than that in December 2009 (Fig. 1. A), possibly due to relatively stronger evaporation of surface water and higher groundwater influx in summer. The maximum concentration of methane remained similar in both seasons, as well as the overall pattern: an opposing gradient at around 80-100 cm depth.

Quantifying abundance of NC10 bacteria in different soil depths. Total bacterial and NC10 phylum abundance in different soil depths was determined in two overlapping cores by qPCR using primers targeting the 16S rRNA genes. The highest cell numbers ($1.3\text{-}3.2\times10^{7}$ g^{-1} wet soil) of NC10 bacteria were found at 80-85 cm depth (Fig. 1 C, D), coinciding with the concomitant decrease of methane and nitrate (Fig. 1 A, B), and a peak in abundance of NC10 phylum-characteristic phospho-lipid fatty acids (Fig. 1 G; Kool et al, submitted). In contrast, total bacteria cell numbers, ranging from 0.9 to 11.8×10^{8} cells g^{-1} wet soil, did not show a depth-related pattern (Fig. 1 E, F).

Enrichment and activity. Nitrite dependent methane oxidizing activity was initially determined by measuring the fraction of $^{13}CO_2$ in total CO_2 after supply of $^{13}CH_4$ and nitrite to three soil sections (80-100; 100-120; 120-135 cm). Despite the addition (and permanent presence) of nitrate (0.5 mM), all soil cores produced some methane in the first two weeks of incubation, but no methane oxidation could be detected (detection limit approximately 0.5 nmol d^{-1} g^{-1} soil). After about 3 month's incubation, the 80 - 100 cm section showed methane oxidation activity (9.0 nmol d^{-1} g^{-1} soil, assessed as CO_2 production), and an increase in this rate indicated microbial growth. This incubation (80-100 cm) was used as inoculum to start a sequencing batch reactor for the enrichment of the responsible microorganism. Over the first 9 months of enrichment activity remained low with a nitrite reduction rate of about 50 µmol d^{-1} L^{-1}, and then started to increase to about 1.0 mmol (NO_2^-) d^{-1} L^{-1} in month 15. Batch tests and experience with previous NC10 bacteria enrichment cultures had indicated that nitrite was preferred over nitrate; consequentially the medium, prepared with *in situ* water was not only amended with nitrate, but also nitrite. To test the coupling of nitrite reduction to methane oxidations, both activities were tested in batch experiments after 10 months with ^{15}N- and ^{13}C-labelled substrates during the enrichment period (Fig. 2). Nitrite-N was completely recovered as nitrogen gas, concomitantly methane was fully oxidized to CO_2. The ratio of $^{13}CO_2$ and $^{15,15}N_2$ production was 3:4.3, similar to the theoretical stoichiometry of 3:4 (38). An activity test at different pH values demonstrated that the culture preferred circumneutral conditions, but was active down to the lowest tested value of 5.9 (Fig. 3).

FISH analysis of the enriched bacteria. FISH was performed on biomass of the enrichment culture fixed every month, but no clear hybridization with NC10 specific probes was observed until after 8 months of medium supply. Even though small numbers of NC10 bacteria must

have been present, they remained undectable at first due to strong autofluorescent background and hybridization inhibiton, presumably caused by peat material. Starting at month 9, NC10 cells could be detected (Fig. 4A). With the progression of incubation, both total cell numbers visulized by DAPI stain, and the percentage belonging to the NC10 phylum gradually increased (Fig. 4 B, C) and coincided with an increased activity of the culture. At month 14 about 50%, and at month 19 more than 80% of the population did hybridize with the NC10 sepecific probes (Fig. 4).

16S rRNA and *pmoA* gene phylogenetic analysis. *M. oxyfera*-related 16S rRNA and *pmoA* genes were successfully obtained from both inoculum soil and the enrichment after 1 or 17 months of incubation. Long (>1000 bp) 16S rRNA sequences obtained with primer 8F (universal) and 1043R (NC10 specific) were used for phylogenetic analysis. Results showed that the 16S rRNA sequences belong to the group A of NC10 bacteria (14), forming a cluster (differences between 0.1% to 2.7%) with sequences retrieved from coal-tar contaminated aquifer (AF351214, AF351217, FJ810544) and lake Constance sediment (HQ906524, HQ906538) (9). These sequences share only 94.9% to 95.5% identity with *M. oxyfera* (Fig. 5 A).

The phylogenetic analysis of the *pmoA* gene showed similar results. *pmoA* sequences from both soil and enrichment culture again cluster together with *pmoA* sequences retrieved from Lake Constance sediment (HQ906571, HQ906568, HQ906566) (9). These *pmoA* sequences had an identity with those of *M. oxyfera* of 86.2-90.9% on nucleotide level, but the 95.8-97.9% on amino acid level indicated functional conservation (Fig. 5 B). No significant difference could be observed between the inoculum and the 17 months old enrichment culture, indicating that no population shift within the NC10 phylum had occurred. Both the

16S rRNA and *pmoA* genes phylogenetic results suggested that a new cluster of NC bacteria had been enriched.

Discussion

The Brunssummerheide peatland is a spring fen in an oligotrophic sand valley fed by nitrate-polluted groundwater, and therefore contains nitrate concentrations in the upper peat layer which are uncommonly high for pristine peatlands (52). Also in contrast to many other peatlands (6, 24, 26, 27), methane was not detected in the upper 70-80 cm of the depth profile at 5 sampling occasions in different seasons from 2009 to 2011, even though methane was produced in the deep anoxic zone (below 100 cm, Fig. 1A, B). As roots of vascular plants do not reach that deep in the Brunssummerheide (maximum 60 cm), this suggested the existence of an anoxic methane sink in the peat, independent from oxygen and aerenchymal transport by roots, for which oxidized nitrogen species could serve as electron acceptor. The counter gradient of methane and nitrate at the depth of 80 cm may provide an ideal niche for, and may be at least partly created by the recently characterized n-damo bacteria. Targeting their 16S rRNA gene in DNA extracts from different depths confirmed this: Highest n-damo cell numbers (up to 3.2×10^7 cells g^{-1} soil) and ratios (3 to 8% of total bacterial community) were observed at the depth of 80-90 cm (Fig. 1 C, D), coinciding with the methane-nitrate transition zone (Fig. 1 A, B). At this depth, also a peak in abundance of phospho-lipid fatty acids diagnostic for NC10 phylum bacteria was detected (Fig. 1 G; Kool et al., submitted). The n-damo cell number and lipid profiles also agreed with the finding that among soil samples from 80-100, 100-120 and 120-135 cm depth only the 80-100 cm sample showed anaerobic methane-oxidizing activity upon incubation. Despite the relatively high numbers of n-damo bacteria detected at a depth 80-90 cm, it took several months to obtain an enrichment

culture with measurable activity. Also detection by fluorescence *in situ* hybridization using NC10 phylum-specific probes, hampered by a strongly auto-fluorescent background from the organic-rich inoculum, was only possible after 9 months of continuous cultivation with constant supply of nitrite and methane. This may be due to the "dilution" of the naturally NC10 phylum-enriched soil layer with less active deeper layers (90-100 cm) in the inoculum, and a very low growth rate at the prevailing conditions, especially the pH (6.0 – 6.2). The pH optimum test showed that the NC10 phylum bacteria enriched in the continuous culture were only acidotolerant to a certain extent, not acidiphilic. They were active down to a pH below 6, but their physiological optimum was clearly higher, above 7 (Fig. 3). This is a prime example for the discrepancy between physiological and ecological optimum. In contrast to previous *M. oxyfera* enrichment cultures from neutral, eutrophic sediments (14), which had a similar optimum (around 7.5), but were not active at a pH below 7 (assessed under similar conditions, O. Rasigraf, MSc thesis, 2011, unpublished), a different ecotype was dominant in the more acidic and low nutrient environment. According to the species delineation of 97% identity of the 16S rRNA gene for bacteria in general and 93% of the *pmoA* gene diagnostic for methanotrophic bacteria (35), the NC10 phylum bacterium dominating the Brunssummerheide enrichment culture even constitutes a new species within the genus *Methylomirabilis*.

Like other NC10 enrichment cultures (14, 20, 33), the enrichment period was characterized by a long phase without measureable activity, followed by a period of slow, but exponential increase in nitrite consumption rate. In the present case, nitrite-reducing activity remained low for the first 9 months, and then started to increase to about 1.0 mmol (NO_2^-) d^{-1} L^{-1} in month 15. After this increase it was not possible to stimulate the growth of the culture further and a sort of stationary phase was reached similar to other enrichments of NC10 bacteria (14, 20, 22). The doubling time of the Brunssummerheide *Methylomirabilis* strain was estimated to be

about two months, which is 4 to 8 fold lower than the values reported before (14). It is difficult to predict whether this reflects the growth rate under field conditions. On one hand some factors like a higher temperature (25 ºC), the optimum temperature of methanotrophs in most peat soils (19) in contrast to 10-15 ºC *in situ* and constant substrate supply may be beneficial, but other factors like stirring, use of surface- instead of porewater or a decrease in microbial partner communities may also be disadvantageous for growth in the laboratory.

However, once established, the methanotrophic community does not need to grow fast to constitute a relevant methane sink in the environment. According to previous estimations, *Methylomirabilis* cells in an enrichment culture have an activity of 0.1 to 0.4 fmol CH_4 $cell^{-1}$ d^{-1} (14), indicating that the Brunssummerheide soil of 80 – 85 cm depth with about 1.3 to 3.2×10^7 cells g^{-1} soil may convert between 1.3 and 12.8 nmol CH_4 d^{-1} g^{-1} soil. This range is at the lower end of methane oxidation rates reported for aerobic methanotrophs (41) in wetlands, but apparently high enough to balance the methane diffusing upwards from deeper, methanogenic soil layers.

Nitrite is clearly the preferred electron acceptor of previously reported *M. oxyfera* enrichments (13, 14, 20, 38). When nitrite was depleted in the present *Methylomirabilis* enrichment culture, methane oxidizing activity in the presence of nitrate (1 mM) ceased; upon addition of fresh nitrite, methane consumption started again (data not shown), demonstrating that the methane-oxidizing activity of Brunssummerheide enrichment is also nitrite dependent. Although nitrite was also detected in the depth profile, its concentrations were much lower (max. 4.2 µM, mostly around the detection limit of the colorimetric method) than those of nitrate. There was no depth-related pattern, and values were not constant over time. The nitrite needed by n-damo bacteria active in the soil might be supplied by other microorganisms (e.g denitrifying bacteria) or *Methylomirabilis* itself converting nitrate to

nitrite using organic carbon compounds other than methane. This would explain why nitrate is sufficient as an electron acceptor for methane oxidation *in situ* and in the initial batch incubations, whereas after enrichment, concomitant with a relative loss of other bacteria and a degradation of labile organic carbon, this supply path is insufficient and nitrite addition becomes mandatory for methane oxidation.

The present study shows an additional, so far hardly investigated pathway linking the biogeochemical cycling of nitrogen and methane in peatlands. Given the world-wide increasing groundwater nitrate and atmospheric nitrogen loads (32, 45), this methane sink may become more relevant for mitigating the mobilization of carbon in the form of methane from wetlands in the future.

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Table 1 Primer pairs used for qPCR analysis and clone libraries construction in this study.

	Forward primer			Reverse primer			Annealing	Target
	Designation	Sequence (5' - 3')	Reference	Designation	Sequence (5' - 3')	Reference	temp. (^{o}C)	
qPCR	p1F	GGGCTTGACATCCCACGAACCTG	(14)	p1R	CGCCTTCCTCCAGCTTGACGC	(14)	65	NC10 bact. 16S rRNA
	p2F	GGGGAACTGCCAGCGTCAAG	(14)	p2R	CTCAGCGACTTCGAGTACAG	(14)	65	NC10 bact. 16S rRNA
	533F	GTGCCAGCMGCCGCGGTAA	(49)	805R	GACTACCAGGGTATCTAATC	(28)	58	All bact. 16S rRNA
	1100F	YAACGAGCGCAACCC	(10)	1492R	GGTTACCTTGTTACGACTT	(53)	58	All bact. 16S rRNA
Clone library	8F	AGAGTTTGATYMTGGCTCAG	(21)	1043R	TCT CCA CGC TCC CTT GCG	(14)	55-65	NC10 bact. 16S rRNA
	193F	GACCAAAGGGGGCGAGCG	(14)					
	A189b	GGNGACTGGGACTTYTGG	(34)	cmo682	AAAYCCGGCRAAGAACGA	(34)	55-65	NC10 bact. *pmoA*
	cmo182	TCACGTTGACGCCGATCC	(34)					

Figure captions

Fig. 1 Depth profiles of the Brunssummerheide peatland. (A, B), Nitrate (filled square) and methane (open circle) concentrations in porewater sampled in December 2009 (A) and in June 2010 (B). (C-F) Bacterial cell numbers (cells g^{-1} wet soil) as assessed by qPCR on DNA extracted from two overlapping soil cores from 51 to 102 cm (open circles), and from 77 to 127 cm (open triangles). NC10 bacteria abundance was determined with primer pairs p1F & p1R (C), andp2F & p2R (D). Total bacterial abundance was determined with primer pair 535F & 805R (E), and 1100F & 1492R (F). (G) Relative abundance of the phospho-lipid fatty acids 10-methyl-hexadecanoic acid (10MeC16:0, open diamonds) and 10-methyl-hexadecanoic acid (10MeC16:1Δ7, multiplied by 3, closed squares) diagnostic of NC10 bacteria (data from Kool et al., [24A]).

Fig. 2 Activity test of the enrichment culture at month 10 with $^{15}NO_2^-$ and $^{13}CH_4$. Nitrite (filled circle) was consumed, $^{15,15}N_2$ (filled square), $^{14,15}N_2$ and $^{13}CO_2$ (filled triangle) were produced. The $^{13}CO_2$ production rate was 20.2 µmol d^{-1}, and the rate of $^{15,15}N_2$ production was 29.0 µmol d^{-1}.

Fig. 3 Methane-oxidizing activity of the n-damo enrichment culture incubated at different pH values.

Fig. 4 Fluorescence in situ hybridization of the enrichment culture at different times of incubation. A: month 9; B: month 14; C: month 19. NC10 bacteria appear in pink, due to co-hybridization of NC10 bacteria specific probes 193-Cy3 and 1027-Cy3 (red) and a mixture of probes EUBI-III, IV-Cy5 (light blue) for most eubacteria and DAPI (dark blue). (Scale bars: 5µm).

Fig. 5 Phylogenetic trees of the 16S rRNA (A) and the *pmoA* genes (B, including *amoA* and *pxmA* sequences) of the enrichment culture. The trees were calculated in Mega5 using the neigbor-joining method. Bootstrap support values (1000 replicates) greater than 50% are indicated at the nodes. The sequences obtained in this study from inoculum soil and enrichment after 1 or 17 months of incubation are shown in bold. **References**

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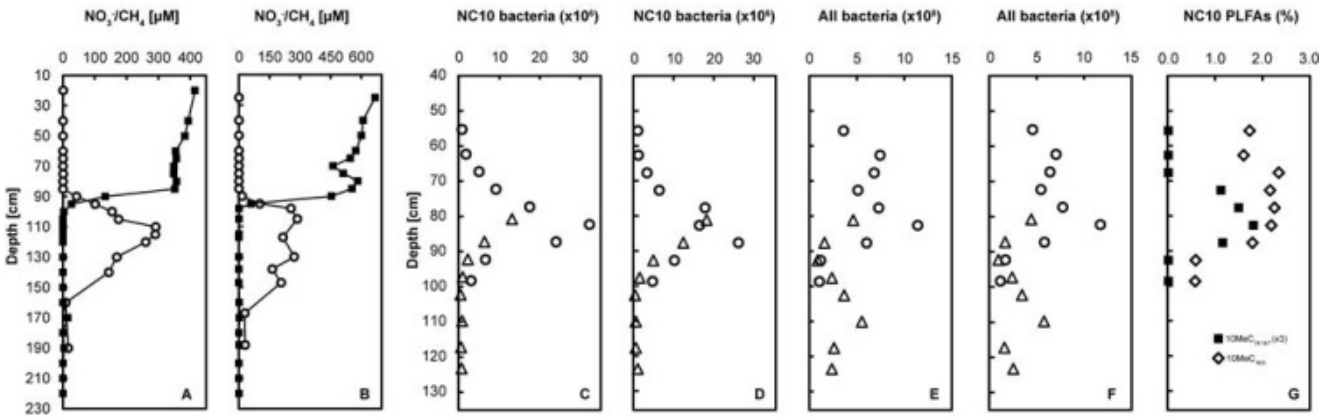
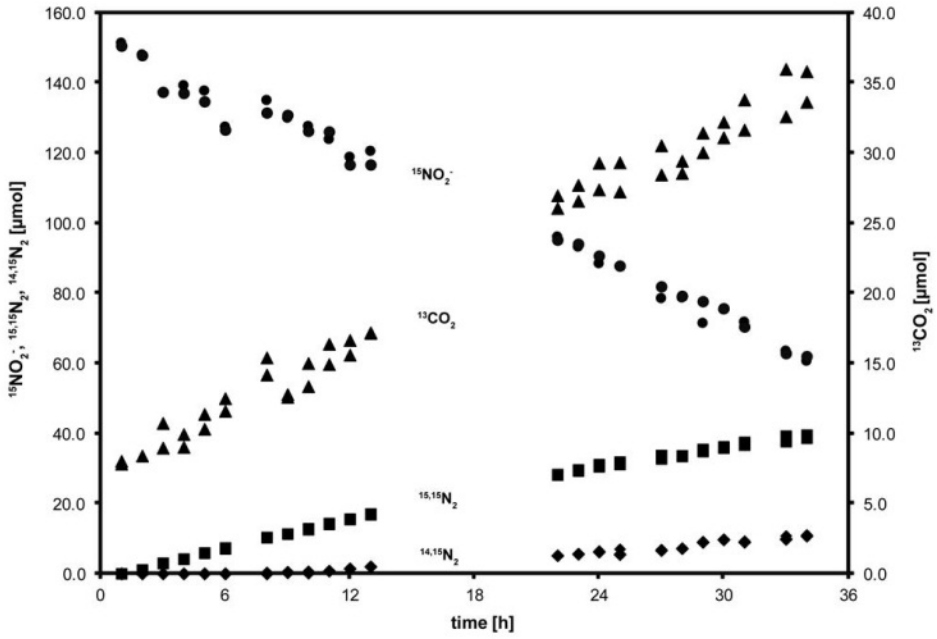


Fig.1



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591 Fig. 2

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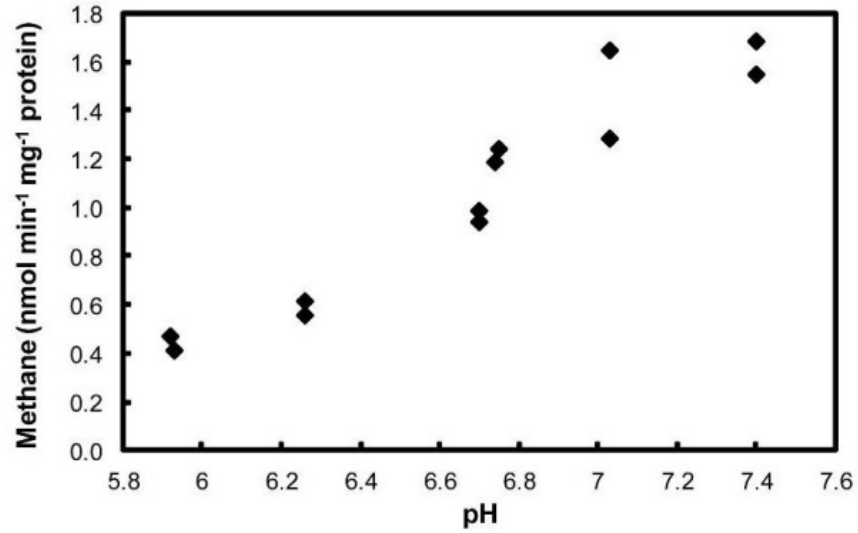

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594 Fig.3

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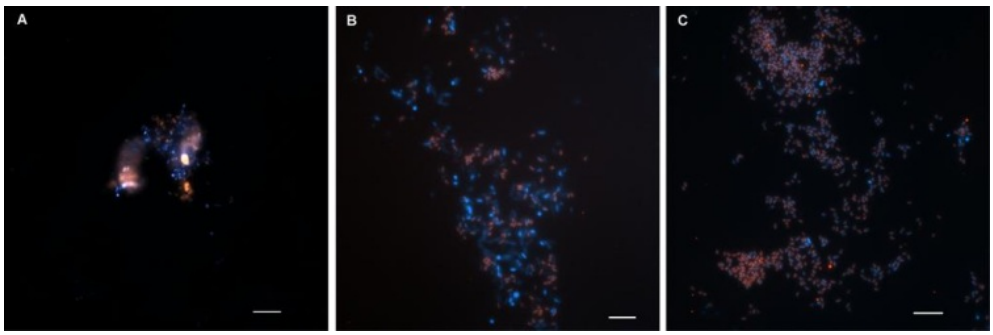

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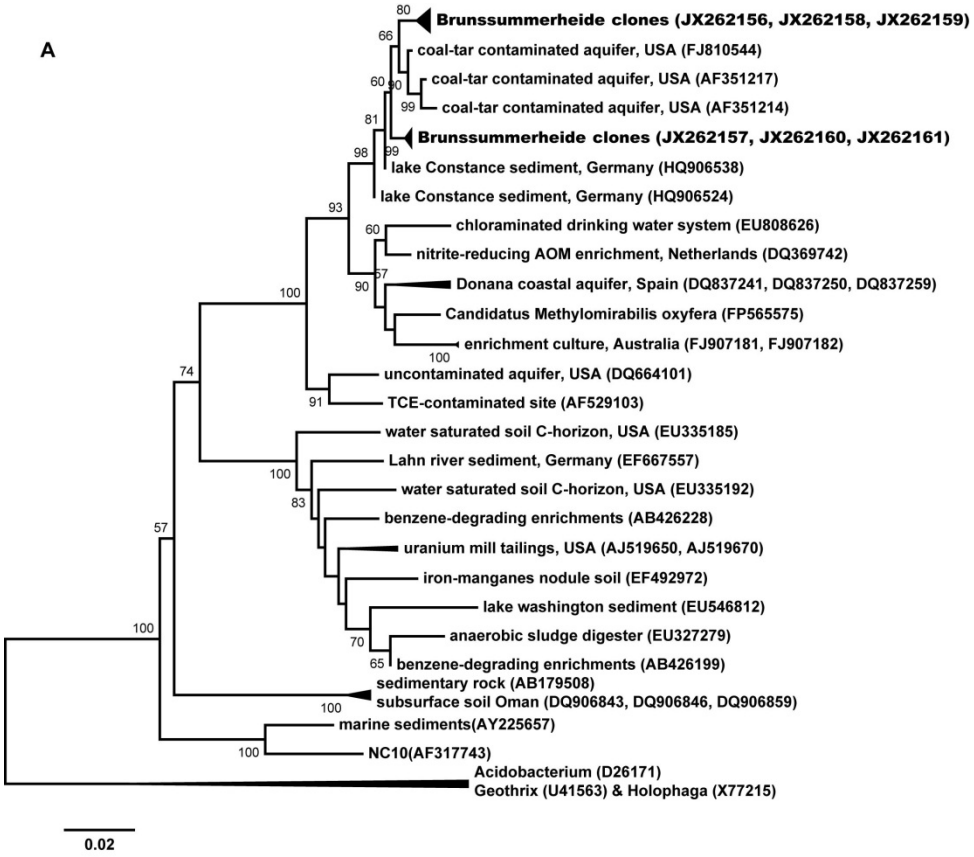
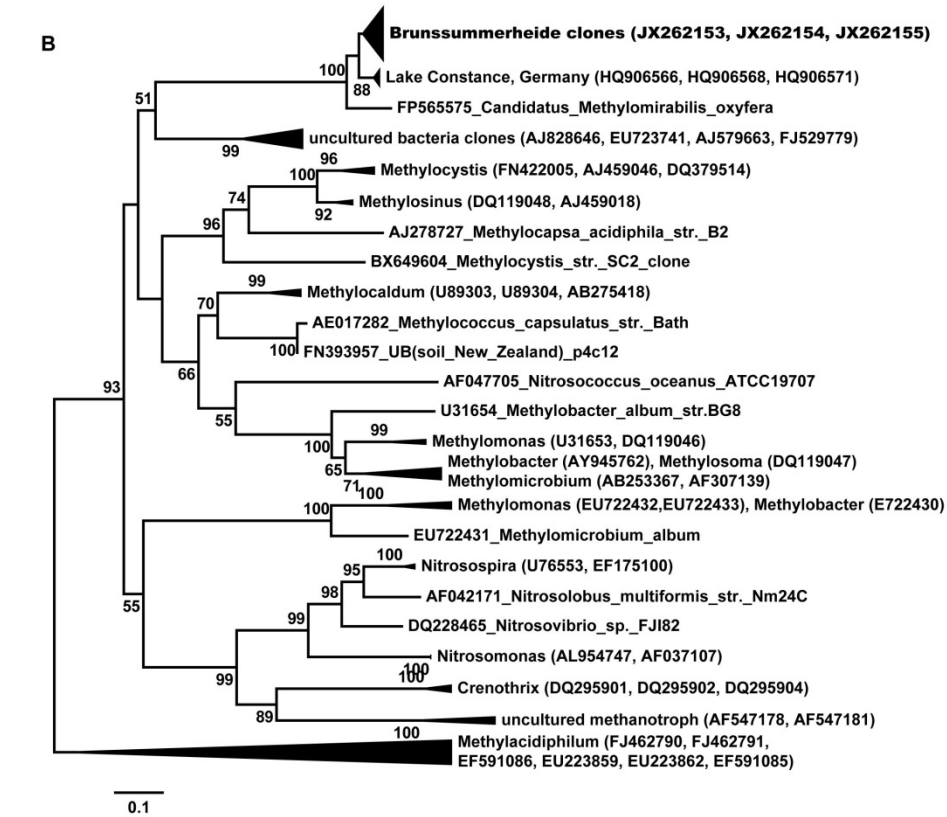
597 Fig. 4


A
Brunssummerheide clones (JX262156, JX262158, JX262159)
coal-tar contaminated aquifer, USA (FJ810544)
coal-tar contaminated aquifer, USA (AF351217)
coal-tar contaminated aquifer, USA (AF351214)
Brunssummerheide clones (JX262157, JX262160, JX262161)
lake Constance sediment, Germany (HQ906538)
lake Constance sediment, Germany (HQ906524)
chloraminated drinking water system (EU808626)
nitrite-reducing AOM enrichment, Netherlands (DQ369742)
Donana coastal aquifer, Spain (DQ837241, DQ837250, DQ837259)
Candidatus Methylomirabilis oxyfera (FP565575)
enrichment culture, Australia (FJ907181, FJ907182)
uncontaminated aquifer, USA (DQ664101)
TCE-contaminated site (AF529103)
water saturated soil C-horizon, USA (EU335185)
Lahn river sediment, Germany (EF667557)
water saturated soil C-horizon, USA (EU335192)
benzene-degrading enrichments (AB426228)
uranium mill tailings, USA (AJ519650, AJ519670)
iron-manganes nodule soil (EF492972)
lake washington sediment (EU546812)
anaerobic sludge digester (EU327279)
benzene-degrading enrichments (AB426199)
sedimentary rock (AB179508)
subsurface soil Oman (DQ906843, DQ906846, DQ906859)
marine sediments(AY225657)
NC10(AF317743)
Acidobacterium (D26171)
Geothrix (U41563) & Holophaga (X77215)
0.02


B
Brunssummerheide clones (JX262153, JX262154, JX262155)
Lake Constance, Germany (HQ906566, HQ906568, HQ906571)
FP565575_Candidatus_Methylomirabilis_oxyfera
uncultured bacteria clones (AJ828646, EU723741, AJ579663, FJ529779)
Methylocystis (FN422005, AJ459046, DQ379514)
Methylosinus (DQ119048, AJ459018)
AJ278727_Methylocapsa_acidiphila_str._B2
BX649604_Methylocystis_str._SC2_clone
Methylocaldum (U89303, U89304, AB275418)
AE017282_Methylococcus_capsulatus_str._Bath
FN393957_UB(soil_New_Zealand)_p4c12
AF047705_Nitrosococcus_oceanus_ATCC19707
U31654_Methylobacter_album_str.BG8
Methylomonas (U31653, DQ119046)
Methylobacter (AY945762), Methylosoma (DQ119047)
Methylomicrobium (AB253367, AF307139)
Methylomonas (EU722432,EU722433), Methylobacter (E722430)
EU722431_Methylomicrobium_album
Nitrosospira (U76553, EF175100)
AF042171_Nitrosolobus_multiformis_str._Nm24C
DQ228465_Nitrosovibrio_sp._FJI82
Nitrosomonas (AL954747, AF037107)
Crenothrix (DQ295901, DQ295902, DQ295904)
uncultured methanotroph (AF547178, AF547181)
Methylacidiphilum (FJ462790, FJ462791,
EF591086, EU223859, EU223862, EF591085)
0.1

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599 Fig. 5