Nitrite-dependent methane oxidation

Katharina Friederike Ettwig
2010

Ettwig, Katharina Friederike (2010): Nitrite-dependent methane oxidation. PhD thesis, Radboud University Nijmegen, 120 p. With summaries in English, Dutch and German.

This PhD project was financially supported by NWO.

Frontpage: Sampling site in the Ooijpolder near Persingen and sediment incubations. Design by B. Kartal and K. Ettwig.

Print: Ipskamp Drukkers, Enschede.

ISBN/EAN 978-90-9025631-3

Nitrite-dependent methane oxidation

Een wetenschappelijke proeve op het gebied van de Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus
prof. mr. S.C.J.J. Kortmann, volgens
besluit van het college van decanen
in het openbaar te verdedigen
op maandag 4 october 2010
om 15:30 uur precies

door

Katharina Friederike Ettwig
geboren op 17 april 1978 te Duisburg

Promotores:

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

Prof. dr. ir. Marc Strous

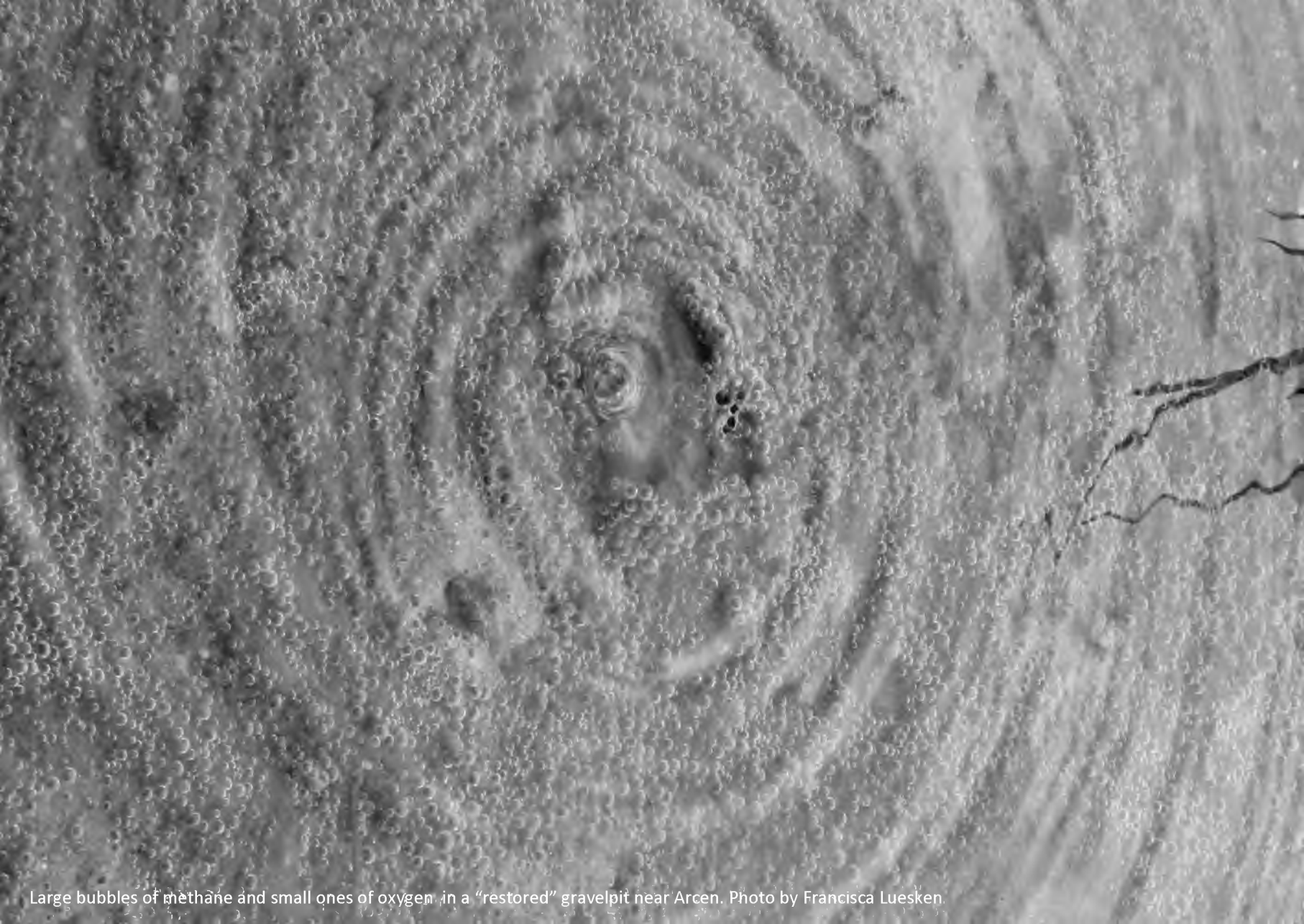
Manuscriptcommissie:

Prof. dr. Hans de Kroon

Prof. dr. ir. Alfons J.M. Stams
Wageningen Universiteit

Prof. dr. J. Colin Murrell
University of Warwick

Contents



Large bubbles of methane and small ones of oxygen in a “restored” gravelpit near Arcen. Photo by Francisca Luesken

Chapter 1

Introduction

*"*Wuhrmann*: Is there any information that nitrate or nitrite may be used by methane oxidizers?* - Rudd*: There is some indication that these methane oxidizers have the capability of using electron acceptors other than oxygen. However, we have not been able to prove this to our satisfaction."* (Published discussion from "Methane oxidation in a eutrophic Canadian shield lake"; Rudd & Hamilton, 1975)

"We have attempted without success over the course of 2 years to obtain methylotrophs capable of using nitrate as an electron acceptor. " (Panganiban et al., 1979)

Considerable speculation and unclear reports have long been the only traces of anaerobic methane oxidation coupled to nitrate (NO_3^-) or nitrite (NO_2^-) reduction in the scientific literature. From two angles this process is of great interest: for a better understanding of biogeochemical cycles, notably those of methane and nitrogen; and for a better insight into the functional diversity of microorganisms, as they seem to exploit nearly every thermodynamically feasible reaction of abundant natural substances (Lane, 2006). On this basis, Broda (1977) could postulate the existence of anaerobic ammonium oxidation with nitrite as electron acceptor and of phototrophic ammonium oxidation. The first of these predicted chemolithotrophic metabolisms was indeed described later (Mulder et al., 1995; Strous et al., 1999). This was followed by several more discoveries in the basic elemental cycles, like the phototrophic, anoxygenic oxidation of nitrite to nitrate (Griffin et al., 2007), of arsenite to arsenate (Kulp et al., 2008), or the anaerobic oxidation of arsenite coupled to denitrification (Sun et al., 2008). In all cases, the aerobic equivalent, for example the aerobic oxidation of ammonium (Schloesing & Müntz, 1877; Winogradsky & Omeliansky, 1899), was already known for a long time – presumably because of greater agricultural or biotechnological interest or the faster growth and consequentially easier culturing of aerobic microorganisms. Also the observation of the microbial consumption of methane (Käserer, 1905), the simplest organic molecule (CH_4) and the isolation of aerobic methane-oxidizing bacteria (Söhngen, 1906) dates back to more than 100 years.

Denitrification

Research on denitrification began during the late 19^{th} century when loss of plant-available nitrogen – notably nitrate and ammonium (NH_4^+) – from soils was noticed as a threat to agricultural productivity (Gayon & Dupetit, 1882). Microorganisms that could use nitrate instead of oxygen as electron acceptor were isolated, and anaerobic conditions identified as the cause of biotic loss. Consequentially, advice was given how to *"empêcher les microbes anaérobies de se développer et d'exercer leur fâcheuse influence réductrice"* (Gayon & Dupetit, 1882). Already at that time it was noticed, that not only dinitrogen (N_2), but also nitrous oxide (N_2O) was formed as gaseous product of nitrate reduction. Nevertheless, the role of nitrous oxide as an obligatory, free intermediate, first claimed by Kluyver & Donker (1926) was questioned for a long time (Allen & van Niel, 1952; Sacks & Barker, 1952). It was finally firmly established by isotope labeling experiments (Delwiche, 1959; Matsubara & Mori, 1968; St. John & Hollocher, 1977). The role of nitric oxide remained controversial for much longer (e.g. Weeg-Aerssens 1988), until finally the pathway formulated already by Payne (1971) was generally accepted (reviewed in Zumft, 1993). This pathways is now also well-characterized on the enzymatic level (Berks et al., 1995; Zumft, 1997). Each step from

the ionic nitrate or nitrite (NO_2^-) via the intermediate nitric oxide to the gaseous end products nitrous oxide and nitrogen are carried out by specialized enzymes: nitrate (nar or nap), nitrite (the copper-containing nirK and the cytochrome c-type nirS), nitric oxide (norB or norZ) and nitrous oxide (nos) reductases (see also figure 1 in chapter 4). This understanding is based largely on the species available in pure culture (Shapleigh, 2006), some of which are model organisms accessible to detailed genetic and physiological studies, e.g. *Pseudomonas* (Philippot et al., 2001), *Bradyrhizobium* (Bedmar et al., 2005) and *Paracoccus* (Baker et al., 1998).

It is generally assumed that nitrate-respiring organisms are also capable of aerobic respiration (Allen & van Niel, 1952), but metagenomic studies on uncultivated bacteria suggest that this may not be always the case (Walsh et al., 2009). Also the denitrification pathway in archaea (Philippot, 2002; Cabello et al., 2004) and some eukaryotes (Kobayashi et al., 1996; Risgaard-Petersen et al., 2006) is much less understood than in proteobacteria. The quantitative contribution of denitrification and denitrifying organisms in the global nitrogen cycle is intensely debated. Controversial issues include the relative importance of anaerobic ammonium oxidation (anammox), denitrification and dissimilatory nitrate reduction to ammonium (DNRA) for oceanic nitrogen losses (Kuypers et al., 2005; Burgin & Hamilton, 2007; Lam et al., 2009; Ward et al., 2009), the factors controlling N_2O production by nitrifying and denitrifying bacteria (Bremner, 1997; Stüven & Bock, 2001; Wrage et al., 2001), and the significance of chemical vs. biological denitrification (Wullstein & Gilmour, 1966; Luther et al., 1997; van Cleemput, 1998; Mccalley & Sparks, 2009). Denitrification is common among both heterotrophic and autotrophic microorganisms. The latter use inorganic compounds as electron donors, like various sulfur compounds (Beijerinck, 1904, 1920), ferrous iron (Straub et al., 1996) and arsenite (Sun et al., 2008). In heterotrophs, a wide range of organic substrates is known to support denitrification: chicken broth, sugars and alcohols, diverse complex organic molecules (Burford & Bremner, 1975), wastewater organic carbon (Gayon & Dupetit, 1886; Matějů et al., 1992), aromatic hydrocarbons (Rabus & Widdel, 1995), and methanol (Sperl & Hoare, 1971; Kalyuzhnaya et al., 2009), to name a few.

Alkane oxidation

Methane, however, is not just another organic substrate. A very high activation energy of 439 kJ/mol is required to break the C-H bond, which makes it second only to benzene among organic compounds (Thauer & Shima, 2008). The ratio of activation energy to free energy yield of the reaction decreases with the carbon chain length in alkanes (C_nH_{2n+2}). This makes multi-carbon alkanes slightly 'easier' microbial substrates than methane under anaerobic conditions (Widdel & Musat, 2010). Hexane (C_6H_{14}) and

longer-chain alkane degradation without oxygen is known for two decades (Aeckersberg et al., 1991; Rueter et al., 1994; Zengler et al., 1999; Ehrenreich et al., 2000; So & Young, 2001), but anaerobic oxidation of short-chain alkanes is a very recent discovery: In 2007, Kniemeyer and coworkers isolated a propane-(C_3H_8) and butane-(C_4H_{10}) degrading, sulfate-reducing bacterium that belongs to the δ-proteobacterial *Desulfococcus/Desulfosarcina* cluster. Additionally, they obtained an enrichment culture oxidizing ethane (C_2H_6) under sulfate reducing conditions. The very slow growth of the latter – measurable activity occurred only after about 2 years – has so far hampered its phylogenetic characterization. Also the activation mechanism for ethane has not been elucidated so far. For other alkanes, two enzymatic solutions for the anaerobic conversion of the substrate to a more reactive intermediate have been demonstrated: The relatively well-characterized fumarate-addition to the C1- or C2-position of the carbon skeleton by a glycyl radical enzyme (Rabus et al., 2001; Selmer et al., 2005; Kniemeyer et al., 2007; Savage et al., 2010), and a carboxylation of the carbon-3 position (So et al., 2003; Heider, 2007). The fumarate-addition is energetically costly, and thought to be not feasible for small molecules like ethane and methane (Widdel & Musat, 2010).

In aerobic bacteria, oxygen is not only the electron acceptor, but also plays a crucial role in the initial activation of alkanes by a variety of mono-and dioxygenases (Berthe-Corti & Fetzner, 2002; van Beilen & Funhoff, 2007). Methanotrophic bacteria employ two distinct methane monooxygenases (MMOs) for the initial step of methane oxidation (Hakemian & Rosenzweig, 2007). Both the soluble, iron containing MMO (sMMO), and the membrane bound, copper-iron MMO (pMMO) form with the aid of O_2 a radical metal-oxo species in their catalytic centers. This radical attacks the C-H bond, and one of the oxygen atoms is then added to the remaining methyl group, yielding methanol as the first intermediate. The other oxygen is reduced to water (Figure 1a).

Under anoxic conditions, methane was long believed to be stable. Its anaerobic oxidation was first reported on ecosystem level without knowledge of the involved microorganisms (Barnes & Goldberg, 1976; Reeburgh, 1976; Panganiban et al., 1979; Devol & Ahmed, 1981). Theoretical considerations and inhibitor studies soon led to the hypothesis that archaea related to methanogens and sulfate-reducing bacteria were together responsible for this methane sink (Zehnder & Brock, 1980; Hoehler et al., 1994). Around the turn of the century, the combination of new analytical techniques and the discovery of naturally enriched communities enabled the characterization of the microorganisms involved in anaerobic methane oxidation coupled to sulfate reduction (Pimenov et al., 1997; Hinrichs et al., 1999; Boetius et al., 2000). Since then it has been established that three distinct groups of Archaea, one of the order *Methanomicrobiales* (tentatively named ANME-I) and two within the *Methanosarcinales* (ANME-II and -III) perform reverse methanogenesis in syntrophy with sulfate-reducing bacteria of the

Desulfosarcina/Desulfococcus or the *Desulfobulbus* branch of the δ-Proteobacteria (Orphan et al., 2002; Knittel et al., 2005; Niemann et al., 2006), but the way of shuttling electrons between the two consortia members is still unclear (Figure 1b, reviewed in Knittel & Boetius, 2009).

The initial activation of methane by methanotrophic archaea is most likely performed by the same enzyme that produces methane in methanogens: the methyl-coenzyme M-reductase (MCR), acting in reverse and producing methyl-coenzyme M as the first intermediate (Shima & Thauer, 2005). This activity was recently demonstrated in vitro for purified MCR from the methanogen *Methanothermobacter marburgensis* (Scheller et al., 2010). Different lines of evidence support the reverse-methanogenesis hypothesis: MCR was found in high amounts in ANME-I-dominated microbial mats (Krüger et al., 2003), it is present in the genomes of all ANME-archaea investigated so far (Hallam et al., 2003; Hallam et al., 2004; Meyerdierks et al., 2005; Meyerdierks et al., 2010) and its specific inhibitor, bromo-ethanesulfonate (BES, a coenzyme M-analogue) also inhibits anaerobic methane oxidation (Zehnder & Brock, 1980; Hoehler et al., 1994; Nauhaus et al., 2007).

The free energy change associated with the oxidation of methane under sulfate-reducing conditions is very low: although variable over a range of environmental conditions, it usually does not exceed -15 to -40 kJ/mol CH_4, largely depending on partial pressure of methane (Valentine, 2002; Larowe et al., 2008). This is close to the thermodynamic limit of around -20 kJ/mol necessary for the translocation of one proton for ATP production (Schink, 1997), and to make matters more complicated, this energy also has to be shared between the partners in the consortia.

Anaerobic methane oxidation coupled to the reduction of electron acceptors further up in the redox ladder releases substantially more energy to sustain microbial metabolism (table 1), but the evidence for its existence is still preliminary (Miura et al., 1992; Beal et

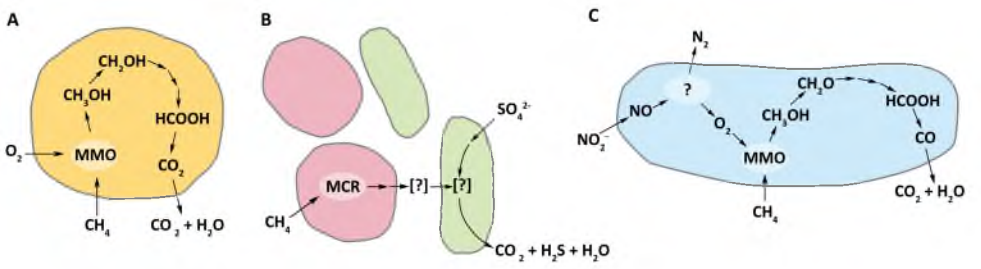


Figure 1 | The three different pathways of microbial methane oxidation known to date. **A** Aerobic methane oxidation by *Proteobacteria* and *Verrucomicrobia*. **B** Anaerobic methane oxidation coupled to sulfate reduction. Archaea are depicted in red, sulfate-reducing bacteria in green. **C** methane oxidation coupled to nitrite reduction by '*Candidatus* Methylomirabilis oxyfera'. MMO - methane monooxygenase; MCR - Methyl-coenzyme M-reductase. Picture design: Johannes Zedelius.

Table 1 l Net reactions of methane oxidation coupled to the reduction of various terminal electron acceptors.

Reaction	Free energy change ΔG°' (kJ/mol CH_4)
$CH_4 + 2O_2 \rightarrow CO_2 + 2\ H_2O$	-818
$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$	-929
$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O$	-765
$CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$	-528
$CH_4 + 8Fe(OH)_3 + 7CO_2 \rightarrow 8\ FeCO_3 + 14H_2O$	-344
$CH_4 + SO_4^{2-} + 2H^+ \rightarrow CO_2 + H_2S + 2H_2O$	-21.2

al., 2009). The reactions of methane with nitrogen oxides should constitute an even better temptation for microorganisms to exploit, as their energy yield is much closer to aerobic than to sulfate-dependent methane oxidation (table 1). This, however, does not automatically mean that an enzymatic solution to overcome the initially high activation energy has evolved.

Past research on anaerobic methane oxidation coupled to denitrification

Until the first unambiguous demonstration of microbial anaerobic methane oxidation coupled to denitrification was published in 2006 (Raghoebarsing et al.), the level of understanding of this process was more like in the citations preceding this introduction: vague. In the case of the eutrophic Canadian shield lake mentioned in the above citation (Rudd & Hamilton, 1975), methane oxidation in the water column was confined to a narrow layer close to the thermocline, where oxygen concentrations were not exceeding 1 mg/l (31 µM). Higher oxygen levels inhibited methane oxidation, leading to the question if alternative electron acceptors could be involved. Later it was shown that aerobic methane oxidizers were responsible for the observed activity, and that their requirement for low oxygen concentrations was due to their dependence on nitrogen fixation (Rudd et al., 1976). In a similar freshwater lake study which reported the peak of methane oxidation at the thermocline, methane oxidation co-occurred with nitrate depletion, but because oxygen was also present in sufficient quantity, the latter was assumed to be the responsible electron acceptor (Bédard & Knowles, 1997).
The association of aerobic methanotrophs with denitrifying bacteria has often been observed (Amaral et al., 1995; Eisentraeger et al., 2001; Knowles, 2005), and can be explained by oxygen consumption by methanotrophs and the use of some of their

metabolic intermediates - methanol and formate – as carbon and energy source for the denitrifying bacteria. Co-occurrence of complete denitrification and methane-oxidizing ability was assessed for 136 strains of aerobic methanotrophic bacteria, but was not found (Bowman et al., 1993), and an early report on the isolation of such bacteria (*Alcaligenes sp.*; Davies, 1975) was discredited later due to considerable ethanol load supplied with the vitamin solution (Mason, 1977). Some aerobic methane-oxidizing bacteria can tolerate high nitrite concentrations. In *Methylomicrobium album*, for example, methane-oxidizing activity and growth is even slightly stimulated by nitrite (Nyerges & Stein, 2009; Nyerges et al., 2010). The consumption of NO and the reduction of nitrate or nitrite to NO and N_2O has been shown in several aerobic methanotrophs, but neither was complete denitrification to N_2 observed, nor did the bacteria couple this reduction to energy metabolism (Krämer et al., 1990; Ren et al., 2000; Nyerges et al., 2010).

Ambiguous results were obtained in several wetland studies. Kumaraswamy *et al.* (2001) demonstrated anaerobic methane oxidation in rice field soil under nitrate- and iron-reducing conditions, but observed also considerable activity in controls without added electron acceptors. Smemo & Yavitt (2007) measured simultaneous methane production and consumption in different peat soils and did find a net consumption after addition of nitrate, but could not clearly differentiate between the effects of methanogen inhibition and methanotroph stimulation. Also, nitrate reduction in their incubations could not fully account for the inferred oxidation rates, so that other electron acceptors must have played a role, too. Oxygen and sulfate as electron acceptors can be excluded for the anoxic hypolimnion of meromictic Lake Lugano, where concentration profiles, isotopic composition of methane and ^{13}C-depleted biomarkers point to anaerobic oxidation of methane (Niemann et al., 2009), but whether nitrate is involved in that case still awaits more detailed studies. Several other cases of anaerobic oxidation of methane in lakes can be attributed to sulfate reduction; this has been documented for the salty Lake Mendota (Panganiban et al., 1979) and Big Soda Lake (Iversen et al., 1987), and the meromictic freshwater lakes Lago di Cadagno (Schubert et al., 2007) and Plußsee (Eller et al., 2005).

The possibility of anaerobic methane oxidation coupled to denitrification has also been considered in the field of hydrogeochemistry (Griffioen, 1999). In aquifers, especially in groundwater bodies influenced by contaminant sources like landfills, methane is often the most abundant carbon source. In several studies on landfill leachate plumes circumstantial evidence for nitrate-driven methane oxidation was obtained. Profiles of methane, sulfate, ferrous iron, nitrate and oxygen in a landfill in Denmark showed a clear decrease of methane originating from the landfill in the denitrifying zone, before it reached the oxic part of the aquifer (Bjerg et al., 1995). Similarly, the analysis of several hydrogeochemical parameters indicated anaerobic oxidation of methane in a Dutch

landfill leachate plume (Banisveld), where residual dissolved methane in the denitrifying zone showed a clear enrichment of the heavy carbon isotope ^{13}C (van Breukelen et al., 2003; van Breukelen & Griffioen, 2004). In a tracer study on a contaminated aquifer on Cape Cod, USA, Smith et al. (1991) demonstrated the occurrence of anaerobic methane oxidation in anoxic, nitrate-rich (650 µM) groundwater by injection of ^{13}C-labeled methane and subsequent measurement of its disappearance and the formation of ^{13}C-CO_2. In view of the very low in situ oxygen concentration of < 0.3 µM, and the absence

Methane and nitrogen cycles

Methane is a trace greenhouse gas in earth's atmosphere and showed a similar, but more drastic development compared to CO_2 during the last two centuries. Because of its strong relative radiative forcing, 25-30 times the global warming potential of CO_2 on a 100 year-scale (Denman, 2007; Shindell et al., 2009), it is the second most important greenhouse gas, contributing 14-19 % to direct radiative forcing - despite its low concentration (Denman, 2007). Mainly due to land use changes, cattle breeding and fossil fuel burning, methane concentrations have more than doubled since industrialization to currently 1.8 ppm*v*.

Methane is naturally produced by three processes: by entirely abiotic serpentinization reactions occurring at high pressure and temperature via H_2 and CO_2, thermal decomposition of buried organic material (Reeburgh, 2007), and the activity of strictly anaerobic microorganisms, the methanogenic archaea (Mah et al., 1977). The first two sources are confined to deep sediment layers, with contact to biosphere and atmosphere largely restricted to seeping of methane from volcanic areas and deep-sea gas hydrates (Etiope & Klusman, 2002). For the emissions to the atmosphere, biological methane production is the most important, with the largest single sources being natural wetlands (20-39 %), and the most significant anthropogenic ones ruminants (~15 %) and rice fields (11-19 %; Forster et al. (2007) and references therein). Methanogenesis is one of the final steps in the anaerobic degradation of organic matter, using CO_2 or the methyl groups of organic compounds as electron acceptors (Thauer, 1998). The energy yield of this metabolism is low, and the methanogens are usually outcompeted by respiring microorganisms for substrates (Winfrey & Zeikus, 1977; Lovley & Klug, 1983; Conrad, 1999). Together with the requirement for anaerobiosis, this restricts methanogens to niches where oxygen is absent, and also alternative electron acceptors are depleted. On its way to the atmosphere methane diffuses through the anaerobic sediments, and passes zones dominated by different regimes of anaerobic respiration (i.e. sulfate, iron, manganese and eventually nitrate reduction) before reaching the aerobic sediment or water column (Nealson, 1997; Brune et al., 2000).

Similar to the carbon cycle, also the nitrogen cycle has been heavily altered by humans since the industrial revolution (Vitousek et al., 1997; Galloway et al., 2004; Galloway et al., 2008). The single most important anthropogenic interference is the production of ammonia that is since 1913 produced from atmospheric nitrogen via the Haber-Bosch process. Now approximately 25-30 % of globally produced reactive nitrogen is derived from this industrial process, and finds its way to the biosphere by direct application as fertilizer to agricultural land and atmospheric deposition of ammonia and nitrogen oxides (Galloway et al., 2008; Robertson & Vitousek, 2009). Especially in nitrogen-limited ecosystems (Vitousek & Howarth, 1991), this additional

of sulfate or iron reduction, the authors concluded that nitrate was the most likely electron acceptor.

Besides biogeochemists and microbiologists, civil engineers were also interested in the coupling of anaerobic methane oxidation to denitrification, as methane would constitute an inexpensive electron donor, often produced on-site, for wastewater denitrification (Sollo et al., 1976; Mason, 1977; Thalasso et al., 1997). All studies reporting rates comparable to methanol-fed denitrification systems are based on

input can translate to acidification (Guo et al., 2010), enhanced greenhouse gas emissions (notably N_2O), and increased primary production, with all the consequences typically associated with eutrophication: Shifts in species composition and loss of biodiversity (Bobbink et al., 1998; Tomassen et al., 2003) and oxygen depletion due to decomposition (partly to methane) of increased amounts of organic matter (Rabalais et al., 2002). The net effect on greenhouse gas emissions is difficult to predict, because presence of nitrogen oxides can also inhibit methane production, and stimulate methane consumption (Aerts & De Caluwe, 1999; Bodelier & Laanbroek, 2004; Barnard et al., 2006).

The process described in this thesis provides another, direct link of the carbon with the nitrogen cycle: Methane is oxidized in an anaerobic environment with the aid of nitrite, and carbon dioxide and nitrogen gas are produced. The bacteria that have been found to perform this reaction use enzymes similar to aerobic methane-oxidizing bacteria. They produce the oxygen needed for methane activation themselves from nitrite, presumably via the intermediate nitric oxide (figure 1).

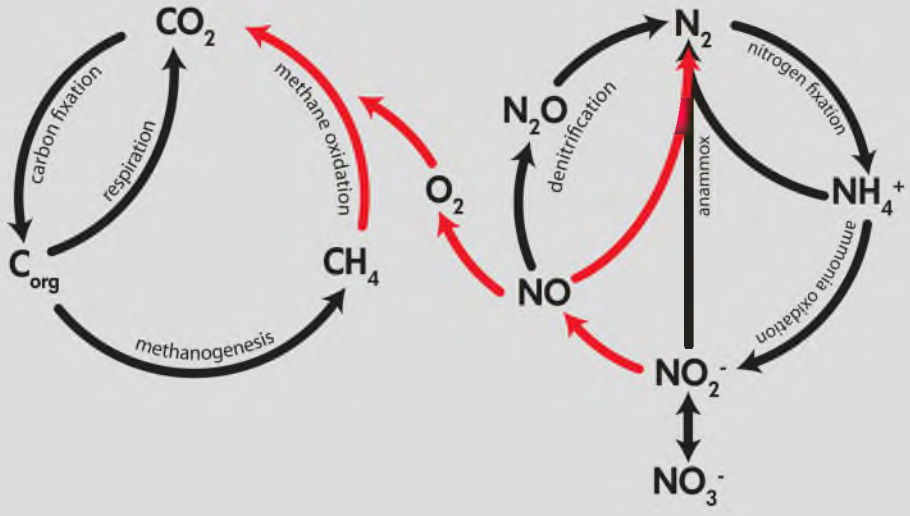


Figure 1 l Basic processes in the biological carbon and nitrogen cycles and the transformations catalyzed by '*Candidatus* Methylomirabilis oxyfera' (in red).

oxygen-limited reactors, which relied on the oxygen-dependent methane oxidation to methanol or another intermediate (Thalasso et al., 1997; Costa et al., 2000; Modin et al., 2007). These methanotrophic intermediates are subsequently used by denitrifiers as electron donors (Osaka et al., 2008). Only one of the published studies rigorously excluded oxygen, but reported low specific rates (~0.63 nmol mg^{-1} (protein) min^{-1}) and did not provide controls for the dependence of denitrification on the presence of methane (Islas-Lima et al., 2004).

Taken together, the research field of anaerobic methane oxidation coupled to denitrification was not entirely unworked, but the documentation of the process in the environment was sparse, and an understanding on the organismic and biochemical level was entirely missing.

Outline of this thesis

This thesis describes the progress that was made in the understanding of anaerobic methane oxidation coupled to nitrite reduction since its initial description. Raghoebarsing et al. (2006) had incubated an inoculum from a eutrophic Dutch canal with an anaerobic, mineral medium under the continuous supply of methane, nitrite and nitrate. Over an extended period of time (more than a year), a microbial enrichment culture was obtained and characterized. This culture reduced nitrite and nitrate to dinitrogen gas and oxidized methane in a stoichiometry expected from the equations in table 1. Nitrite was preferred over nitrate. Phylogenetic analysis and subsequent fluorescence in situ hybridization of biomass showed that the microbial community was dominated (70-80%) by a bacterium of the uncultivated NC10 phylum, and archaea of the order *Methanosarcinales* made up about 10-20 %. Carbon from ^{13}C-methane was mainly incorporated in bacterial lipid biomarkers, and to a minor extent also in archaea. In view of the analogy to the consortium of ANME-archaea and sulfate-reducing bacteria that catalyze the sulfate-dependent anaerobic methane oxidation, these findings were interpreted as an indication that also in this case the archaea activated the methane in a reverse-methanogenesis mechanism, and that electrons were shuttled to the denitrifying bacterial partner.

In the introduction chapter, anaerobic methane oxidation coupled to denitrification is placed in the context of global carbon and nitrogen cycles, and previous research on the process and its occurrence in nature is summarized. The second chapter follows up on the development of the original enrichment culture described by Raghoebarsing et al. (2006), and documents the decline and finally disappearance of the archaeal member of the community. Apparently, neither archaea nor other organisms using MCR were necessary for the process. The still dominant NC10 bacterium was identified as the main player in nitrite-driven anaerobic methane oxidation. Chapter 3 describes the

enrichment of very similar bacteria from a different inoculum source, and provides more evidence on their specific role in methane oxidation by correlating their abundance with activity. Additionally, it provides molecular tools for the environmental detection of NC10 bacteria, compiles the diversity of this new phylum manifested by sequences deposited in databases and those obtained by screening the sediment inoculum.

The enrichment cultures described in chapters 2 and 3 formed the basis for the investigation of the genome and the central metabolic pathways conducted in chapter 4. Several complementary approaches were combined to characterize the NC10 bacterium, now named '*Candidatus* Methylomirabilis oxyfera'. In a collaborative effort, the complete genome of '*M. oxyfera*' was assembled and key catabolic pathways annotated. On this basis, a biochemical mechanism for nitrite-reducing methane oxidation was proposed and subsequently experimentally tested with stable-isotope labeling techniques. The results indicated that nitrite reduction in '*M. oxyfera*', although leading to the production of nitrogen gas, did not involve the denitrification intermediate nitrous oxide, but instead proceeded via conversion of NO into nitrogen and oxygen. Oxygen was subsequently used in the canonical pathway of aerobic methane oxidation, with the initial, methanol-yielding step catalyzed by pMMO. Possible implications of this new pathway for the ordering of events around the oxygenation of the atmosphere are briefly discussed. The final outlook chapter (5) summarizes the main findings and identifies some of the questions on '*M. oxyfera*', its role in biogeochemical cycles and its metabolism, *which we have not been able to prove to our satisfaction.*

Chapter 2

Denitrifying bacteria anaerobically oxidize methane in the absence of archaea

Published previously as:
Katharina F. Ettig, Seigo Shima, Katinka T. van de Pas-Schoonen, Jörg Kahnt, Marnix H. Medema, Huub J.M. op den Camp, Mike S.M. Jetten and Marc Strous (2008) in *Environmental Microbiology* **10** (11): 3164-3173.

Summary

Recently, a microbial consortium was shown to couple the anaerobic oxidation of methane to denitrification, predominantly in the form of nitrite reduction to dinitrogen gas. This consortium was dominated by bacteria of an as yet uncharacterized division and archaea of the order *Methanosarcinales* The present manuscript reports on the upscaling of the enrichment culture, and addresses the role of the archaea in methane oxidation. The key gene of methanotrophic and methanogenic archaea, *mcrA*, was sequenced. The associated cofactor F_{430} was shown to have a mass of 905 Da, the same as for methanogens and different from the heavier form (951 Da) found in methanotrophic archaea. After prolonged enrichment (> 1 year), no inhibition of anaerobic methane oxidation was observed in the presence of 20 mM bromoethane sulfonate, a specific inhibitor of MCR. Optimization of the cultivation conditions led to higher rates of methane oxidation and to the decline of the archaeal population, as shown by fluorescence in situ hybridization and quantitative MALDI-TOF analysis of F_{430}. Mass balancing showed that methane oxidation was still coupled to nitrite reduction in the total absence of oxygen. Together our results show that bacteria can couple the anaerobic oxidation of methane to denitrification without the involvement of archaea.

Introduction

In 2006, Raghoebarsing and colleagues obtained an enrichment culture that coupled the anaerobic oxidation of methane (AOM) to denitrification. Freshwater canal sediment was incubated for more than a year in a bioreactor with biomass retention (sequencing batch reactor) until measurable methane and nitrite turnover was obtained. In the enrichment culture, one bacterial phylotype belonging to the candidate division "NC10" made up ~80% of the population. This division had been defined until then only by environmental sequences (Rappé and Giovannoni, 2003). A smaller fraction of the population (up to 10%) consisted of archaea. These belonged to the order *Methanosarcinales* and were distantly related to *Methanosaeta* and ANME-II (anaerobic methanotrophs) (86-88 % 16S rRNA gene identity). Labelling experiments and $\delta^{13}C$ measurements indicated that both these microorganisms were involved in the anaerobic oxidation of methane, although the bacterial lipids were more strongly labelled (Raghoebarsing et al., 2006). On this basis, it was hypothesized that the process was performed via reverse methanogenesis by an "ANME archaeon" with electron shuttling to a denitrifying bacterium.

Denitrification is the reduction of nitrate and nitrite to nitrous oxide and dinitrogen gas (Payne, 1973; Knowles, 1982). Many bacteria and archaea have the potential to denitrify (Philippot, 2002), and numerous organic and inorganic compounds can be used as an electron donor for denitrification. Although methane is a favourable electron donor for

both nitrate and nitrite reduction (-765 and -928 kJ/mol CH_4, respectively; Raghoebarsing et al. 2006), experimental evidence for its completely anaerobic oxidation was only found recently.

Though energetically much less favourable, sulphate-dependent AOM has been known for decades from marine sediment profiling and ^{14}C-CH_4 incubation studies (reviewed in Valentine and Reeburgh, 2000; Strous and Jetten, 2004). The hypothesis, that it involves methanogens operating in reverse (Zehnder and Brock, 1980) was supported by inhibition studies with the methanogenic inhibitor bromoethane sulfonate (BES; Hoehler et al., 1994), and later by the finding that AOM was performed by methanogen-like Archaea (ANME) living in close association with sulphate-reducing delta-Proteobacteria (Hinrichs et al., 1999; Boetius et al., 2000). Until now, occurrence and environmental significance of AOM has been demonstrated in numerous sulphate-rich environments, e.g. deep marine gas hydrates (Michaelis et al., 2002) and cold seeps (Orcutt et al., 2005), shallow marine sediments (Treude et al., 2005), terrestrial (Alain et al., 2006) and marine mud volcanoes (Niemann et al., 2006). In all cases, the presence of one ore more of the methane-oxidising euryarchaeal groups, ANME-I, -II or -III, and the use of sulphate as electron acceptor have been shown. Even though the overall process stoichiometry and the involvement of the ANME archaea are well understood, the biochemical mechanism of sulphate-dependent AOM is still unclear. Environmental genomic studies of ANME archaea support the dominant view, that the archaeal partner carries out reverse methanogenesis, but not sulphate reduction (Hallam et al., 2004), and that electrons are shuttled to a sulphate-reducing bacterium (SRB). Recently, it was suggested that methanethiol is the archaeal product serving as electron donor for the SRB (Moran et al., 2007). On the other hand, a general role of the SRB is still debated, since ANME archaea are sometimes found in the absence of or without close physical association with the SRB (Orphan et al., 2002; Orcutt et al., 2005; Treude et al., 2005).

In reverse methanogenesis, the methane-activating step is bioenergetically the most difficult, and is thought to be performed by a modified methyl-coenzyme M-reductase (MCR) operating in reverse (Hallam et al., 2004). MCR contains the nickel-cofactor F_{430} (Friedmann et al., 1990), and the only MCR from methanotrophic archaea characterized so far revealed a modification in this otherwise very conserved methanogenic cofactor: MCR purified from a microbial mat naturally enriched in ANME-I archaea had a cofactor F_{430} with an increased molecular mass (Krüger et al., 2003). Therefore, it was speculated that, together with the existence of a unique cysteine-rich sequence at the active site region, this modification might change the catalytic properties of the enzyme (Shima and Thauer, 2005). Recently, it was found that MCR from ANME-2 archaea harbors the normal F_{430} and it does not contain such a cysteine-rich sequence (A. Meyerdierks, S. Shima, J. Kahnt & M. Krüger, unpublished results; cited by Thauer & Shima, 2008). The discovery of another group of putative ANME archaea in the consortium oxidizing

methane under denitrifying conditions (Raghoebarsing et al., 2006) posed the question, if their characterization would shed light on the biochemical mechanism of AOM.
In the present study, the enrichment culture described previously was scaled up and continued, providing more biomass to support the experiments of the present study. The MCR-gene was partially amplified and the mass of the MCR-cofactor F_{430} was determined. Interestingly, while the activity of the culture increased, the archaeal population declined over time until it was no longer detectable, with the "NC10" bacterium remaining the dominant microorganism in the enrichment. The culture still oxidized methane anaerobically, and this activity was not inhibited by BES, a specific inhibitor of MCR. We conclude that AOM coupled to denitrification proceeded by a biochemical mechanism other than reverse methanogenesis and an archaeal partner was not required.

Results

Development of a microbial community that couples the AOM to denitrification in a continuous culture l A 16 l bioreactor with an external settler for biomass retention was inoculated with biomass from the 1.5 l enrichment culture described by Raghoebarsing et al. (2006). Fluorescence in situ hybridization (FISH) showed that upon inoculation the population was dominated by a bacterium affiliated with the phylum "NC10" (approximately 80 %) and an archaeon of the order *Methanosarcinales* (approximately 10 %). Medium containing nitrite and nitrate was supplied continuously, and the activity of the culture was monitored daily by the measurement of nitrite concentration (Figure 1). For a test phase of 2 months, nitrite was omitted, but methane-oxidizing activity with nitrate alone was very low (data not shown). After that period, nitrite was again added with the medium. It was clearly preferred as an electron acceptor over nitrate, which was only consumed at a rate less than 0.08 mmol d^{-1} in the presence of nitrite. Nitrite-reducing activity remained fluctuating around only 3.5 mmol day^{-1} and showed no general upward trend for more than a year. This indicated either inhibition by products of the microorganisms or the deficiency of an unknown growth factor. For that reason the medium flow rate was varied (to increase the washout of potentially toxic by-products of the process), and the following additions to the medium were tested: 0.1 mM NH_4Cl (after 11 months, as an alternative nitrogen source), 0.1 mM cysteine (after 13.5 months, as an alternative sulphur source), 1 ml l^{-1} vitamin solution (after 14 months, according to Balch et al., 1979) and 0.3 g l^{-1} sea salt (after 15 months, as a complex mixture of potentially missing trace elements). The additions were included in the medium preparation from the specified time onward, but did not show clear effects on overall activity (Figure 1). After 17 and 19 months, 450 ml anaerobic, filtered, but not sterile interstitial water from freshwater ditch sediment was added, and activity

increased. Presence of electron donors for nitrite reduction in the interstitial water as a cause for this positive effect can be excluded, since no stimulation of denitrifying activity was detectable in batch incubations without methane (data not shown)

Monthly FISH analysis of the culture showed that at all times at least 70 % of the biomass belonged to bacteria of the "NC10" phylum, which had already been prevalent in the inoculum (Figure 2a-c). These bacteria were thin rods, approximately 1 µm long and 0.3 µm wide, with the DNA concentrated in the centre of the cell as judged by the localization of the DNA-binding DAPI stain (4,6-diamidino-2-phenylindole, pictures not shown). Also, in the first 10 months, the archaea of the order *Methanosarcinales* found in the primary enrichment remained enriched to up to 10 % of cells (Figure 2a). These coccus-shaped cells were always present in round clusters of 3-6 µm diameter. After 10 months, they gradually became less abundant, and their fluorescence less bright. After 12 months, they could only occasionally be found with FISH (<1 % of cells), and were no

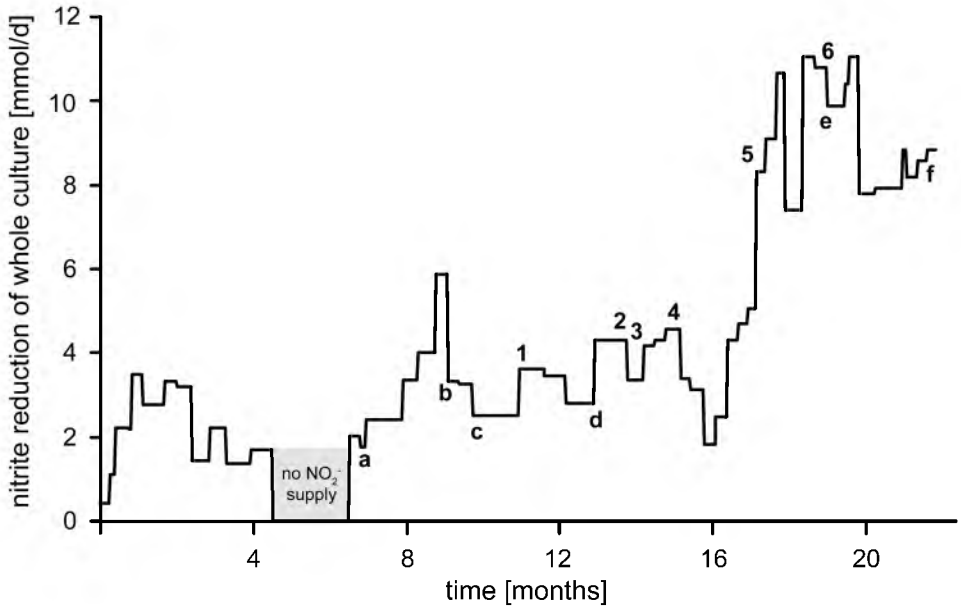
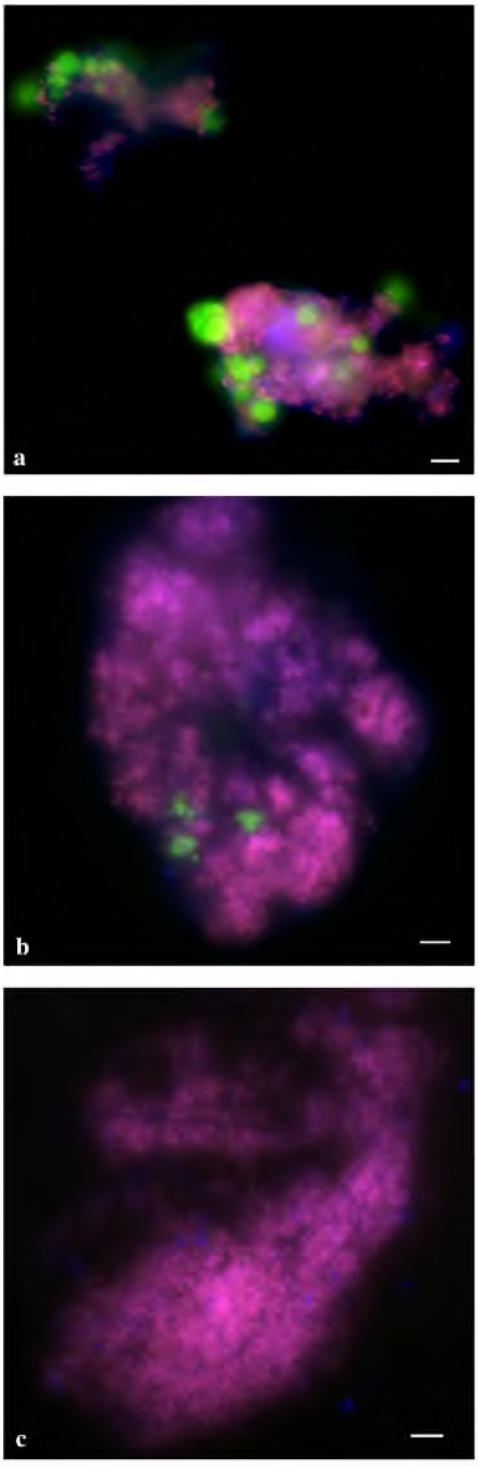


Figure 1 | Development of nitrite-reducing activity in the continuous culture. Numbers indicate additions or modifications of the medium, letters indicate experiments described in the text. During the period indicated by shading, no nitrite, but only nitrate was supplied to the reactor.
1: addition of 0.1 mM NH_4Cl to the medium; 2: addition of 0.1 mM cysteine to the medium; 3: addition of 1 ml/l vitamin solution (Balch et al., 1979) to the medium; 4: addition of 0.3 g/l sea salt; 5: addition of 450 ml interstitial water, 6: second addition of 450 ml interstitial water.
a: FISH analysis (Figure 2a); b: DNA extraction for mcr*A* amplification; c: qualitative MALDI-TOF analysis of cofactor F_{430} (Figure 3); d: FISH analysis (Figure 2b) and BES inhibition experiment (Figure 5); e: FISH analysis (Figure 2c, archaea not detectable) and quantitative MALDI-TOF analysis of cofactor F_{430} (Figure 4); f: whole-culture batch experiment (Figure 6).



longer detectable from month 15 onwards (Figure 2b & c).

mcrA gene and cofactor F430 detection and quantification I After 9 months, when the archaeal population still accounted for about 10% of the biomass, PCR-amplification and phylogenetic analysis of a 760 bp fragment of the *mcrA* gene that encodes the α-subunit of the MCR, revealed the presence of two similar genes (95.6 % identity on amino acid (aa) level). They formed a monophyletic group together with some environmental sequences (87- 99 % identity on aa level) within the *Methanosarcinales*, and were distantly related to both the *mcrA* group e supposedly corresponding to ANME-IIa (70 - 75 % identity; Hallam et al., 2003; Alain et al., 2006) and cultivated *Methanosaeta* and *Methanosarcina* species (64 - 70 % identity on aa level).

After 10 months MALDI-TOF analysis of biomass from the enrichment culture revealed the presence of the cofactor F_{430} associated with the methanogenic enzyme methyl-coenzyme M reductase (MCR). Of the two known forms, only the well

Figure 2 I Fluorescence in situ hybridization of biomass from the bioreactor at different times: **a)** after 7 months **b)** after 13 months **c)** after 19 months of enrichment. Epifluorescence micrographs taken with 1000x magnification after hybridization with probes S-*-DBACT-0193-a-A-18 or S-*-DBACT-1027-a-A-18 (Cy3, red), probe S-D-Arch-0915-a-A-20 (Fluos, green) and a mixture of EUB I-III & V (Cy5, blue). "NC10"-bacteria appear pink due to double hybridization with both specific and general bacterial probes. Scalebar = 5 µm.

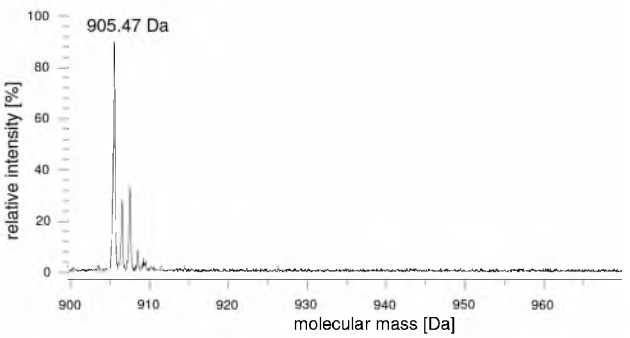


Figure 3 I MALDI-TOF mass spectrum of biomass from the enrichment culture after 10 months showing the presence of cofactor F_{430} with a monoisotopic molecular mass of 905 Da.

described variety from methanogens with a molecular mass of 905 Da (Friedmann et al., 1990) was found, and not the heavier modification (951 Da, Krüger et al., 2003) associated with ANME I archaeal methanotroph communities (Figure 3).

After 19 months, the aforementioned method failed to detect cofactor F_{430}. By applying ^{15}N-labelled F_{430} as an internal standard, the concentration of F_{430} was determined to be less than 0.67 pmol mg^{-1} (protein) (Figure 4). Assuming that the archaea in the culture had a methyl coenzyme M-reductase with a molecular weight of 303 kDa and with two molecules of F_{430} bound to it (Friedmann et al., 1990), and MCR making up 10% of cell protein (Rouviere and Wolfe, 1987), it can be concluded that they accounted for less than 0.1 % of whole culture protein.

Inhibition experiment with bromoethane sulfonate (BES) I The enrichment culture responded very sensitively to manipulations, and simple batch incubations after transfer to culture flasks (40-80 ml liquid volume in 60-120 ml serum bottles capped with black butyl rubber stoppers, with and without pH buffer) showed very little (<10 % of continuous culture) or no activity (data not shown). Reproducible results, with activities in the same range as the continuous culture, were obtained after pumping biomass from the enrichment culture into 300 ml subculture bottles (see experimental procedures). The addition of BES (final concentration 10 mM) to a subculture oxidizing

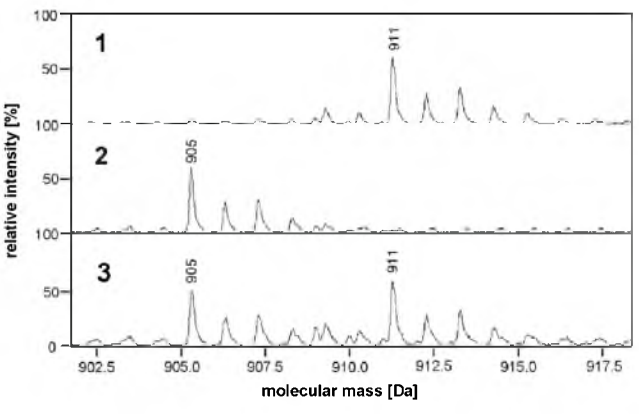


Figure 4 I Estimation of the F_{430} content of concentrated cell suspension (6 mg (protein) ml^{-1}) using ^{15}N-labelled F_{430} as an internal standard. **(1)** **F**ully ^{15}N-labelled F430; **(2)** cell suspension without added standard; **(3)** cell suspension with 2 nM ^{15}N-labelled standard. Peak height comparison shows that the sample contains ca. 2 nM F_{430}.

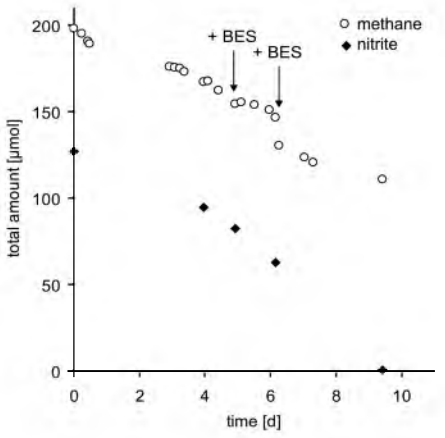


Figure 5 I Lack of inhibition by BES addition on the consumption of methane and nitrite in a 220 ml subculture after 13 months of enrichment. Arrows indicate the addition of BES to final concentrations of 10 and 20 mM, respectively. Nitrite was measured by HPLC using the conductivity detector, methane with a GC-FID.

methane and reducing nitrite had no measurable effect on the activity, neither had another addition of the same dose (Figure 5).

Demonstration of activity of the culture after disappearance of archaea I The activity of the whole culture did not decrease after the apparent disappearance of the archaea, but nitrite reduction reached a level on average nearly 3 times higher than before (9.1 ± 1.3 mmol nitrite reduced per day; Figure 1, months 18-22). A batch experiment with the whole culture after 22 months demonstrated the simultaneous consumption of methane and the reduction of nitrite and nitrate to dinitrogen gas and minor amounts of N_2O in the total absence of oxygen (Figure 6). The consumption of nitrite and nitrate was matched by the recovery of gaseous nitrogen compounds, indicating that no significant amount of air could have entered the system. Methane and nitrite reduction rates were 1.7 and 3.7 nmol $min^{-1} \cdot mg^{-1}$ (protein), respectively.

Discussion

The key conclusion of the presented data is that, unlike previously hypothesized, archaea are not essential for the anaerobic oxidation of methane with nitrite as electron acceptor. Both the lack of inhibition by the specific inhibitor BES and the disappearance of the archaea from the culture (as demonstrated by several independent methods) point to a biochemical mechanism other than the symbiosis of a 'reverse methanogen' with a denitrifying bacterium. In reverse methanogenesis, methyl-coenzyme M reductase (MCR) is thought to be the methane-activating enzyme (Krüger et al., 2003; Hallam et al., 2004). Although failure of the methyl-coenzyme M analogue BES to inhibit AOM has been observed in two previous studies, one with marine sediments (Alperin and Reeburgh, 1985) and one with lake water (Iversen et al., 1987; no inhibition of methanogenesis by 20 mM BES either), most studies do report a potent inhibition at

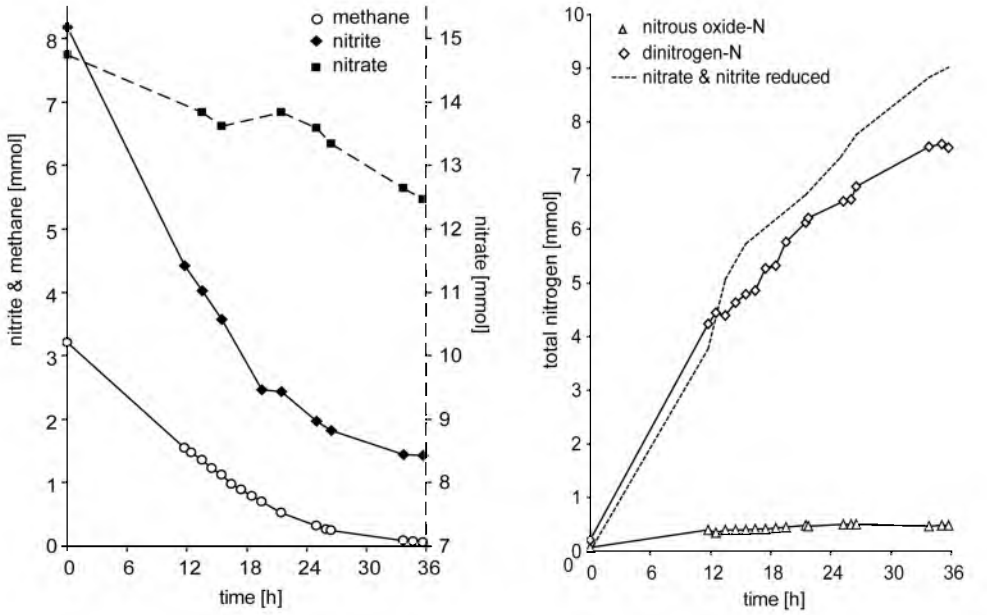


Figure 6 | Coupling of methane oxidation and denitrification in the enrichment culture after 22 months of enrichment. At the start of the experiment, concentrations of methane, nitrite and nitrate in solution were 0.39, 0.63 and 1.13 mM; total amounts in liquid and headspace together are given in the figure. The culture contained 1130 mg protein in 13 l (± 40 mg). **Left:** Consumption of methane, nitrate and nitrite. Nitrate and nitrite were separated using HPLC and quantified by a UV detector. **Right:** Production of dinitrogen and nitrous oxide gas and calculated amount of nitrite and nitrate reduced.

concentrations of 1-5 mM BES (Zehnder and Brock, 1980; Hoehler et al., 1994; Nauhaus et al., 2005). In long-term sediment incubations, degradation of BES by SRB can easily explain a lack of inhibition. However, in the present study degradation could be excluded, since BES was visible and stable on the HPLC chromatograms used to determine nitrate and nitrite. Additionally, the BES solution was tested on *Methanosarcina barkeri* cultures growing on methanol, with 20 mM final concentration resulting in an immediate and total inhibition of methane production.

The lack of inhibition is in line with the gradual disappearance of the archaea from the culture, as shown by FISH (Figure 2). The accuracy of FISH counts was constrained by growth of microorganisms in dense cell clusters, small size of the "NC10" bacterial cells and patchy distribution of archaeal cell clusters. It was nevertheless obvious that archaea became increasingly rare and, finally, apparently extinct, while the "NC10" bacteria remained the dominant phylotype in the culture. To back these results, the essential cofactor F_{430} of MCR was quantified at the time when no archaeal cells could be detected anymore by FISH. The MALDI-TOF-method using ^{15}N-labeled F_{430} as internal

standard did reveal minute amounts of F_{430} in the culture (< 0.67 pmol mg^{-1} protein). However, assuming typical amounts of MCR in archaeal cells these values corresponded to <0.1 % of protein originating from methanotrophic or methanogenic archaea. Since F_{430} is a very stable molecule that could persist in dead cells trapped in biofilm aggregates (Diekert et al., 1980; Pfaltz et al., 1982), the value of less than 0.1% might still be an overestimate. The decline of the archaeal population was accompanied by an increase in the nitrite-consumption rate of the culture (Figure 1), making it clear that bacteria alone were responsible for the observed activity. Although methane consumption could not be monitored continuously, the high methane consumption rate measured in the whole-culture batch experiment (see below) indicated that nitrite consumption did not become uncoupled from methane oxidation after the decline of the archaeal population. This experiment, performed after 22 months, clearly showed the simultaneous disappearance of methane and nitrite and the formation of dinitrogen gas (Figure 6). The oxygen concentration was always under the detection limit of the electrode used, and even if this could be due to its rapid consumption, the diffusion of a significant amount of air into the culture could still be excluded, as this would have been apparent from an increase in dinitrogen gas concentration. Opposed to that, dinitrogen gas development (7.5 mmol N_2-N) was fully accounted for by nitrite and nitrate reduction (9 mmol).

Nitrite was preferred over nitrate as an electron acceptor both in the continuous culture and in the above experiment, consistent with the observations of Raghoebarsing et al. (2006). In the linear phase of the batch experiment (21 h) the ratio of NO_2^- to NO_3^- consumption was 5:1. If all the nitrite reduction in this period (5.75 mmol) was coupled to methane oxidation according to the following equation,

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O \quad (1)$$

then nitrite accounted for 80 % of total methane oxidation (2.68 mmol). The remaining 20 % of methane oxidation could be explained by nitrate reduction (1.1 mmol) with the following stoichiometry

$$5\ CH_4 + 8\ NO_3^- + 8\ H^+ \rightarrow 5\ CO_2 + 4\ N_2 + 14\ H_2O \quad (2)$$

leaving 0.35 mmol NO_3^- consumption uncoupled to methane oxidation. This denitrification might be due to decay processes leading to internal production of substrates for denitrifiers or due to contamination of the demineralised water and chemicals used in the medium with trace amounts of organic electron donors. Measurement inaccuracies also could be a possible explanation, since nitrite and nitrate consumption together account for slightly more nitrogen than recovered as N_2 and N_2O (Figure 6).

Although it is clear from the presented data that archaea are dispensable for AOM with nitrite as electron acceptor, their role in the initial enrichment is still an intriguing question. Both methanogenesis and reverse methanogenesis at the high redox potential of denitrifying conditions are surprising given the requirement of very reduced conditions (around -600 mV intracellularly) for the functioning of the MCR (Shima & Thauer, 2005). It could still be possible that the archaea in the initial enrichment (Raghoebarsing et al., 2006) did oxidize methane in syntrophy with denitrifying bacteria (not necessarily the dominant "NC10" phylotype) or without a partner. This possibility was supported by the facts that they were enriched over a period of 16 months and that archaeal lipids were clearly depleted in ^{13}C (-67‰) in comparison with the supplied methane (-40.7‰). In this case, their disappearance would likely be due to a lost competition with the dominant bacteria after change from the sediment-containing sequencing batch reactor used initially to the continuous culturing-system described in the present paper. Also the additions to the medium (NH_4Cl, cysteine, vitamins and sea salt), though not sufficient to influence overall activity, might have shifted the competitive advantage towards the bacteria. On the other hand, the archaea may have been methanogenic, as the ^{13}C-CH_4-labelling experiment performed with the initial enrichment culture (Raghoebarsing et al., 2006) suggested. In that experiment the archaea showed only a minor incorporation of ^{13}C from methane into archaeal lipids (+18 ‰ within 6 days), whereas some bacterial lipids were strongly labelled (+4426 ‰). The small amount of ^{13}C incorporated into archaeal lipids might result from using waste products, intermediates or dead cells of bacteria as substrates for methanogenesis. It has since been suggested (Thauer and Shima, 2006) that the low affinity constant for methane reported there (< 0.6 µM) was not consistent with MCR being the methane-activating enzyme accounting for the bulk of methane oxidation. A low affinity constant was confirmed in the present study. From the data presented here, an affinity constant for methane of approximately 5 µM could be determined, which is in the same order of magnitude as in field studies reporting circumstantial evidence for the occurrence of AOM coupled to denitrification in an anoxic aquifer (9 µM, Smith et al., 1991). In contrast, the affinity of AOM with sulphate as electron acceptor was in the mM range (Nauhaus et al., 2002), consistent with a different initial enzymatic step.

When it was discovered that the MCR-cofactor F_{430} of the methanotrophic ANME-I archaea had an unusual mass (951 vs. 905 Da, Krüger et al., 2003), it was speculated that this modification was involved in the reversal of the enzyme function, from methane formation to methane activation. The archaea from the culture described here did not have this modified cofactor F_{430}, but the conventional, 'methanogenic' type. Circumstantial evidence, however, indicates that also ANME-II archaea harbour the conventional version (A. Meyerdierks, S. Shima, J. Kahnt & M. Krüger, unpublished, reported in Thauer & Shima, 2008). Therefore the type of cofactor does not seem to be

of predictive value for the enzyme function. Also, neither of the *mcrA* sequences obtained in this study could be clearly assigned to a group of well-characterized methanogens or methanotrophs. Instead, together with environmental sequences, they formed their own distinct group that is slightly closer related to the *mcr* group e supposedly belonging to ANME-IIa (Hallam et al., 2003; Alain et al., 2006) than to the methanogenic genera *Methanosarcina* and *Methanosaeta*, corresponding to the 16S rRNA gene-derived phylogeny (Raghoebarsing et al., 2006). Recently, first physiological data were reported for members of a group of environmental *mcr A* sequences (e.g. EU276007) clustering with the sequences presented in this study. These organisms, enriched from Tibetan wetlands, were shown to be versatile methanogens, able to use methanol, acetate, hydrogen and TMA as substrates (Zhang et al. 2008).

Unfortunately, due to their disappearance from the enrichment culture, the physiology of the archaea found in the earlier stages of the culture remains elusive. However, the cultivation set-up described in the present study proved successful to sustain and increase the biomass of bacteria presumably oxidizing methane without a partner. This opens up the way to explore the biochemical mechanism employed by these bacteria.

Materials and methods

Enrichment of microorganisms

Microorganisms were grown in continuous culture in a 16 l glass bioreactor (Applikon, The Netherlands; liquid volume 13 l) inoculated with biomass from an enrichment culture anaerobically oxidizing methane (Raghoebarsing et al., 2006). The culture was stirred at 200 rpm, the temperature was controlled at 30 °C and medium was continuously supplied (0.2-0.8 l day^{-1}). The liquid volume in the culture vessel was maintained by a level-controlled effluent pump, and biomass was retained within the system by an external settler (Figure 7). The medium contained (per liter): $KHCO_3$ 1.25 g, KH_2PO_4 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $FeSO_4$ 0.0025 g, Na-EDTA 0.0025 g, $NaNO_3$ 0.255 g (3 mM), trace element solution 0.5 ml. The trace element solution contained (g l^{-1}): EDTA 15, $ZnSO_4 \cdot 7H_2O$ 0.43, $CoCl_2 \cdot 6H_2O$ 0.24, $MnCl_2 \cdot 4H_2O$ 0.99, $CuSO_4$ 0.25, Na_2MoO_4 0.242, $NiCl_2 \cdot 6H_2O$ 0.19, SeO_2 0.067, H_3BO_3 0.014 and $Na_2WO_4 \cdot 2H_2O$ 0.050. After one year of enrichment the EDTA in medium and trace element solution was omitted and trace metals and iron were supplied in an acidic solution containing Fe, Zn, Co, Mn, Cu, Ni, B and an alkaline solution containing Se, W and Mo at the concentrations specified above. The medium was continuously sparged with a mixture of Ar/CO_2 (95:5). The culture was continuously sparged with CH_4/CO_2 (95:5 vol/vol, purity >99.995 %, Air Liquide, The Netherlands) at a flow rate of 10 ml min^{-1} as carbon and energy source and to maintain anaerobiosis. The CO_2 concentration was sufficient to buffer the culture liquid in a pH range of 7.3-7.6 as monitored by a pH electrode. Absence of oxygen was monitored by a Clark-type oxygen electrode. To prevent growth of photosynthetic organisms, the culture vessel was wrapped in black foil and black tubing with low oxygen permeability (Viton and Norprene, Cole Parmer, USA) was used. Nitrite concentration in the culture was maintained between 0 and 1 mM by varying

the flow rate (0,2-0.8 l d^{-1}) and the nitrite concentration (3-15 mM) of the influent medium, dependent on the nitrite-reducing activity of the culture. For 2 months (Figure 1), nitrite was omitted and medium containing only 10 mM nitrate as electron acceptor was supplied, but as methane-oxidizing activity in a batch test was much slower with only nitrate compared to with nitrite (data not shown), the medium was again prepared with nitrite as the main electron acceptor. Further modifications of the medium composition are specified in the results sections. The demineralized water for medium preparation was autoclaved, but the culture was not operated aseptically. Interstitial water was extracted by centrifugation from freshwater ditch sediment collected in the Ooijpolder, The Netherlands, filtered through a paper filter and made anaerobic by sparging with dinitrogen gas before addition to the reactor.

Fluorescence in situ hybridization (FISH)

Biomass (2-4 ml culture liquid) was harvested monthly from the enrichment culture, centrifuged, washed with phosphate-buffered saline (PBS; 10mM Na_2HPO_4/NaH_2PO_4 pH 7.2 and 130 mM NaCl) and fixed for 2-3 h with 3 % (w/v) paraformaldehyde in PBS on ice. After washing with PBS, samples were stored in PBS/ethanol (1:1) at -20 °C.

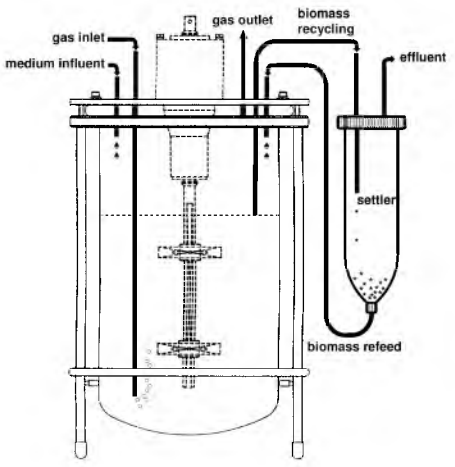


Figure 7 | Continuous-culture set-up with biomass retention in an external settler.

For microscopy, fixed biomass was spotted in 10 µl volumes onto the wells of teflon-coated microscope slides and embedded in 0.1 % agarose (w/v). After sequential dehydration in 50%, 80% and 96 % ethanol for 3 min each probes were hybridized for 1.5 h at 46 °C in hybridization buffer containing 900 mM NaCl, 2 mM Tris/HCl pH 8.0, 0.2 ‰ sodium dodecyl sulphate, 40 % formamide and a combination of the following oligonucleotide probes: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 (dominant bacteria affiliated with the "NC10" phylum; Raghoebarsing et al., 2006); a mixture of EUB I-III & V (most bacteria; Daims et al., 1999), S-D-Arch-0915-a-A-20 (most archaea ; Stahl and Amann, 1991). For image acquisition, a Zeiss Axioplan 2 epifluorescence microscope equipped with a CCD camera was used together with the Axiovision software package (Zeiss, Germany). Percentages of phylogenetic groups were estimated based on visual inspection of at least one complete well.

Amplification and phylogenetic analysis of the mcrA gene

After 9 months of enrichment, DNA was extracted from 2 ml of concentrated biomass withdrawn from the bottom of the external settler. A procedure based on phenol/chloroform/isoamylalcohol modified from Juretschko et al. (1998) was used. From the initial enzymatic steps described therein only a proteinase K digest was performed. An approximately 760 bp long fragment of the *mcrA* gene was PCR-amplified using the ME1&2 primers (Hales et al., 1996) with an annealing temperature of 55 °C. A PCR product of the correct length was cloned into *E. coli* with the pGEM T-easy cloning vector system (Promega). Plasmid DNA was isolated from overnight cultures of single white

colonies using the FlexiPrep kit (Amersham Biosciences) and digested with the EcoRI restriction enzyme. For each restriction type, two purified plasmid DNA samples were sequenced using both the M13 forward and reverse primers. Sequences were translated and aligned to close BLAST hits in BioEdit using the muscle algorithm (Edgar, 2004) followed by manual adjustments. For calculating the percentage of amino acid identities additional reference sequences were added to the alignment, and calculations were based on 157-260 positions. Sequences have been submitted to Genbank (accession numbers EU495303 and EU495304).

Analytical procedures

Nitrate and nitrate | Nitrite concentration in the enrichment culture was estimated daily with Merckoquant test strips (0-80 mg l^{-1}; Merck, Germany). Medium and culture liquid concentrations of nitrate and nitrate were occasionally verified by colorimetric measurements as described previously (Kartal et al., 2006). In batch experiments, nitrate and nitrite were measured with high performance liquid chromatography (HPLC) on a Hewlett Packard 1050 system. 10 µl of liquid sample were injected with an autosampler. A 30 mM sodium hydroxide solution was used as the liquid phase at a flow rate of 1.5 ml min^{-1}. Anions were isocratically separated on a 4×250 mm Ionpac AS11-HC (Dionex, UK) column at 30 °C and detected using the built-in UV detector or a CD25 conductivity detector (Dionex, UK).

Gaseous nitrogen compounds and methane | Gas samples of 100 µl volume were withdrawn with gas-tight glass syringes (Hamilton, Switzerland) through a rubber septum on top of the reactor or the incubation bottle, respectively. Dinitrogen gas and nitrous oxide were measured on an Agilent 6890 series gas chromatograph (Agilent, USA) equipped with a Porapak Q column, a molecular sieve (Hewlett Packard, USA) and a thermal conductivity detector working at 200 °C. The carrier gas was helium at a flow rate of 21 ml min^{-1}. The injection temperature was 125 °C, the oven temperature 65 °C. The inlet area of the GC was flushed with helium to minimize entry of N_2 from air.

Methane was measured on a HP 5890 gas chromatograph equipped with a Porapak Q column (80/100 mesh) and a flame ionization detector (Hewlett Packard, USA). The carrier gas was helium at a flow rate of 28 ml min^{-1}. The injection and detection temperature was 150 ° C, the oven temperature 120 °C. The gases were quantified by comparing the peak areas to those of a commercial calibration gas mixture (Air liquide, precision ± 2 %) and self made standards.

Total protein | Total protein content was measured after sonication or bead-beating of 2-4 times concentrated cell suspensions with the bicinchoninic acid (BCA) assay (Pierce, USA), according to the manufacturer's instructions. Briefly, the Biuret method producing Cu^{+1} ions is combined with the subsequent reaction of these ions with BCA yielding a colored complex colorimetrically detectable at 562 nm. Bovine serum albumine was used as a standard.

Identification and quantification of cofactor F430 | After 10 months of enrichment, 2 ml culture liquid were pelleted at 10 000 *g* and resuspended in 50 µl matrix solution containing 10 mg ml^{-1} α-cyano-4-hydroxy-cinnamic acid in a 50:50 mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid (Farhoud et al., 2005). The mixture was spotted on a target plate and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Biflex III, Bruker, Germany).

After 19 months of enrichment, the above-mentioned method failed to detect cofactor

F_{430} anymore. In order to quantify trace amounts of cofactor F_{430}, highly concentrated culture liquid was spiked with ^{15}N-labeled cofactor F_{430} as an internal standard. Specifically, culture liquid was pelleted at 10 000 *g*, resuspended in water to a concentration of 6 mg ml^{-1} protein and mixed 1:1 with matrix solution (50% saturation of α-cyano-4-hydroxy-cinnamic acid in a solution of 70% acetonitrile, 30% H_2O and 0.1% trifluoroacetic acid (v/v)). The mixture was spotted on a target plate and analysed by MALDI-TOF MS (Ultraflex, Bruker, Germany). After ultrasonication for 5 min at room temperature, the mixture was supplemented with a MCR standard containing ^{15}N labeled F_{430} to final concentrations of 2, 4 and 8 nM and subsequently analyzed by MALDI-TOF MS. ^{15}N labeled cofactor F_{430} was extracted from *M. thermoautotrophicum* grown with $^{15}NH_4^+$ as sole nitrogen source. Incorporation of ^{15}N increases the molecular mass of cofactor F_{430} resulting in a main peak at 911 Da compared to 905 Da in unlabelled cofactor. Comparison of peak height allowed estimation of the maximum concentration of F_{430} in the sample.

Activity measurements

Inhibition experiment | Bromoethane sulfonate (BES, Sigma-Aldrich, Germany) inhibition experiments were carried out after 13 months of enrichment in a subculture consisting of a 300 ml glass bottle capped with a modified stainless steel plate with three ports, held in place by a screw cap. One port was closed with a septum allowing gas-tight sampling of the headspace, the other two were connected to Norprene tubes (Cole-Parmer, USA) reaching into the bottle for liquid supply and withdrawal. After being made anoxic by thorough flushing with a mixture of Ar/CO_2 (95:5), culture liquid from the enrichment culture was being pumped through at a flow rate of 17.5 ml min^{-1}. The gas headspace of the bottle was set at 80 ml by the height of the effluent tube. The subculture bottle was stirred by a magnetic stirrer and kept at 30 °C. After ca. 24 h (accounting for at least 110 volume changes) it was disconnected from the main culture vessel by closing the influent and effluent tubes. By flushing with Ar/CO_2 (95:5) methane concentration in the headspace was decreased to about 6 %, and gas samples of 100 µl were measured 3-4 times daily. Once a day a 1 ml liquid sample was withdrawn, centrifuged and the supernatant stored at -20 °C until analysis of nitrite. After 119 and 148 h BES was added from anaerobic 2 M stock solution to final concentrations of 10 and 20 mM, respectively. The data shown are from one experiment, two separate ones carried out yielded essentially the same result.

Whole culture batch experiment | To measure methane consumption and development of gaseous nitrogen compounds in the continuous culture, medium and gas supply were stopped and headspace methane concentration decreased to ca. 2.5 % by flushing with Ar/CO_2 (95:5). At each sampling time, headspace gas samples of 100 µl each were taken for immediate analysis by gas chromatography (see above), and 1 ml liquid sample was centrifuged and the supernatant stored at -20 °C until analysis. Assuming that liquid and gas phase were in equilibrium, the total amount of gaseous compounds in the culture vessel was calculated based on the measured headspace concentration and the solubility coefficients for methane, nitrous oxide and dinitrogen gas at 30 °C in water (Wilhelm et al., 1977). Rates of methane and nitrite consumption were calculated from the linear part of the respective graphs (11.75 - 19.5 h).

Acknowledgements

We are grateful to Ashna Raghoebarsing for initial work on the described setup and for providing biomass for inoculation. We would like to thank Jan Keltjens, Boran Kartal and Rolf Thauer for helpful discussions, Wouter Wanders and John Hermans for assistance with HPLC measurements and Margaret Butler for critically reading the manuscript. K.F.E. and M.S. were supported by a VIDI grant from the Dutch Science Foundation (NWO).

Chapter 3

Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum

Published previously as:
Katharina F. Ettwig, Theo van Alen, Katinka T. van de Pas-Schoonen, Mike S.M. Jetten and Marc Strous (2009) in *Applied and Environmental Microbiology* **75** (11): 3656-3662.

Abstract

Anaerobic methane oxidation coupled to denitrification was recently assigned to bacteria belonging to the uncultured phylum NC10. In this study, we incubated sediment from a eutrophic ditch harboring a diverse community of NC10 bacteria in a bioreactor with a constant supply of methane and nitrite. After 6 months, fluorescence in situ hybridization showed that NC10 bacteria dominated the resulting population. The enrichment culture oxidized methane and reduced nitrite to dinitrogen gas. We assessed NC10 phylum diversity in the inoculum and the enrichment culture, compiled the sequences currently available for this bacterial phylum, and showed that of the initial diversity, only members of one subgroup had been enriched. The growth of this subgroup was monitored by quantitative PCR and correlated to nitrite-reducing activity and the total biomass of the culture. Together, the results indicate that the enriched subgroup of NC10 bacteria is responsible for anaerobic methane oxidation coupled to nitrite reduction. Due to methodological limitations (a strong bias against NC10 bacteria in 16S rRNA gene clone libraries and inhibition by commonly used stopper material) the environmental distribution and importance of these bacteria could be largely underestimated at present.

Introduction

Atmospheric concentrations of methane have risen 2.6-fold since preindustrial times (Intergovernmental Panel on Climate Change, 2007). After several years of stagnation, there was again a clear increase in the methane concentration in 2007 (Rigby et al., 2008). Currently, it is uncertain whether an increase in the number of sources and production or a decrease in the number of sinks and consumption is responsible for this reversal of the trend.

Freshwater habitats like natural wetlands and rice fields are a major source (38%; Intergovernmental Panel on Climate Change, 2001) of atmospheric methane. In the absence of other documented electron donors, aerobic methane oxidation is assumed to be the most important sink in these habitats, but the role of alternative electron donors is not well understood (Kumaraswamy et al., 2001; Smemo & Yavitt, 2007). Anaerobic methane oxidation coupled to denitrification is energetically favorable, but evidence that it occurs is scarce. In marine, methane-containing sediments, nitrate and nitrite are usually not quantitatively important electron acceptors; in freshwater sediments the denitrifying and aerobic zones are in close proximity (Christensen et al., 1989; Sweerts & De Beer, 1989; Laverman et al., 2006), possibly masking the process from detection. To our knowledge, concomitant methane and nitrate profiles of sediments have never been published.

So far, methane oxidation coupled to denitrification has received the most attention in the field of hydrogeology. In groundwater, contamination with nitrate and nitrite occurs frequently, whereas electron donors are limiting. Methane plumes often form around landfills, and their attenuation has sometimes been attributed to denitrification (Bjerg et al., 1995; Van Breukelen & Griffioen, 2004). So far, a single previous study unambiguously demonstrated anaerobic oxidation of methane coupled to denitrification in a contaminated freshwater aquifer (Smith et al., 1991). The first in vitro observation of anaerobic methane oxidation coupled to denitrification came from a laboratory-scale sludge digestor (Islas-Lima et al., 2004). The use of a laboratory enrichment culture also eventually resulted in identification of the organisms involved; bacteria of the NC10 phylum and archaea of the order *Methanosarcinales* dominated a mixed culture carrying out anaerobic methane oxidation coupled to denitrification (Raghoebarsing et al., 2006). This culture was enriched from a freshwater canal sediment after 1 year of continuous supply of methane and nitrite. Subsequently, the archaea were shown to be dispensable, as they disappeared after prolonged incubation of the same culture (chapter 2). Mass balance calculations showed that methane oxidation was coupled to the reduction of nitrite with a 3:8 stoichiometry, in accordance with theoretical expectations. The bacteria that dominated the mixed culture and apparently oxidized methane anaerobically are members of the NC10 phylum, one of the many phyla having no members in pure culture (Rappé & Giovannoni, 2003; Fuerst, 2007). The 16S rRNA gene sequences of such organisms, however, have been found in a number of environmental surveys of aquatic environments; e.g., the most closely related sequences have been found in aquifers (Bakermans & Madsen, 2002; Lopez-Archilla et al., 2007) and lake sediments (Koizumi et al., 2003; Kalyuzhnaya et al., 2008). Sequence similarity and phylogenetic affiliation may indicate similar metabolic capacities of organisms, but by itself this is not sufficient to infer similar metabolism (Dedysh et al., 1998). This is especially true for denitrifying methanotrophs, because only a single enrichment culture has been described so far (chapter 2; Raghoebarsing et al., 2006).

The objective of the present study was to generalize the previous finding that NC10 bacteria were associated with anaerobic methane oxidation, a necessary step forward in addressing the significance of this poorly understood process as a methane sink in freshwater habitats.

Results and discussion

Using previously described FISH probes (Raghoebarsing et al., 2006) as primers in a nested PCR approach, we screened several freshwater sediments for the presence of phylum NC10 sequences. Of those, sediments from a ditch draining agricultural land in a

Table 1 | Primers and PCR conditions for the 16S rRNA gene libraries.

clone library	forward primer		reverse primer		annealing temp. (°C)	amplicon positions[4]
		Sequence (5'–3')		Sequence (5'–3')		
Ino-nFR	202F[1]	GAC CAA AGG GGG CGA GCG	1043Ra[1]	TCT CCA CGC TCC CTT GCG	69	202-1060
Ino-Ra	8F[2]	AGA GTT TGA TYM TGG CTC AG	1043Ra[1]	TCT CCA CGC TCC CTT GCG	57	8-1060
Ino-Rb	8F[2]	AGA GTT TGA TYM TGG CTC AG	1043Rb	TCT CCA CGT TCC CTT GCG	57	8-1060
Ino-F	202F[1]	GAC CAA AGG GGG CGA GCG	1492R[3]	GGT TAC CTT GTT ACG ACT T	57	202-1529
Enr-Ra	8F[2]	AGA GTT TGA TYM TGG CTC AG	1043Ra[1]	TCT CCA CGC TCC CTT GCG	59	8-1060
Enr-Rb	8F[2]	AGA GTT TGA TYM TGG CTC AG	1043Rb	TCT CCA CGT TCC CTT GCG	57	8-1060
Enr-F	202F[1]	GAC CAA AGG GGG CGA GCG	1492R[3]	GGT TAC CTT GTT ACG ACT T	60	202-1529

[1] Based on previously described FISH probes (Raghoebarsing et al., 2006)
[2] See Juretschko et al. (1998)
[3] See Lane (1991)
[4] The positions are based on the gap-free sequence of the NC10 bacterial clone D-BACT (GenBank accession no. DQ369742), starting at *E. coli* position 8.

floodplain of the River Rhine yielded an amplicon of the correct length. The sequences obtained after cloning (library Ino-nFR) (Table 1 and Figure 1) were related to the bacterium dominating an enrichment culture performing anaerobic methane oxidation coupled to denitrification (Raghoebarsing et al., 2006) and fell into two distinct groups (groups a and b) of the NC10 phylum. However, these bacteria were not detected in the sediment with FISH using the same probes. Because of the strong background fluorescence of sediment particles, only a few bacteria were visible, and no archaea could be detected. The sediments were subsequently used to inoculate an anaerobic continuous culture bioreactor. The culture was sparged with CH_4-CO_2 as the sole carbon sources and supplied with mineral medium containing nitrite and nitrate as electron acceptors. In the first 3 weeks, the nitrite-reducing activity in the culture decreased from 4.6 mmol day^{-1} to zero (Figure 2). After a 110-day period without measurable activity, nitrite reduction resumed, and methane oxidation activity, although still low, could be measured in a batch experiment with the whole culture from day 136 to day 142 (Figure2) (methane oxidation activity, 0.18 nmol min^{-1} mg $protein^{-1}$, as determined using previously described methods (chapter 2). Until day 217, nitrite reduction increased in a roughly linear fashion up to a maximum of 33.5 mmol day^{-1} (Figure 2). During this period, the nitrite concentration in the medium could be increased from 0.5 to 20 mM with nearly complete consumption by the culture. The $KHCO_3$ concentration

was decreased from 15 to 0.5 mM to compensate for H^+ consumption associated with denitrification. The stagnation of activity at values between 27 and 32 mmol day^{-1} starting around day 230 may have been caused by the removal of about 2.3 l of culture liquid with biomass (corresponding to ca. 1.1 g protein) for experiments over a 2-month period. In this period, the culture contained 4.75 ± 0.8 g protein, and the specific activity was 3.4 to 5.6 nmol NO_2^- min^{-1} mg protein.

Methane oxidation activity and its coupling to denitrification was further demonstrated in different batch experiments (Figure 3). Initially, all attempts to detect activity in serum bottles failed, which was also observed in a previous study (chapter 2). This was apparently due to the use of black butyl rubber stoppers, which caused total inhibition of the activity (Figure 3a). Anoxic handling of the biomass, in contrast, was not crucial; exposure to oxygen during transfer to the bottles did not lead to lower activity.

Repeated boiling of black stoppers in water and diluted HCl did not eliminate the

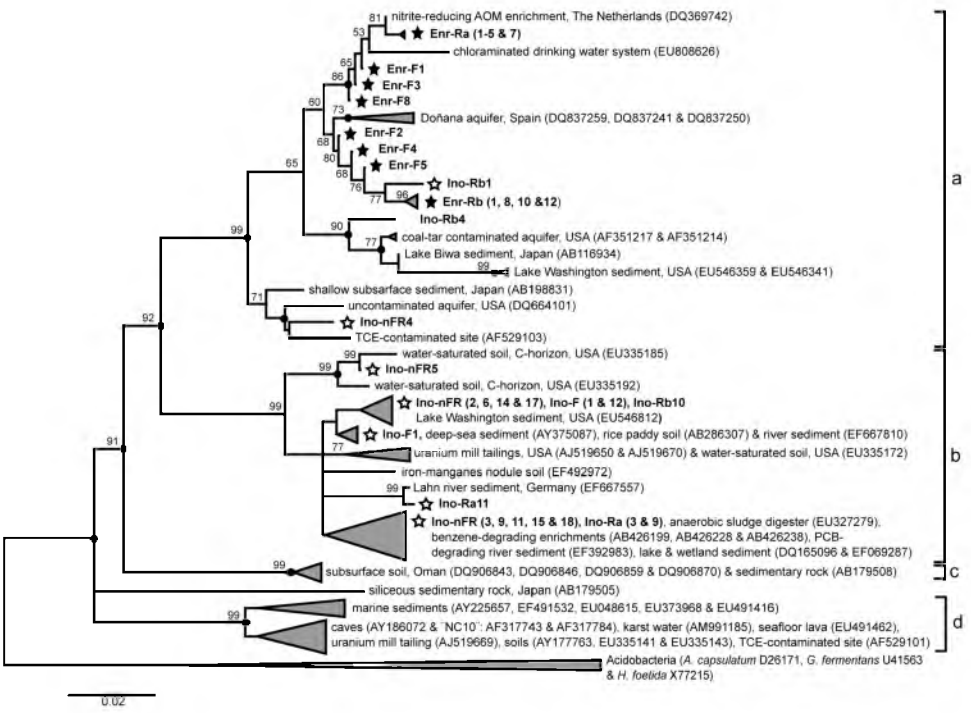


Figure 1 | Phylogenetic tree of the NC10 phylum with Acidobacteria as the outgroup. Sequences obtained from the inoculum material of the enrichment culture (libraries Ino-nFR, Ino-F, Ino-Ra & Ino-Rb) are indicated by an open star, those from the enrichment culture after six months (Enr-F, Enr-Ra & Enr-Rb) are indicated by a filled star. The tree was calculated with the minimum evolution method and the Tajima-Nei correction. Bootstrap support values >50 % (n = 1000) are indicated at the nodes, and branches supported by all treeing methods (see Material and Methods) are indicated with a circle.

inhibition. It is generally known that some aerobic methanotrophs can be inhibited by such stoppers, but for anaerobic methanotrophs this was completely unexpected. This observation makes it clear that in future studies of denitrification coupled to methane oxidation it is essential to choose a suitable stopper material. Using thick (ca. 0.8 mm) red butyl rubber stoppers manufactured for blood collection tubes or gray butyl rubber stoppers, we obtained specific activities of 1.6 to 2.2 nmol CH_4 min^{-1} mg $protein^{-1}$ and 4.7 to 5.1 nmol NO_2^- min^{-1} mg $protein^{-1}$ in small-batch incubations, which were indistinguishable from the values for the continuous culture (3.4 to 5.6 nmol NO_2^- min^{-1} mg $protein^{-1}$). The specific activities observed here also compare well with previously described activities of similar enrichment cultures (3.7 nmol NO_2^- min^{-1} mg $protein^{-1}$ (Raghoebarsing et al., 2006; chapter 2) and cultures of anaerobic, archaeal methanotrophs (1.7 nmol CH_4 min^{-1} mg $protein^{-1}$; Nauhaus et al., 2007). The observed stoichiometry of nitrite consumption versus methane consumption (8:3.5) was similar to

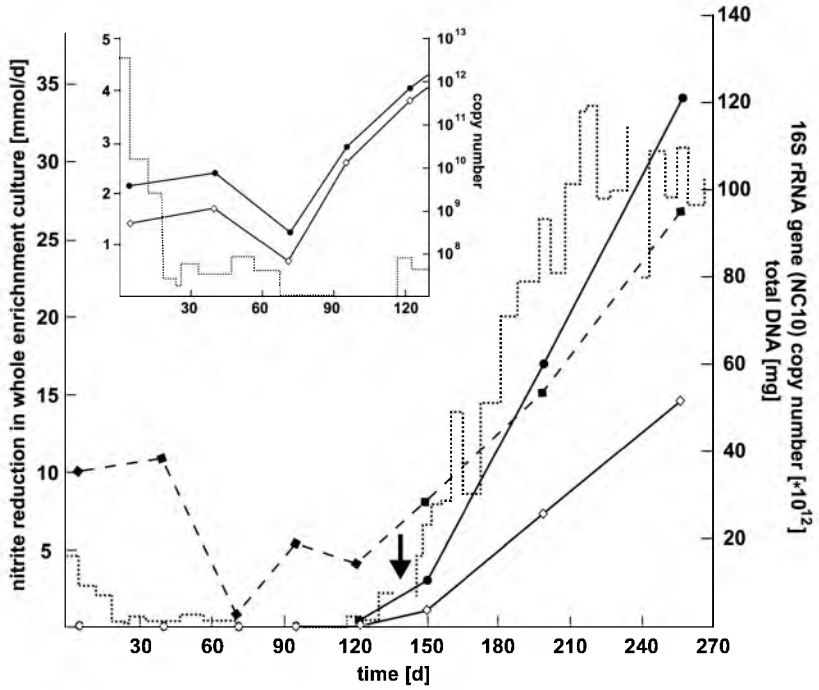


Figure 2 I Nitrite reduction (dotted line), DNA concentration (dashed line) and abundance of 16S rRNA genes of NC10 bacteria (solid lines) in the enrichment culture. The copy number of 16S rRNA genes of NC10 bacteria was assessed with the primer pairs qP1F/R (filled circles) and qP2F/R (open diamonds). The liquid volume of the culture was 10 to 13 l. From day 136 to 142 (arrow) the bioreactor did not receive medium and gas to assess methane oxidation activity. The insert shows a zoom on the first four months demonstrating the greater sensitivity of the qPCR approach as compared to activity measurements. At the end of the enrichment period (day 243) the culture contained 4.75 g protein (± 0.8 g).

the theoretical value, 8:3 (Raghoebarsing et al., 2006; chapter 2). No ammonium formation was detected (detection limit 20 µM).

After 5 months of enrichment, FISH was successful using the previously described probes targeting denitrifying methanotrophic bacteria of the NC10 phylum. The amount of bacterial biomass relative to the amount of sediment particles had strongly increased, decreasing the autofluorescence. Individual NC10 cells were visible in a matrix of other bacteria. One month later, the NC10 bacteria had become the dominant bacteria, and the level of enrichment was about 70% after 7 months. In the small rods (ca. 0.8 to 1 by 0.3 to 0.5 µm) the 4',6'-diamidino-2-phenylindole (DAPI) signal was concentrated in the center of each cell, and the cells occurred both as aggregates and as single cells (Figure 4). Archaea were not detected at any time, confirming that they are dispensable for the nitrite-reducing, anaerobic oxidation of methane (chapter 2).

Even though NC10 phylum bacteria already accounted for more than 50% of the population in the enrichment culture, they were not detected in a clone library (31 clones) obtained after 6 months with general 16S rRNA gene primers (primers 8F and 1545R; Juretschko et al., 1998). Instead, this library was dominated by uncultured *Acidobacteria* (11 clones) and *Chloroflexi* (10 clones, data not shown). In order to explore

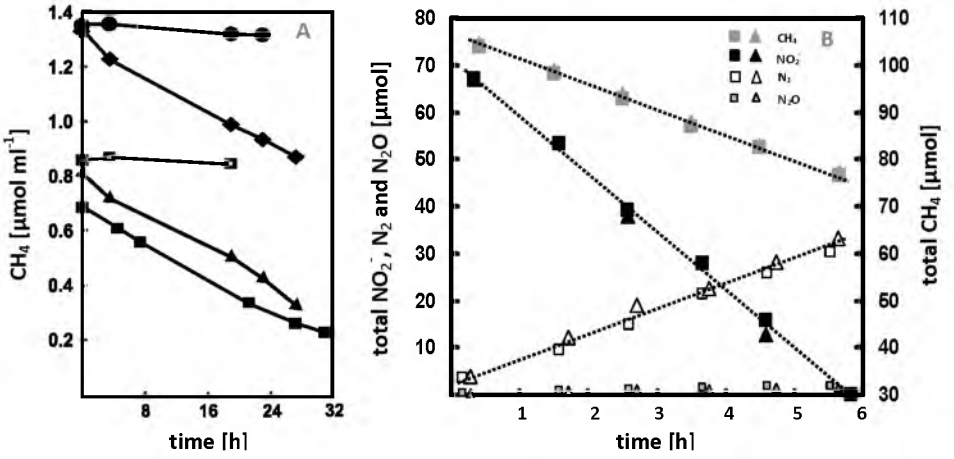


Figure 3 l a) Influence of stopper material on methane oxidation activity in the presence of nitrite and control for methane loss through alternative stopper material: Black (filled circles), red (filled squares) and gray butyl rubber (filled diamond: 40 ml biomass, filled triangle: 8 ml biomass, open square: water). The error margin of triplicate measurements is within the symbols. The experiments were carried out in duplicate yielding essentially the same result.

b) Methane oxidation and nitrite reduction to dinitrogen gas in duplicate batch incubations (40 ml two-fold concentrated biomass, 40 (±4) mg protein, gray butyl rubber stopper). Filled black symbols: ^{15}N-nitrite (average of triplicate measurements, error bars within symbols); gray symbols: methane (average of duplicate measurements); open symbols: ^{15}N-dinitrogen gas (single measurement); small, outlined symbols: ^{15}N-nitrous oxide (single measurement).

the diversity of NC10 bacteria, we used combinations of general bacterial 16S rRNA gene primers and specific primers (Table 1) with DNA extracted from the inoculum and the enrichment culture. With this approach, most sequences obtained were affiliated with the NC10 phylum. As expected, their diversity in the inoculum was much greater than that in the enrichment culture after 6 months. The sequences amplified from the inoculum represented two groups, *a* and *b* (Figure 1), but after 6 months only group *a* sequences were retrieved. This group also includes the strain previously enriched from the Twentekanaal (Raghoebarsing et al., 2006) and several sequences from other freshwater environments, e.g. the denitrifying zones of Lake Biwa sediments (Koizumi et al., 2003) and the Doñana aquifer (Lopez-Archilla et al., 2007) and methane-bearing sediment from Lake Washington (Kalyuzhnaya et al., 2008). Thus, it seems to be associated with anaerobic methane oxidation. Whether this is also the case for group *b*, many members of which were also found in the inoculum used, remains to be determined. Still, all sequences of the NC10 phylum known to date (Figure 1) come from aquatic, potentially anoxic, methane-bearing environments. Very likely, these bacteria are underrepresented in biodiversity surveys performed with general primers, since they were not detected with 16S rRNA gene clone libraries even when they made up the majority of the microbial community. The cause of this strong negative bias is unclear. Our results indicate that the general primers used did not have mismatches with the targeted sequences. Also, there was no overrepresentation of the nucleotides A and T at the priming sites binding to the wobble bases of the degenerate forward primer, another factor shown to decrease amplification efficiency (Polz et al., 1998). For the

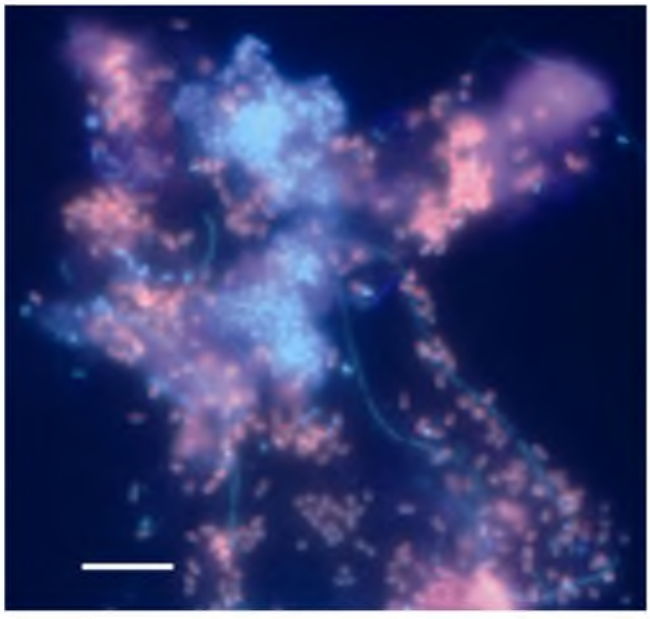


Figure 4 | Fluorescence in situ hybridization of biomass from the enrichment culture after seven months. The cells were hybridized with probes S-*-DBACT-1027-a-A-18 (Cy3, red) specific for NC10 bacteria (group a and partly b), a mixture of EUB I-III and V (Fluos, dark blue) detecting nearly all Eubacteria and the DNA stain DAPI (cyan). NC10 bacteria appear pink due to double hybridization with specific (red) and general (dark blue) probes. The DAPI signal is concentrated in the center of the cells. Essentially identical results were obtained with the probe S-*-DBACT-0193-a-A-18. Scale bar = 5 µm.

enriched group *a*, two primer pairs for qPCR were developed and used with samples collected throughout the enrichment procedure (Figure 2). In negative controls, the cycle threshold value was more than 40 cycles, whereas the cycle threshold value of all samples was less than 29. The melting curve for the PCR products from the first 2 months had several peaks, indicating that there was formation of multiple products. This can be attributed to the high concentration of nontarget DNA in these samples. The first two measurements, therefore, are upper estimates and are less reliable than quantifications after day 71, when only one peak was observed. Nevertheless, the reproducible, twofold difference between the two primer sets remains difficult to explain. Both primer sets had no mismatches with the template 16S rRNA gene sequence, and the PCR efficiencies of the dilution curves were identical (100%). Other authors have found differences of up to 26% even with the same primer set (Smith et al., 2006), illustrating the limits of the qPCR approach. Multiple primer sets, like those used in the present study, do not necessarily generate more reliable results, but they can provide a more realistic view of the uncertainties of this technique and underline the need to confirm the findings with other non-PCR-based methods. However, the growth of the target population could be demonstrated by qPCR approximately 1 month before its activity became detectable (Figure 2, inset). Assuming one or two copies of the 16S rRNA gene per cell (a realistic estimate for slowly growing bacteria; Klappenbach et al., 2000), the specific per-cell activity was approximately 0.09 fmol CH_4 day^{-1} $cell^{-1}$ (for one copy) or 0.18 fmol CH_4 day^{-1} $cell^{-1}$ (for two copies) assessed with qPCR primer pair qP1. With primer pair qP2, this value was 0.20 fmol CH_4 day^{-1} $cell^{-1}$ (for one copy) or 0.40 fmol CH_4 day^{-1} $cell^{-1}$ (for two copies). These values are low, but given the tiny cell size (volume of roughly 0.05 to 0.2 μm^3), they are still on the same order of magnitude as the values for other slowly growing, anaerobic bacteria (e.g., 2.6 to 6.36 fmol NH_4 day^{-1} $cell^{-1}$ for anaerobic ammonium-oxidizing bacteria; Kuypers et al., 2003). We also assessed the agreement between the number of cells and the protein content of the culture using the estimates of cell volume (0.05 to 0.2 μm^3) to determine the approximate protein content using the formula of Norland et al. (1987) (for the relationship between cell volume and dry weight). If we multiplied the dry weight by the cell number obtained by qPCR (see above) and assumed that protein accounts for 50% of the dry weight, we obtained a theoretical content of 0.3 to 4.5 g for the enrichment culture. This matches the measured protein content, 4.75 ± 0.8 g, well, especially when the presence of other nontargeted bacteria is considered.

The steady increase in copy number from day 71 onward coincides with an increase in nitrite-reducing activity and biomass. Together, these results support the hypotheses that the denitrification observed in the first month of enrichment can be attributed to the oxidation of endogenous electron donors (decaying biomass, also indicated by the decreasing DNA content; Figure 2) present in the sediment, whereas the increase in

biomass and nitrite-reducing activity observed from month 4 onward was predominantly due to the growth of bacteria belonging to NC10 group *a* using methane as an electron donor. Together with the results of previous studies (chapter 2; Raghoebarsing et al., 2006), these findings strongly support a general role for these organisms in the biochemically enigmatic process of anaerobic methane oxidation coupled to denitrification.

Materials and Methods

Sampling and enrichment

Sediment samples (upper 5 cm) were obtained in July 2006 from four ditches draining agricultural land in the Ooijpolder (51°50'40'''N, 5°54'44'''E), a floodplain of the River Rhine in The Netherlands. The samples were transported to the lab within 1 h and mixed with ambient water to obtain a homogeneous slurry (2 liters) used for inoculation. The sediment was incubated in a 16-liter glass bioreactor (Applikon, Schiedam, The Netherlands), which was operated aseptically in a sequencing-batch mode (Strous et al., 1998) to prevent loss of biomass. Initially, a cycle consisted of 3 to 7 days of continuous supply of medium, a settling period of 1 to 2 h, and 30 to 60 min to draw off liquid from above the settled sediment and biomass. As activity increased, after 6 months the cycle was shortened to 22.5 h of supply of medium, 1 h of settling, and 30 min of drawing off of liquid. During the supply period, the culture was stirred gently at 100 rpm, sparged with CH_4-CO_2 (95:5, vol/vol; purity, >99.995%; flow rate, 10 ml/min; Air Liquide, France), and supplied with mineral medium (flow rate, 0.5 to 2 liters day^{-1}). The medium was continuously sparged with Ar-CO_2 (95:5, vol/vol) to maintain anoxic conditions and contained the following components (per liter): $KHCO_3$, 0.5 to 1.5 g (see Results); KH_2PO_4, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $NaNO_3$, 0.425 g (5 mM); $NaNO_2$, 0.0345 to 1.38 g (0.5 to 20 mM) (see Results); an acidic trace element solution, 0.5 ml; and an alkaline trace element solution, 0.2 ml. The acidic (100 mM HCl) trace element solution contained (per liter) 2.085 g $FeSO_4 \cdot 7H_2O$, 0.068 g $ZnSO_4 \cdot 7H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CuSO_4$, 0.095 g $NiCl_2 \cdot 6H_2O$, and 0.014 g H_3BO_3. The alkaline (10 mM NaOH) trace element solution contained (per liter) 0.067 g SeO_2, 0.050 g $Na_2WO_4 \cdot 2H_2O$, and 0.242 g Na_2MoO_4. All medium components were sterilized, either by 0.2-µm filtration (trace metal solution) or by autoclaving, and mixed aseptically.

The minimum liquid volume of the enrichment culture was kept at 10 liters by a level controller, and the maximum volume at the end of a filling period was 13 liters. A gas buffer (ca. 13 l liters) filled with the exhaust gas and additionally flushed with Ar (100 ml min^{-1}) prevented entry of air during drawing off. The oxygen concentration was continuously monitored with a Clark-type oxygen electrode. The culture vessel was wrapped in black foil, and black tubing with low oxygen permeability (Viton and Norprene; Cole Parmer, United States) was used. The nitrite concentration in the bioreactor effluent was estimated daily, and the medium flow was adjusted if the concentration was <0.2 or >1.5 mM. Also, the $KHCO_3$ and $NaNO_2$ contents of the medium were varied depending on the denitrifying activity of the culture (see Results), and together with the CO_2 in the gas supplied, they resulted in a pH of the culture liquid between 6.9 and 7.5, as monitored with

a pH electrode. The temperature was controlled at 30°C.

Molecular analysis

DNA isolation / Samples (2 ml) for quantitative PCR (qPCR) were taken every 2 to 4 weeks in triplicate. For the clone libraries, samples were taken from the slurry used for inoculation and from the enrichment culture after 6 months and stored at –20°C until DNA isolation. After bead beating in 120 mM sodium phosphate buffer, DNA was extracted and purified as described by Kowalchuk et al. (2004) (sodium dodecyl sulfate lysis, DNA precipitation with NaCl-polyethylene glycol 6000, protein precipitation with ammonium acetate, DNA precipitation with ethanol). The DNA was dissolved in water. DNA quality was checked by agarose gel electrophoresis, and the DNA concentrations of samples for qPCR were measured at 260 nm with a Smartspec 3000 spectrophotometer (Bio-Rad).

Phylogenetic analysis / To assess the diversity of bacteria affiliated with the NC10 phylum, 16S rRNA gene libraries were constructed after PCR amplification of DNA isolated from the inoculum (library Ino) and the enrichment culture (library Enr) after 6 months of incubation. One library (library Ino-nFR) was obtained using a nested PCR approach: a PCR with general bacterial primers 8F and 1545R (Juretschko et al., 1998) was followed by a second amplification using a specific primer pair and a high annealing temperature. The other libraries were obtained with combinations of a specific primer with a general primer and relatively low annealing temperatures (Table 1). PCR products were amplified in *Escherichia coli* with the pGEM-T Easy cloning vector (Promega, United States). Plasmids were isolated from 10 to 15 randomly selected clones per library using a GeneJet miniprep kit (Fermentas, Lithuania). Sequencing was performed at the DNA Diagnostics Center of Nijmegen University Medical Center using both the M13 forward and reverse primers. The quality of sequences was checked with the FinchTV program, and a BLAST search was performed to obtain related sequences (>90% similarity) from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/, accessed October 2008). Additionally, all sequences assigned to the NC10 phylum by the taxonomies of Hugenholtz and Pace (http://greengenes.lbl.gov [August 2008]) were added. To cover the full NC10 phylum, short sequences (minimum length, 548 bp) were also included. Three species of *Acidobacteria*, a closely related phylum (Fuerst, 2007), served as an outgroup. The sequences were aligned with the MUSCLE algorithm (Edgar, 2004) and imported into the MEGA4 software (Tamura et al., 2007), where the alignment was manually checked and trimmed. Phylogenetic trees were calculated on basis of 1,394 and 800 aligned positions (positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons) with MEGA's neighbor-joining, maximum parsimony, and minimum evolution (Figure 1) methods, which yielded the same overall topology. The robustness of tree topology was also tested by bootstrap analysis (1000 replicates). Evolutionary distances were computed with the Tajima-Nei correction. The phylum was divided into four well-supported groups (groups *a* to *d*).

qPCR / Based on the 16S rRNA gene sequences obtained from the enrichment culture, the sequence of a previously enriched bacterium (Raghoebarsing et al., 2006), clone D-BACT (GenBank accession no. DQ369742), and closely related sequences (DQ837241, DQ837250, and AF351217), two primers pairs for qPCR were designed; qP1F (5'-GGG CTT GAC ATC CCA CGA ACC TG-3') and qP1R (5'-CGC CTT CCT CCA GCT TGA CGC-3') amplify positions 1001 to 1201, and qP2F (5'-GGG GAA

CTG CCA GCG TCA AG-3') and qP2R (5'-CTC AGC GAC TTC GAG TAC AG-3') amplify positions 1169 to 1460. The primers and their optimal annealing temperature were tested with the enrichment culture (chapter 2), and PCR products obtained with both primer pairs were cloned and sequenced as described above. All of the sequences retrieved were the correct length and were very similar (97.6 to 98.5% identity) to the reference sequence (accession no. DQ369742). As a negative control, DNA from different mixed cultures dominated by anammox bacteria (Kartal et al., 2007; Van De Vossenberg et al., 2008) was used as a template, and it did not yield a PCR product. For each primer pair, standard curves (for 70 to 7 x 10^8 copies) were constructed, taking into account the molecular mass of the plasmid including the amplicon (Mongo Oligo mass calculator, version 2.06 [http://library.med.utah.edu/masspec/mongo.htm]) and the plasmid concentration determined by spectrophotometric measurement with a Smartspec 3000 (Bio-Rad) at 260 nm. qPCR for the standard curves and the samples was performed with the Bio-Rad IQTM 5 cycler and real-time detection system using IQTM SYBR green Supermix (Bio-Rad, United States), 800 nM forward primer, and 800 nM reverse primer. An initial denaturing step of 95°C for 3 min was followed by 40 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min. After a final extension for 5 min at 72°C, a melting curve analysis was carried out at temperatures from 60°C to 95°C, increasing at a rate of 0.5°C/30 s. The calculated efficiency was 100% for both primer pairs. 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 liquid volume of the bioreactor during sampling.

FISH / Biomass from the enrichment culture was harvested monthly, fixed, stored, and subjected to fluorescence in situ hybridization (FISH) as described previously (chapter 2), using a concentration of 40% formamide. The following probes were used: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 for dominant bacteria affiliated with the NC10 phylum (Raghoebarsing et al., 2006), a mixture of EUB I-III and EUB V for most bacteria (Daims et al., 1999), and S-D-Arch-0915-a-A-20 for most archaea (Stahl & Amann, 1991). The percentages of phylum NC10 bacteria were estimated based on visual inspection of at least one complete well.

Activity measurements

For batch incubations, biomass was transferred aerobically to glass bottles (volume, 8, 40, or 60 ml). Nitrite and 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.3) were added to final concentrations of 1 to 2 and 10 mM, respectively. The bottles were capped with either black (Rubber B.V., Hilversum, The Netherlands), gray (Helvoet Pharma, Alken, Belgium), or red (Terumo, Leuven, Belgium) butyl rubber stoppers, and anaerobic conditions were created by six cycles of vacuuming and subsequent gassing with Ar-CO_2 (95:5), followed by 5 min of flushing with Ar-CO_2, leaving an overpressure of 0.5 x 10^5 Pa. Methane was added to final headspace concentrations of 2.5 to 10%. Samples were incubated on a magnetic stirrer at 30°C. To assess formation of gaseous N compounds, twofold-concentrated samples were preincubated anaerobically with methane until the residual nitrite and nitrate were exhausted, and ^{15}N-nitrite (99.6% ^{15}N; Isotec, United States) was used as the sole electron acceptor.

Analysis of nitrogen compounds, methane, and protein

The nitrite concentration in the enrichment culture was estimated daily with Merckoquant test strips (0 to 80 mg/liter; Merck, Germany).

For batch experiments, ammonium and nitrite were measured colorimetrically as described previously (Kartal et al., 2006). Methane was quantified by gas chromatography, and the total protein content was determined by the bicinchoninic acid assay (Pierce, United States) using a bovine serum albumin standard (Thermo Scientific, United States) as described previously (chapter 2). For quantification of gaseous nitrogen compounds, 50 µl samples were withdrawn with a gas-tight glass syringe and injected into a gas chromatograph while the inlet area was flushed with helium to prevent entry of air. Additionally, use of ^{15}N-labeled nitrite in the incubations enabled distinction between contaminating ^{14}N-N_2 (air) and ^{15}N-N_2 produced in the experiment. Gases were separated by gas chromatography (6890 series; Agilent, United States) using a Porapak Q column at 80°C (5 min) with helium as the carrier gas (flow rate, 24 ml min^{-1}). The gas chromatograph was coupled to a mass spectrometer (Agilent 5975C inert MSD; Agilent, United States) to quantify the molecular masses 28 to 30 Da (N_2) and 44 to 46 Da (N_2O). Data were analyzed using the software Enhanced MSD Chem Station (version E.02.00.493; Agilent). Calibration was performed with standards consisting of $^{28}N_2$ in helium. Additionally, the initial and final concentrations of N_2 and N_2O were verified by gas chromatography as described previously (chapter 2).

Nucleotide sequence accession numbers

The sequences of 16S rRNA genes have been deposited in the GenBank database under accession no. FJ621531 to FJ621562.

Acknowledgements

We thank Erwin van der Biezen and Marnix Medema for screening sediment samples for the presence of NC10 sequences and Francisca Luesken for help with mass spectrometry. K.F.E. and M.S. were supported by a VIDI grant from the Dutch Science Foundation (NWO). T.V.A. was supported by a grant from the Dutch Foundation for Applied Research (STW).


exp. 1
1.4.08
15:30
exp. 1
1.4.08
15:35
exp. 1
1.4.08
15:37
exp. 1
1.4.08
15:45
exp. 2
1.4.08
16:28
exp. 2
1.4.08
16:33
exp. 2
1.4.08
16:36
exp. 2
1.4.08
16:33
exp. 6
3.4.08
15:55
exp. 6
3.4.08
16:03
exp. 6
3.4.08
16:06
exp. 6
3.4.08
16:06
exp. 6
3.4.08
16:31
exp. 6
3.4.08
16:34
exp. 6
3.4.08
16:37
exp. 6
3.4.08
16:40
exp. 8
3.4.08
17:30
exp. 8
3.4.08
17:43
exp. 8
3.4.08
17:48
exp. 8
3.4.08

Chapter 4

Nitrite-driven anaerobic methane oxidation by oxygenic bacteria

Published previously with supplementary material as:
Katharina F. Ettwig, Margaret K. Butler, Denis Le Paslier, Eric Pelletier, Sophie Mangenot, Marcel M.M. Kuypers, Frank Schreiber, Bas E. Dutilh, Johannes Zedelius, Dirk de Beer, Jolein Gloerich, Hans J.C.T. Wessels, Theo van Alen, Francisca Luesken, Ming L. Wu, Katinka T. van de Pas-Schoonen, Huub J.M. Op den Camp, Eva M. Janssen-Megens, Kees-Jan Francoijs, Henk Stunnenberg, Jean Weissenbach, Mike S.M. Jetten and Marc Strous (2010) in *Nature* **464**: 543-548.

Abstract

Only three biological pathways are known to produce oxygen: photosynthesis, chlorate respiration and the detoxification of reactive oxygen species. Here we present evidence for a fourth pathway, possibly of considerable geochemical and evolutionary importance. The pathway was discovered after metagenomic sequencing of an enrichment culture that couples anaerobic oxidation of methane with the reduction of nitrite to dinitrogen. The complete genome of the dominant bacterium, named '*Candidatus* Methylomirabilis oxyfera', was assembled. This apparently anaerobic, denitrifying bacterium encoded, transcribed and expressed the well-established aerobic pathway for methane oxidation, whereas it lacked known genes for dinitrogen production. Subsequent isotopic labelling indicated that "*M. oxyfera*" bypassed the denitrification intermediate nitrous oxide by the conversion of two nitric oxide molecules to dinitrogen and oxygen, which was used to oxidize methane. These results extend our understanding of hydrocarbon degradation under anoxic conditions and explain the biochemical mechanism of a poorly understood freshwater methane sink. Because nitrogen oxides were already present on early Earth, our finding opens up the possibility that oxygen was available to microbial metabolism before the evolution of oxygenic photosynthesis.

Introduction

With the ubiquitous use of fertilizers in agriculture, nitrate (NO_3^-) and nitrite (NO_2^-) have become major electron acceptors in freshwater environments (Galloway et al., 2008). The feedback of eutrophication on the atmospheric methane (CH_4) budget is poorly understood, with many potential positive and negative feedback loops acting in concert (Bodelier & Laanbroek, 2004). This previously prompted us to investigate the possibility of anaerobic oxidation of methane (AOM) coupled to denitrification (reduction of NO_3^- and NO_2^-, via nitric oxide (NO), to nitrous oxide (N_2O) and/or dinitrogen gas [N_2]), and microbial communities that perform this process were enriched from two different freshwater ecosystems in the Netherlands (Raghoebarsing et al., 2006; chapter 3), and recently from mixed Australian freshwater sources by others (Hu et al., 2009). All independent enrichment cultures were dominated by the same group of bacteria representing a phylum (NC10) defined only by environmental 16S rRNA gene sequences (Rappé & Giovannoni, 2003). While many surveys have found these sequences in a variety of aquatic habitats worldwide, reports on the natural activity of these bacteria are scarce (summarized in chapter 3).

Methane is one of the least reactive organic molecules (Thauer & Shima, 2008). Aerobic methanotrophs overcome its high activation energy by a reaction with molecular oxygen (Trotsenko & Murrell, 2008). Anaerobic sulphate-reducing microbial consortia

activate methane by a reversal of its biological production, using a homologue of the methane-releasing enzyme (methyl-coenzyme M reductase; MCR) of methanogens (Krüger et al., 2003). These consortia usually consist of distinct archaea related to methanogens (ANME) and sulphate-reducing bacteria (Knittel & Boetius, 2009). Initially, AOM coupled to denitrification was hypothesized to proceed in a similar manner, with archaea carrying out reverse methanogenesis in association with denitrifying bacterial partners (Raghoebarsing et al., 2006). However, it was subsequently shown that the complete process could also be performed by the bacteria in the total absence of archaea (chapter 2 and 3). The overall reaction of methane with nitrite (and nitrate) is thermodynamically feasible:

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ = 3\ CO_2 + 4N_2 + 10\ H_2O\ (\Delta G^{O\prime} = -928\ kJ\ mol^{-1}\ CH_4)$$

Yet, so far no known biochemical mechanism could explain the activation of methane in the absence of oxygen or (reversed) methanogens.

Genome assembly from enrichment cultures

We addressed the unknown mechanism of nitrite-dependent anaerobic methane oxidation by metagenomic sequencing of two enrichment cultures described previously; one enriched from Twentekanaal sediment (Raghoebarsing et al., 2006; chapter 2), designated "Twente" herein, and a culture from Ooijpolder ditch sediment (chapter 3), designated "Ooij". Both enrichments were 70 to 80 % dominated by populations of the same bacterial species (minimum 97.5 % 16S rRNA gene identity, for microdiversity in culture "Ooij" see chapter 3). In the present study we propose to name this species

Table 1 | Databases of genomic, proteomic and transcriptomic data

Culture	Approach	Molecule type	Amount of data obtained	NCBI-database, accession number
Enrichment culture "Twente"	454 Pyrosequencing	DNA	90,353,824 nt	Short Read Archive, SRR023516.1
	Illumina sequencing	DNA	196,814,368 nt	Short Read Archive, SRR022749.2
	Assembled genome	DNA	2,752,854 nt	Genbank, FP565575
	Paired-end plasmid (10 kb) sequencing	DNA	16,440,000 nt (trimmed)	*project ID 40193 (linked to FP565575)*
	Illumina sequencing	RNA	198,977,152 nt	Gene Expression Omnibus, GSE18535
	LC-FT-MS-MS	Protein		Peptidome, PSE127
Enrichment culture "Ooij"	Illumina sequencing	DNA	188,099,392 nt	Short Read Archive, SRR022748.2
	LC-FT-MS-MS	Protein		Peptidome, PSE128

Table 2 | General genome properties

Characteristic	Value
Size (bp)	2,752,854
GC content (%)	58.58
Coding density (%)	89.24
rRNAs	3
tRNAs	47
miscRNAs	4
Repeat content (%)	2.97

'*Candidatus* Methylomirabilis oxyfera' gen. nov. sp. nov. The metagenome of culture "Twente" was obtained by 454 pyrosequencing, Illumina sequencing and paired-end Sanger sequencing of a 10 kb insert plasmid library (Table 1). Binning based on GC content and coverage indicated that almost 60 % of the metagenomic data from pyrosequencing were associated with *'M. oxyfera'*. These data were initially assembled into five scaffolds that could then be joined into a single circular chromosome (2,753 kb; see Table 2 for more properties) by long-range PCR amplification.

Consistent with previous work (chapter 2), combined metagenomic data contained no evidence for the presence of the archaea that were originally suggested to form a consortium with the dominant bacterium (Raghoebarsing et al., 2006). Out of approximately 365,000 reads obtained by pyrosequencing, only 78 gave a blast hit with a bacterial 16S or 23S rRNA gene sequence distinct from *'M. oxyfera',* and none matched archaeal sequences. No other single species constituted a numerically significant part of the overall enriched community to enable assembly of more than very small (most less than 2 kb) fragments.

Short read (32 bp) Illumina sequencing of culture "Ooij" revealed that, in contrast to the near-clonal population dominating culture "Twente" a more diverse population of *'M. oxyfera'* inhabited this culture, as commonly observed by metagenomic sequencing of microbial populations (Wilmes et al., 2009). In the present case the estimated number of SNPs was over three times higher in culture "Ooij" than in culture "Twente". Because of this microdiversity, assembly of larger contigs for culture "Ooij" was impossible and the short reads were mapped directly onto the complete genome of *''M. oxyfera"*. Although the two enrichment cultures were dominated by the same species, the sequences were apparently too dissimilar to enable mapping by the presently available approaches (Farrer et al., 2009). Therefore, a new mapping algorithm based on iterated blast searches was developed (Dutilh et al., 2009). This allowed us to construct consensus sequences for genes of the *'M. oxyfera'* populations dominating enrichment culture "Ooij". By proteomic detection of peptides predicted from this consensus, the procedure was validated experimentally. The average identity of the partial genome obtained from enrichment culture "Ooij" to the complete genome of *''M. oxyfera"* was 91.1 % at the DNA level (ORFs and RNAs), and the SNP frequency among the Ooij populations was ≥ 3.45 %.

To facilitate the mechanistic interpretation of the genomic sequence information, the transcription and expression of predicted genes was investigated by Illumina sequencing

of RNA and by liquid chromatography tandem mass spectrometry (LC-MS/MS) of extracted proteins (Table 1).

Paradoxical predictions from the genome

Both enrichment cultures were grown anoxically and performed methane oxidation coupled to the complete denitrification of nitrite to N_2 (Raghoebarsing et al., 2006; chapter 2 and 3). Therefore, we inspected the genome, transcriptome and proteome for homologues of known genes involved in denitrification (Zumft, 1997). Suprisingly, *'M. oxyfera'* apparently lacked some genes necessary for complete denitrification (Figure 1a, and Table 3). Genes for the reduction of nitrate to nitrite (*narGHJI*, *napAB*), nitrite to NO (*nirSJFD/GH/L*) and NO to N_2O (*norZ*=q*nor*) were present in the genome, and expression as proteins could be demonstrated for Nap, Nir and Nor. However, with the exception of the accessory gene *nosL*, the gene cluster encoding enzymes for the reduction of N_2O to N_2 (*nosZDFY*) was missing. At the same time, previous studies have

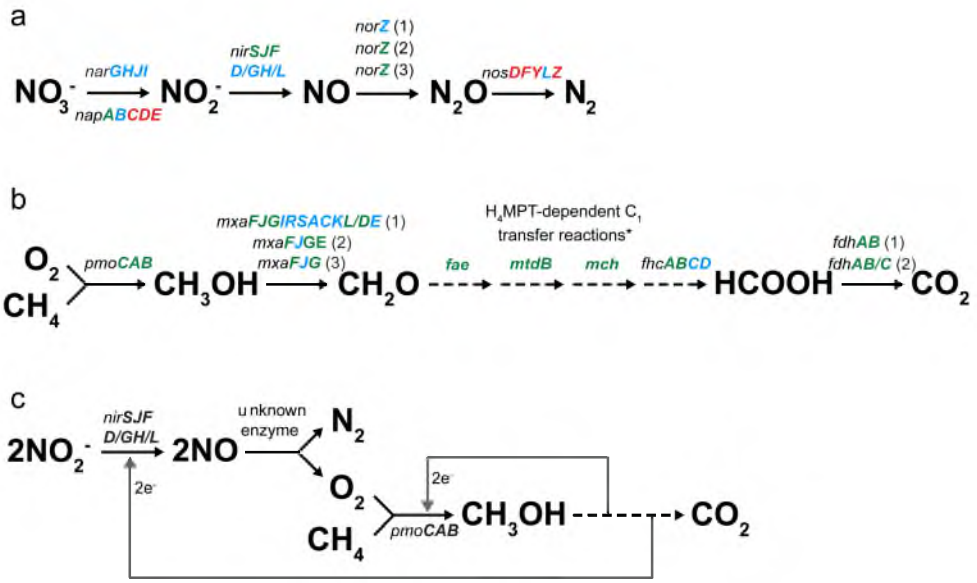


Figure 1 I Important pathways of *'Methylomirabilis oxyfera'*. Canonical pathways of (**a**) denitrification, (**b**) aerobic methane oxidation and (**c**) proposed pathway of methane oxidation with nitrite. Nitrate reductase (*narGHJI*); periplasmic nitrate reductase (*napABCDE*); nitrite reductase (*nirSJFD/GH/L*); nitric oxide reductase (*norZ*); nitrous oxide reductase (*nosDFYLZ*); particulate methane monooxygenase (*pmoCAB*); methanol dehydrogenase (*mxaFJGIRSACKL/DE*); formaldehyde activating enzyme (*fae*); methylene-H4MPT dehydrogenase (*mtdB*); methenyl-H_4MPT cyclohydrolase (*mch*); formyltransferase/hydrolase (*fhcABCD*); formate dehydrogenase (*fdhABC*). Genes in red are absent from the genome, those in blue are present in the genome and those genes in green are present in the proteome and genome. *H_4MPT-dependent reactions involve the intermediates methylene-H_4MPT, methenyl-H_4MPT and formyl-H_4MPT.

clearly shown that N_2O was not the main product of denitrification, but was only produced in trace amounts (chapter 2 and 3). However, based on the analysis of the datasets outlined in Table 1 and the fact that the *'M. oxyfera'* genome sequence appears complete, we judge it highly unlikely that genes encoding canonical N_2O reductase were overlooked and escaped proper assembly. Since complete denitrification can also be achieved by the combined action of multiple species, we could not yet rule out the possibility that the missing catalytic activity was complemented by other bacteria. In a similar fashion, we searched for homologues of anaerobic alkane activation enzymes, such as fumarate-adding glycyl-radical enzymes (Heider, 2007) and the MCR of reverse methanogens (Krüger et al., 2003). Consistent with the absence of archaea, the metagenome contained no homologue of MCR. Perhaps more surprisingly however, alkane-activating glycyl radical enzymes, which had been proposed to activate methane in these organisms (Thauer & Shima, 2008), were also missing. Instead, the genome did encode the complete pathway for aerobic methane oxidation (Figure 1b and Table 4). This well-known pathway proceeds via methanol (CH_3OH), formaldehyde (CH_2O), and formate (HCOOH) to carbon dioxide (CO_2) (Trotsenko & Murrell, 2008). In the first step of this pathway, methane is hydroxylated by reaction with oxygen, yielding methanol and water. This reaction is catalysed by the enzyme methane monooxygenase (MMO). Both metagenomes contained one set of *pmoCAB* genes encoding the particulate (membrane bound) form of this enzyme complex (pMMO); genes encoding the soluble form were absent. Although the amino acid sequences were phylogenetically distant from all homologous sequences presently in the databases (Figure 2), *pmoA* signature residues and those important for function were well conserved. The complete aerobic methanotrophic pathway was found to be transcribed and expressed in both anaerobic enrichment cultures (Table 4), including the complete tetrahydromethanopterin (H_4MTP)-dependent C_1 transfer module. The phylum NC10 is thus only the fifth phylogenetic group known to harbour this potentially primordial metabolic module (Chistoserdova et al., 2005). Phylogenetic analysis indicated that *'M. oxyfera'* represents a deeply-branching lineage of this C1 pathway (Butler et al., in preparation).

Thus, we were faced with two anaerobic, denitrifying microbial communities that were dominated by the same species, an apparently aerobic methanotroph incapable of complete denitrification. To resolve this paradox, we investigated whether *'M. oxyfera'* produced N_2 via a novel mechanism. Figure 1c shows a possible mechanism that could resolve the conflict between the genetic and experimental evidence; it is based on the conversion of two molecules of NO into O_2 and N_2. This reaction is thermodynamically favourable ($\Delta G^{0'}$ = -173.1 kJ mol^{-1} O_2), but kinetically difficult (Howard & Daniels, 1958). No catalyst operating at biologically relevant temperatures (0-100 °C) is known, though for higher temperatures, several catalysts (e.g. copper zeolites) have been developed

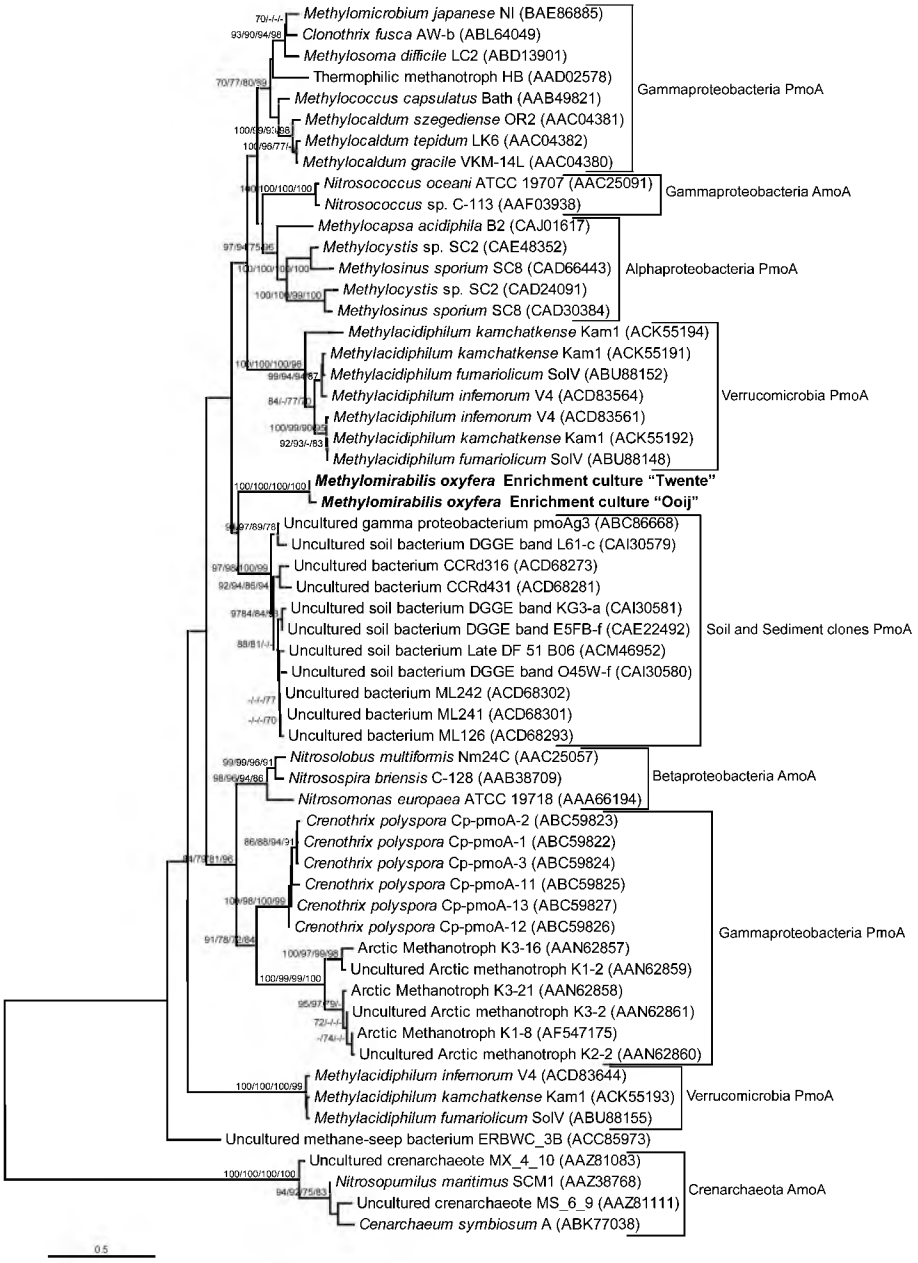


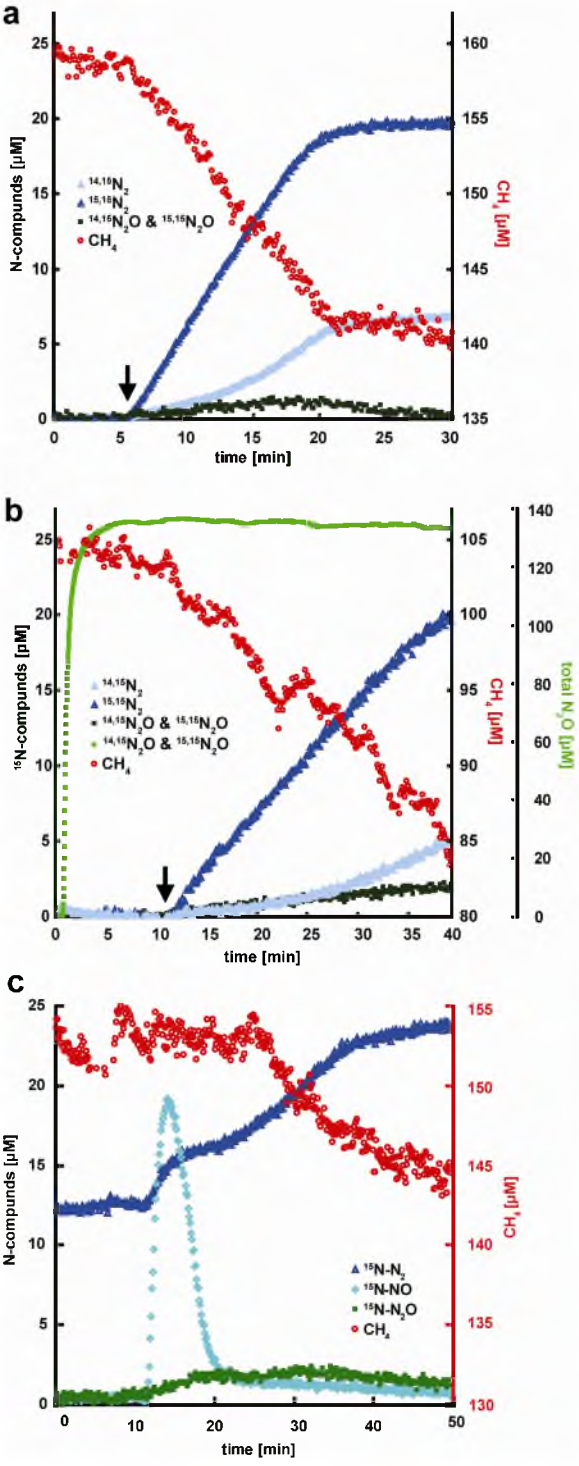
Figure 2 | Phylogenetic relationship among PmoA and AmoA proteins. The distance tree was computed using the Dayhoff matrix method. Bootstrap values (>70%, 100 replicates) calculated within the Neighbor-Joining, Minimum-Evolution, Maximum-Parsimony and Maximum-Likelihood evolutionary methods are shown at branch points from left to right. The scale bar represents 0.5 changes per amino acid.

that decompose NO from industrial and automobile exhaust fumes (Parvulescu et al., 1998). The production of oxygen as a metabolic intermediate is not completely new to biology: dismutation of the toxic intermediate chlorite ($ClO_2^- \rightarrow Cl^- + O_2$) by chlorate-reducing bacteria prevents cell damage and yields oxygen for chemoorganotrophic respiration (Van Glinkel et al., 1996), or possibly for monooxygenase-dependent biosynthesis (Bab-Dinitz et al., 2006).

The pathway outlined in Figure 1c would only require one new enzyme, an "NO dismutase", to catalyse a thermodynamically feasible reaction and replace N_2O reductase. The oxygen produced would become available to oxidize methane aerobically, explaining the presence of genes for aerobic methane oxidation in the *'M. oxyfera'* genome and the insensitivity of the cultures towards oxygen (chapter 3 and unpublished results). In this scenario, the role of the putative quinol-dependant NO reductases (norZ) could be detoxification of NO rather than respiration, consistent with its function in most other known bacteria (Zumft, 2005). Alternatively, together with two multi-copper oxidases encoded in the genome, they are hypothetical candidates for catalysing the oxygen production from NO.

Experimental evidence for proposed pathway

The operation of the new pathway was addressed experimentally in a series of experiments performed with culture "Ooij". Compared to culture "Twente", its activity was higher and it was less sensitive to experimental handling. To corroborate the coupling of nitrite reduction to methane oxidation, we first incubated 380 ml of the enrichment culture with ^{13}C labelled methane and with nitrate (2 mM) as the only electron acceptor while monitoring the concentration and isotopic composition of the dissolved gases. With nitrate only, no methane oxidation was detectable and no N_2 was produced. Upon addition of ^{15}N-labelled nitrite, methane oxidation began and labelled N_2 was formed in stoichiometric amounts (Figure 3a). Together, these results showed unambiguously that methane oxidation by *'M. oxyfera'* cultures proceeded in the absence of extracellular oxygen: the oxygen concentration remained below the detection limit (0.3 µM), the activity was dependent on the presence of nitrite and the stoichiometry of the reaction indicated that no electrons were lost to other electron acceptors (theoretical stoichiometry: $3CH_4/4N_2$; measured: 3/3.87). The experiment also confirmed that denitrification was complete, despite the lack of genes encoding N_2O reductase in the genome of *'M. oxyfera'*. Similar results were obtained after the addition of ^{15}N-NO (Figure 3c). This indicated that NO was an intermediate of *'M. oxyfera',* and is consistent with the presence and expression of nitrite reductase.



To test whether N_2-production by *'M. oxyfera'* proceeded via N_2O as an intermediate, the enrichment culture was incubated with ^{13}C-labelled methane, nitrate and N_2O, but without nitrite. Under these conditions, neither methane nor N_2O was consumed (Figure 3b). Consistent with the genomic inventory, N_2O was apparently not a suitable electron acceptor for methane oxidation. Again, methane was only oxidized after addition of ^{15}N-nitrite, and almost all (93%) of the label was recovered as N_2. Only a small amount (7%) of the ^{15}N-nitrite was converted to N_2O, presumably by community members other than *'M. oxyfera'*. Because a large amount of unlabelled N_2O was

Figure 3 | Coupling of methane oxidation and nitrite or nitric oxide reduction in enrichment cultures of *'Methylomirabilis oxyfera'*. Methane is oxidized only after addition of ^{15}N-labelled nitrite (**a** and **b**; 50 µM, arrow) and 15N-labelled NO (**c**), which is converted to ^{15}N-labelled dinitrogen gas in presence of (**a** and **c**) approximately 2000 µM ^{14}N-nitrate or (**b**) 2000 µM ^{14}N-nitrate and 135 µM ^{14}N-N_2O. Experiments were performed with 380 ml anoxic, stirred enrichment culture "Ooij" (protein content 147 ± 11 mg). Red circles: CH_4; dark blue triangles: $^{15,15}N_2$; light blue triangles: $^{15,14}N_2$; green squares: total N_2O; dark green squares: $^{14,15}N_2O$ and $^{15,15}N_2O$; turquoise diamonds: ^{15}NO.

present from the start, it can be assumed to have fully penetrated the microbial cells, even those residing in aggregates. For this reason, we would expect that if N_2O was turned over as an intermediate during nitrite reduction by *'M. oxyfera'*, most ^{15}N label would have been recovered as N_2O, since the cells would mainly reduce the unlabelled N_2O to N_2. Hence, the ^{15}N label would be "trapped" in the N_2O "pool", definitely so for a scenario where the missing genes for N_2O reductase would be complemented by other bacteria in the enrichment culture. However, it is still a possibility that in *'M. oxyfera'* N_2O production and reduction are extremely strictly coupled. Given the absence of genes for a conventional N_2O reductase (Zumft & Kroneck, 2007), N_2 production would depend on the presence and activity of an as-yet unknown functional analogue. Thus, even in this conservative scenario it is likely that a novel enzyme produces N_2 in *'M. oxyfera'*.

N_2O reductase is inhibited by acetylene (C_2H_2) in mM concentration range (Zumft, 1997), and the addition of acetylene would therefore be a straightforward method to provide further evidence for the absence of this enzyme. However, acetylene also inhibits pMMO at much lower concentrations (µM range; Prior & Dalton, 1985). Thus because the genomic and proteomic analyses suggested that pMMO was the methane-activating enzyme in *'M. oxyfera'*, a complete inhibition of total activity by acetylene would be expected for this organism. Indeed, experiments with culture "Twente" suggested complete inhibition of methane oxidation activity at concentrations as low as 10 µM acetylene (data not shown).

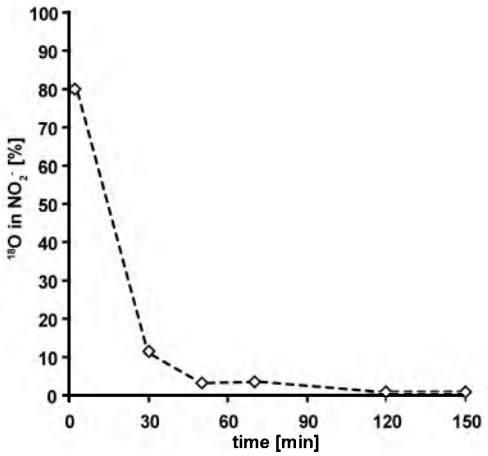


Figure 4 | Oxygen-exchange between ^{18}O-nitrite and (unlabelled) water mediated by *'M. oxyfera'* enrichment culture "Ooij". ^{18}O-labelled NO_2^- was prepared according to Friedman et al. (1986), and the labelling percentage was measured by GC-MS as described in the method section after conversion to N_2O (McIlvin & Altabet, 2005). Control incubations of ^{18}O-NO_2^- in identical medium without biomass showed no measurable O-exchange over a period of 4 h.

To provide more evidence for the potential role of pMMO in anaerobic methane oxidation, we used an established assay for pMMO activity, the oxidation of propylene (propene, C_3H_6; Prior & Dalton, 1985). In this assay, pMMO adds one oxygen atom of dioxygen (O_2) to propylene, yielding propylene oxide (propylene epoxide, C_3H_6O). In incubations of enrichment culture "Ooij" with propylene, formate and oxygen, aerobic pMMO activity could be demonstrated at a rate of 0.54 nmol (C_3H_6O) min^{-1} (mg protein)$^{-1}$. Next,

nitrite was added instead of oxygen. Interestingly, in the presence of nitrite propylene was oxygenated more rapidly (0.94 nmol min^{-1} (mg protein)$^{-1}$) than in the presence of oxygen. No activity above background levels was detected with N_2O or nitrate. Also in this experiment, the pMMO activity was completely inhibited by 0.15 mM acetylene. To exclude the possibility that contaminating oxygen could explain the observed pMMO activity, we used ^{18}O labelling to trace the oxygen atoms of nitrite into propylene oxide. The direct use of ^{18}O-labelled nitrite proved impossible, since the ^{18}O was quickly exchanged with the unlabelled O from water (Figure 4), presumably via the activity of nitrite reductase (Kool et al., 2007). To overcome this problem, we generated ^{18}O-labelled nitrite in the incubations themselves by adding ^{18}O-labelled water. In this way, incorporation of the ^{18}O from nitrite into propylene oxide could be demonstrated (74-88 % originating from nitrite). In line with theoretical expectations, control incubations confirmed that no O exchange occurred between water and O_2 or propylene oxide, and that ^{18}O-labelled water did not lead to propylene oxide formation in the absence of nitrite. Control incubations with aerobic methanotrophs were not active with nitrite and did not incorporate ^{18}O from nitrite. In theory, two mechanisms could explain this result: (1) the direct use of NO by pMMO or (2) the production of oxygen by pMMO or a separate enzyme followed by consumption of the produced oxygen by pMMO (Figure 1c). The divergent position of the *'M. oxyfera'* pMMO (Figure 2) may argue for the first possibility; however most residues typical for pMMOs were conserved. The 3:8 methane:nitrite stoichiometry observed renders the second possibility more likely, as apparently not all NO is reduced via pMMO. With possibility (2) the remaining oxygen (25%) may be consumed by terminal oxidases.

We addressed these two possibilities experimentally by measuring the production of ^{18}O-labelled oxygen in the incubations with propylene. Indeed, a small amount of labelled oxygen was released in anoxic incubations with propylene, nitrite and ^{18}O-labelled water (0.15 nmol min^{-1} (mg protein)$^{-1}$; Figure 5). In control incubations of aerobic methanotrophs in the presence of ^{18}O-water and ^{18}O-nitrite, no labelled oxygen was detectable, ruling out chemical or aspecific reactions with pMMO. Also, production of oxygen by enzymes for the detoxification of reactive oxygen species, e.g catalase or superoxide dismutase, is unlikely, since reactive oxygen species are not known to be produced in the absence of oxygen, even when NO is present (Demicheli et al., 2007). Furthermore, these enzymes are presumably more active in aerobically grown methanotrophs, which did not produce oxygen from nitrite. When methane was used instead of propylene, no oxygen was released by *'M. oxyfera'*. This may be explained by the approximately 2-3-fold higher enzymatic rates with methane (Figure 3 and chapter 3) compared to propylene; the lower activity of pMMO with propylene allowed some oxygen to escape.

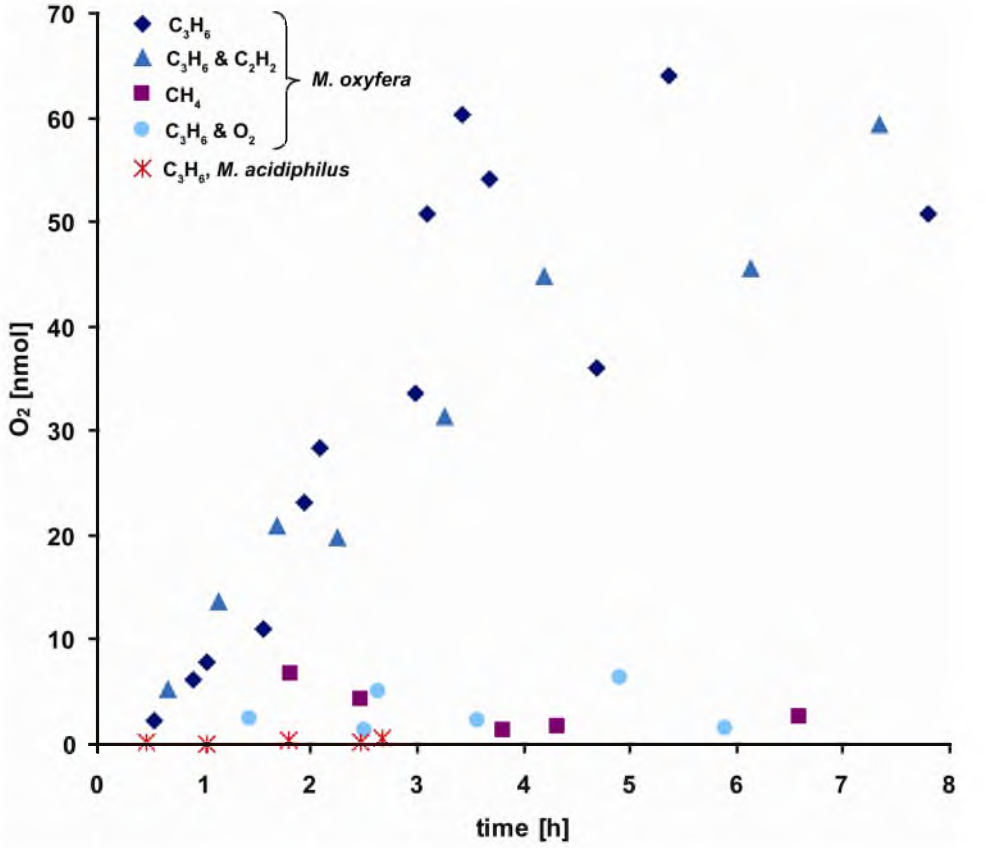


Figure 5 | **Oxygen production from nitrite.** Whole cells of enrichment culture "Ooij" were incubated in buffer containing nitrite and 25 % ^{18}O-water, leading to 90% O-exchange. Total oxygen production from this indirectly labelled $N^{18}O_2^-$ was inferred from the measured concentration of $^{16,18}O2$ and ^{18,18}-O_2 in He headspace with the following additions: propylene (dark blue diamonds), propylene and acetylene (blue triangles), methane (purple squares) and oxygen (light blue circles). Anaerobic control incubations of *Methylosinus acidophilus* (red asterisks) with ^{18}O-nitrite did not produce measurable amounts of oxygen. Cells were concentrated to obtain similar maximum rates of propylene oxidation activity; 1.15 nmol min^{-1} (with NO_2^-, 1.22 mg protein) for '*M. oxyfera*' and 1.68 nmol min^{-1} (with O_2, 0.046 mg protein) for *M. acidophilus*.

Conclusions and perspectives

Here we describe the discovery of a new "intra-aerobic" pathway of nitrate reduction. Interestingly, this pathway resembles the original proposal for the mechanism of denitrification. After its discovery in the nineteenth century, it was generally assumed that denitrification proceeded by the production of oxygen from nitrate, which could subsequently be used for respiration (reviewed in Allen & Van Niel, 1952). Thus, it appears that an old hypothesis may have been brushed aside too easily.

The new pathway of "intra-aerobic denitrification" is not necessarily restricted to methane oxidizing bacteria: with all presently available assays for denitrification the process would either be overlooked (e.g. with the acetylene-block technique) or lumped together with conventional denitrification (e.g. isotope pairing). Under dynamic oxic/anoxic conditions and with recalcitrant substrates (aromatic compounds, alkanes and alkenes) the process may certainly offer ecological advantages.

The production of oxygen from nitrogen oxides is also of interest for ordering the evolution of metabolic pathways on early earth – mostly believed to proceed from 'reduced' (e.g. fermentation) to 'oxidized' (e.g. aerobic respiration; Broda, 1975; Fenchel, 2002) pathways. Following this scenario, the pathway presented here may have evolved to exploit newly formed pools of nitrogen oxides after the oxygenation of the atmosphere. Alternatively, based on enzyme phylogenies, respiration has been discussed as a primordial pathway (Castresana, 2004; Ducluzeau et al., 2009), that originally depended on nitrogen oxides most likely present on early earth (Ducluzeau et al., 2009), though it is debated if they were quantitatively important (Broda, 1975; Chapman & Schopf, 1983). Our study adds a new aspect to this debate, as it is tempting to speculate that intra-aerobic denitrification may have preceded oxygen production by photosynthesis, or extended the niches for the evolution of aerobic pathways in a still predominantly anaerobic environment before the Great Oxidation event approximately 2.45 billion ya (Holland, 2006). The intra-aerobic pathway presented here would have enabled microorganisms to thrive on the abundant methane in the Archaean atmosphere (Pavlov et al., 2000) without the direct dependence of oxygenic photosynthesis, causing ^{13}C-depleted sedimentary carbon that has so far been attributed to aerobic methanotrophs (Hayes, 1983).

Description of 'Candidatus Methylomirabilis oxyfera' gen. nov., sp. nov.

Etymology. Me.thy.lo.mi.ra'bi.lis. M.L. n. *methyl* the methyl group, L. adj. *mirabilis* astonishing, strange. oxy.fe'ra. L.n. *oxygenium* oxygen, L. part adj. *fera* carrying, producing. The name alludes to the substrate methane, which is oxidized by a surprising combination of pathways, involving oxygen as an intermediate.

Locality. Enriched from freshwater sediments of the Twentekanaal and Ooijpolder ditches, The Netherlands.

Properties. Methane-oxidizing and nitrite-reducing bacterium of the candidate division NC10. Grows anaerobically, but produces oxygen for aerobic oxidation of methane. Reduces nitrite to dinitrogen gas without a nitrous oxide reductase. Gram-negative, rod, with a diameter of 0.25 - 0.5 µm and a length of 0.8 - 1.1 µm. Mesophilic with regard to temperature and pH (enriched at 25-30 °C and pH 7-8). Slow growth (doubling time 1-2 weeks).

Materials and Methods

DNA preparation

DNA from both enrichment cultures was isolated as described (Zhou et al., 1996), with modifications. After incubation of biomass in DNA extraction buffer for 2 hours, a 0.7 volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) was added and the mixture incubated for 20 min at 65 °C. The aqueous phase was recovered by centrifugation and another treatment with phenol/chloroform/isoamyl alcohol performed. The aqueous phase was then mixed with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol) and the DNA precipitated and cleaned as described (Zhou et al., 1996).

Sequencing

454 pyrosequencing was performed on total genomic DNA from enrichment culture "Twente" using the Roche Genome Sequencer FLX system by Keygene N.V (The Netherlands; Margulies et al., 2005). The single-end Illumina samples sequencing was performed using total genomic DNA from both enrichment cultures, as described. The samples were prepared according to the manufacturers protocol (Illumina). Subsequent sequencing was carried out on a genome Analyzer II (Illumina). A 10 kb insert plasmid library of total genomic DNA from enrichment culture "Twente" was constructed and clones picked and bi-directionally sequenced by using standard protocols. Sequence data resulting from the plasmid library and pyrosequencing were combined and a preliminary global assembly, performed using both Phrap (Gordon et al., 1998) and Newbler (454 Life Sciences) assembly softwares, resulted in five scaffolds containing seven contigs. Gaps were closed and the genome made circular using a combination of several methods, such as sequencing of a transposon-shotgun library of plasmids overlapping the assembly gaps (with 3229 validated sequences obtained), and PCR amplification and subsequent sequencing of regions between scaffold ends (297 validated sequences). Identified in the genome were 47 tRNAs representing all amino acids, 3 small RNAs and one ribosomal RNA operon, as well as all of the 63 conserved clusters of orthologous groups (COGs), confirming the completeness of the genome. Gene prediction was conducted using AMIGene software. Coding sequences were predicted (and assigned a unique identifier prefixed with "DAMO") and automatic functional annotation performed as previously described with the MaGe system (Vallenet et al., 2006). Annotations for selected genes (Table 3 and 4) were manually confirmed.

Phylogenetic analysis of PmoA and AmoA protein sequences

An alignment of selected bacterial and archaeal PmoA and AmoA amino acid sequences was generated using MEGA version 4 (http://www.megasoftware.net) and conservation of important residues was judged by comparison to previously published alignments (Stoecker et al., 2006; Hakemian & Rosenzweig, 2007). There were a total of 178 amino acid positions in the final dataset. A distance tree was generated in MEGA, using the Neighbor-Joining method. Bootstrap values of 100 replicates were generated for Neighbor-Joining, Minimum Evolution and Maximum Parsimony, with the Dayhoff matrix-based method. PhyML 3.0 (www.atgc-montpellier.fr/phyml/) was used to generate Maximum Likelihood bootstrap values.

Proteomics

A cell-free extract from the biomass of enrichment culture "Twente" was prepared by

concentrating a sample of the biomass in 20 mM phosphate buffer and bead-beating for 2 min. The supernatant was then boiled for 10 min in sample buffer. For a cell-free extract from enrichment culture "Ooij", a sample of the biomass was concentrated in 20 mM phosphate buffer containing 1 mM phenyl-methylsulfonyl fluoride, protease inhibitor cocktail and 1 % SDS, and the sample was boiled for 7 min. Both cell-free extracts were then loaded onto an SDS PAGE gel, prepared using standard methods, with approximately 50 µg protein per lane. After separation of proteins and staining with colloidal Coomassie Blue, the gel lane was cut into 4 slices, and each gel slice was destained with three cycles of washing with successively 50 mM ammonium bicarbonate and 50 % acetonitrile (ACN). Protein reduction, alkylation and digestion with trypsin was performed as described (Wilm et al., 1996). After digestion, the samples were desalted and purified according to Rappsilber et al. (2003). Sample analysis by LC-MS/MS was performed using an Agilent nanoflow 1100 liquid chromatograph coupled on-line via a nano-electrospray ion source (Thermo Fisher Scientific) to a 7T linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT, Thermo Fisher Scientific). The chromatographic column consisted of a 15 cm fused silica emitter (New Objective, PicoTip Emitter, Tip: 8 +/- 1 µm, ID: 100 µm) packed with 3 µm C18 beads (Reprosil-Pur C18 AQ, Dr Maisch GmbH, Germany; Ishihama et al., 2002). After loading the peptides onto the column in buffer A (0.5 % HAc), bound peptides were gradually eluted using a 67 min gradient of buffer B (80 % ACN, 0.5 % HAc). First, the concentration of ACN was increased from 2.4 % to 8 % in 5 min, followed by an increase from 8 % to 24 % ACN in 55 min, and finally an increase from 24 % to 40 % ACN in 7 min. The mass spectrometer was operated in positive ion mode and was programmed to analyse the top 4 most abundant ions from each precursor scan using dynamic exclusion. Survey mass spectra (350-2000 m/z) were recorded in the ICR cell at a resolution of R=5E5. Data dependent collision induced fragmentation of the precursor ions was performed in the linear ion trap (normalized collision energy: 27 %, activation q=0.250, activation time: 30 ms). Mass spectrometric datafiles were searched against a database containing the predicted protein sequences from the '*M. oxyfera*' genome and known contaminants, such as human keratins and trypsin. Database searches were performed using the database search program Mascot (Matrix Science Inc., USA, version 2.2). In order to obtain factors for the recalibration of precursor masses, initial searches were performed with a precursor ion tolerance of 50 ppm. Fragment ions were searched with 0.8 Da tolerance and searches allowed for 1 missed cleavage, carbamidomethylation (C) as fixed modification, and deamidation (NQ) and oxidation (M) as variable modifications. The results from these searches were used to calculate the m/z dependent deviation, which was used to recalibrate all precursor m/z values. After recalibration of the precursor masses, definitive Mascot searches were performed using the same settings as stated above, but with a precursor ion tolerance of 15 ppm. Additionally, reverse database searches were performed with the same settings. Protein identifications were validated and clustered using the PROVALT algorithm to achieve a false-discovery rate (FDR) of <1 % (Weatherly et al., 2005).

Transcriptomics

Total cell RNA was extracted from enrichment culture "Twente" biomass using the RiboPure™-Bacteria Kit (Ambion) following manufacturer's instructions. An additional DNase I treatment was done (provided within the RiboPure™-Bacteria Kit (Ambion)). RNA quality was checked by agarose gel

electrophoresis and the RNA concentration was measured with the Nanodrop® ND-1000 spectrophotometer (Isogen Life science). First-strand cDNA was generated with RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas Life Sciences), according to the manufacturers protocol. Second-strand cDNA synthesis was performed using RNase H (Fermentas Life Sciences) and DNA polymerase I (Fermentas Life Sciences) according to the manufacturer's protocol. Purification of the dsDNA for sequencing was performed with the Qiaquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and single-end Illumina sequencing performed as described above.

Short read sequence mapping

Reads resulting from the Illumina sequencing of enrichment culture "Ooij" were mapped onto the complete genome of '*M. oxyfera*' (enrichment culture "Twente") with an algorithm based on iterated Blast searches (Dutilh et al., 2009). Briefly, the 32 nt Illumina reads were mapped to the "Twente" reference genome by composing a majority-vote consensus. This initial consensus assembly was then taken to iteratively re-map all short reads, improving the assembly coverage and bringing the genome closer to the consensus of the sequenced population "Ooij". Three different programs were used to map the short reads to the reference genome (Maq, Blast and MegaBlast), and with each program at least 12 iterations were constructed. For Blast and MegaBlast also several different word lengths were tried, ultimately yielding a total of 87 potential consensus genomes for the "Ooij" culture Illumina reads (see Supplementary Table in Dutilh et al., 2009). To empirically test which of these assemblies best described the '*M. oxyfera*' "Ooij" population, the ORF coordinates from the complete "Twente" genome were mapped to each of these assemblies (the reads were mapped without gaps, so the genomic coordinates are identical) and all ORFs were translated into protein. Subsequently, the peptides obtained by LC-MS/MS (see above) were mapped to these translated ORFs using Mascot (see above). It was found that the Blast-based assembly with a word length of 8 could explain the largest number of peptides. As the number of peptides plateaued after 7 iterations, we chose iteration 7 as the optimal sequence for the '*M. oxyfera*' "Ooij" consensus genome. Positions where single nucleotide polymorphisms (SNPs) occur were identified by mapping the Illumina reads of the two enrichment cultures to their respective genomes using Maq (default settings). Sites with a polymorphic consensus were called as SNP sites.

Activity measurements

All activity experiments were carried out at 30 °C and pH 7.3 (10 mM MOPS) with whole cells from enrichment culture "Ooij". For measurement of nitrogenous intermediates (Fig. 3), 380 ml enrichment culture containing 147 ± 11 mg protein, approximately 2 mM NO_3^- (to maintain the redox potential) and 100 µM CH_4 were incubated in a modified Schott glass bottle without headspace and stirred with a glass-coated magnet (Figure 6). Microsensors for O_2 (Revsbech, 1989), N_2O (Andersen et al., 2001) and NO (Schreiber et al., 2008), prepared and calibrated as decribed previously, were reaching into the culture through teflon-coated rubber. The concentration and isotopic composition of methane and nitrogenous gases were measured in liquid withdrawn through a sintered glass filter by membrane-inlet mass spectrometry, using a quadrupole mass spectrometer (GAM 200, IP instruments, Germany). To compensate for the liquid loss, the setup was coupled to a He-flushed medium reservoir. N_2O and C_2H_2 were added

in gas-saturated, anaerobic medium, and ^{15}N-NO_2^- as anaerobic stock solution. The ^{15}N-NO stock solution was prepared by adding H_2SO_4 to a solution containing ^{15}N-NO_2^- and KI, and capturing the evolving NO gas in anaerobic water (Schreiber et al., 2008).

Propylene oxidation and oxygen production assays (Fig. 4) were performed (at least in duplicate) with 0.5 ml 10x concentrated whole cells from enrichment culture "Ooij" in 3 ml exetainers (Labco, UK). Biomass was centrifuged anaerobically, washed once and resuspended in anaerobic, MOPS-buffered (pH 7.3, 10 mM) medium (chapter 3) without NO_2^- and NO_3^-. Control incubations were performed with the aerobic, methanotrophic α-Proteobacterium *Methylosinus acidophilus* in NO_3^--free M2 medium (Raghoebarsing 2006, PhD thesis). Samples were preincubated anaerobically for at least 4 h with a He headspace containing 6 % CH_4 to deplete a cellular store of electron acceptors. Pilot experiments had shown that rates of propylene oxidation with and without added electron acceptors were otherwise identical for up to 5 hours. After removing the CH_4 from the headspace by 6 cycles of vacuum and He (0.5 bar) supply, a combination specified in the results section of the following salts (as anaerobic stock solutions) and gases (purity 99 % or higher) were added: ^{15}N-NO_2^- (3 mM final concentration), O_2 (6.5 % in headspace resulting in ca. 84 µM in solution), formate (5 mM final concentration), propylene (16 % in headspace), acetylene (0.4 % in headspace resulting in 150 µM). ^{18}O-NO_2^- was not added directly to '*M. oxyfera*' incubations, because the ^{18}O was quickly equilibrated with water-O by the activity of nitrite reductase (Kool et al., 2007), leading to 96 % equilibration with NO_2^--O within 30 min (Figure 4). Instead, unlabelled NO_2^- was used in combination with medium containing 25 % ^{18}O-labelled water (> 97 % ^{18}O, Cambridge Isotope Laboratories, USA). For control incubations of *M. acidophilus*, ^{18}O-NO_2^- (produced as described in Friedman et al., 1986 and checked by mass spectrometry after conversion to N_2O according to McIlvin & Altabet, 2005) was used in addition to ^{18}O-water, because no nitrite reductase activity can be expected. Samples were horizontally incubated at 30 °C on a shaker (250 rpm). Headspace samples were taken every 30-60 min with a gas-tight glass syringe. Propylene oxide and CH_4 were quantified by gas chromatography as described previously (chapter 2), with increased temperature of oven (150 °C), injection port and detector (180 °C). Oxidation rates were calculated from the linear part of the graphs ($R^2 \geq 0.9$) using at least 3 measuring points. Samples not exceeding the rate of controls without added electron acceptor (see above) were considered negative.

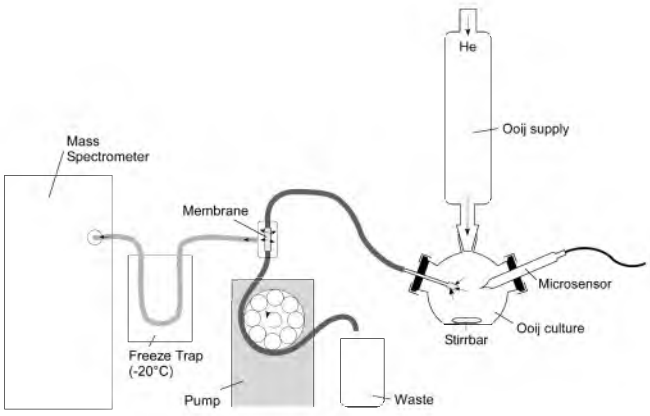


Figure 6 | Schematic setup for the measurement of nitrogenous intermediates by microsensors and membrane-inlet mass spectrometry (see Figure 3).

Formation of ^{18}O-O_2 was measured by coupled gas-chromatography/mass-spectrometry as described previously (chapter 3), detecting the masses 32-36 Da. Calibration for low amounts of oxygen was performed with known amounts of $^{36}O_2$ in He and the $^{34}O_2$ and $^{36}O_2$ content of air, taking into account the average isotopic composition of atmospheric O_2. The lower limit for accurate quantification of $^{36}O_2$ was 1 nmol ml^{-1}. Air contamination was minimized by flushing the inlet area of the GC with He, and the measured values of $^{16,18}O_2$ and $^{18,18}O_2$ were corrected for their abundance in contaminating air, assessed by the amount of $^{16,16}O_2$ and $^{14,14}N_2$. Incorporation of ^{18}O into propylene oxide was measured with a modification of the above-mentioned method at a higher column temperature (150 °C) and detecting the masses 58-60 Da.

Acknowledgements

We thank Fons Stams and Nico Tan for sharing their ideas on NO decomposition, Daan Speth and Lina Russ for pilot experiments, Nardy Kip for providing *M. acidophilus* cultures, Antonio Pierik for EPR analysis, Gabi Klockgether and Gaute Lavik for technical assistance and Boran Kartal, Jan Keltjens, Arjan Pol, Jack van de Vossenberg and Friedrich Widdel for helpful discussions. M.M.M.K., F.S., J.Z. and D.d.B. were supported by the Max Planck Society, M.S.M.J. by ERC grant 232937, M.S., K.F.E. and M.K.B. by a VIDI grant to M.S. from the Netherlands Organisation for Scientific Research (NWO), and M.L.W. and B.D. by a Horizon grant (050-71-058) from NWO.

Author Contributions

Genome sequencing and assembly from enrichment culture "Twente" was performed by D.L.P, E.P., S.M., and J.W. M.S. E.J.-M., K.-J.F. and H.S performed the sequencing and initial assembly of sequence from enrichment culture "Ooij". Mapping of sequences from enrichment culture "Ooij" to "Twente" was performed by B.E.D and M.S. B.E.D. & E.P. performed SNP and coverage analyses. Genome annotation and phylogenetic analysis was carried out by M.K.B. H.C. provided support with alignments. Sample preparation for proteome analysis was performed by M.K.B and M.W, with LC-MS/MS and protein identification performed by J.G. and H.J.C.T.W. Material for transcriptome analysis was prepared by T.A. and F.L., with sequencing performed by E.J.-M., K.-J.F and H.S. Continuous cultures were set up and maintained by K.F.E and K.T.P.-S. Experiments for nitrogenous intermediates were designed and performed by K.F.E., M.M.M.K., F.S., D.d.B., and J.Z., while those for methane activation were designed and performed by K.F.E. Pilot experiments were carried out by K.F.E., F.L., M.K.B., K.T.P.-S, T.A., and M.S. K.F.E., M.K.B., M.S.M.J., and M.S. conceived research. K.F.E., M.K.B. and M.S. wrote the paper with input from all other authors.

Table 3 (p. 70) | Genes encoding denitrification enzymes from '*M. oxyfera*'.

Genes encoding denitrification enzymes were identified by BLASTP search of translated ORFs against the NCBI NR database. Similarity to *P. denitrificans* homologs was determined by BLASTP search at the Genome Project of *P. denitrificans* within NCBI. ND, not detected.
*In the '*M. oxyfera*' *nar* operon, which is organized like that of *N. winogradski*, a different gene with no sequence similarity is present at the position of *narX*; its best BLASTP hit is to kustc0320 (*Candidatus* Kuenenia stuttgartiensis, unknown protein).

Table 4 (p. 71) | Genes encoding selected carbon metabolism enzymes from '*M. oxyfera*'.

Genes encoding enzymes of carbon metabolism were identified by BLASTP search of translated ORFs against the NCBI NR database. Similarity to *M. capsulatus* was determined by BLASTP search at the Genome Project of *M. capsulatus* within NCBI. = indicates that *M. capsulatus* has the best BLASTP hit. (-) indicates that the gene is not present in *M. capsulatus*. ND, not detected.
‡ DAMO_0121 is a fused ORF of two proteins.

Table 3 | Genes encoding denitrification enzymes from '*M. oxyfera*'.

Enzyme	Gene	Best BLASTP hit NCBI (BLASTP search against *Paracoccus denitrificans* between brackets)				Transcript-ome coverage	No. of peptides detected	ORF identifier	Enrichment culture "Ooij" sequences	
		% Identity	% Similarity	Expected (E)-value	GenBank identifier				Coverage/ % identity	No. of peptides
Nitrate reductase	*narG*	67 (46)	80 (62)	0.0 (0.0)	EAR23355 *Nitrococcus mobilis* Nb-231 (Pden_4236)	2	ND	DAMO_0778	34.9/96.8	14
	narH	65 (50)	80 (66)	0.0 (4×10^{-126})	EAR23356 *Nitrococcus mobilis* Nb-231 (Pden_4235)	1	ND	DAMO_0776	34.7/97.5	ND
	narI	57 (38)	71 (56)	8×10^{-64} (3×10^{-27})	Nwi_0778 *Nitrobacter winogradski* Nb-255 (Pden_4233)	1	ND	DAMO_0774	37.3/99.1	ND
	narJ	35 (31)	52 (43)	1×10^{-18} (0.002)	EAR23357 *Nitrococcus mobilis* Nb-231 (Pden_4234)	1	ND	DAMO_0775	41.3/99.3	ND
	narX	Not present in *M. oxyfera**								
Periplasmic nitrate reductase	*napA*	55 (51)	72 (63)	0.0 (0.0)	Glov_1056 *Geobacter lovleyi* SZ (Pden_4721)	1	11	DAMO_2411	33.9/88.2	23
	napB	44 (26)	55 (36)	7×10^{-6} (1.2)	EDO26469 *Nematostella vectensis* (Pden_4722)	1	ND	DAMO_2410	29.9/89.0	ND
	napD	Not present in '*M. oxyfera*'								
	napE	Not present in '*M. oxyfera*'								
	napC	Not present in '*M. oxyfera*'								
Nitrite reductase	*nirS*	58 (54)	72 (70)	0.0 (8×10^{-158})	Rcas_3430 *Roseiflexus castenholzii* DSM 13941 (Pden_2487)	18	96	DAMO_2415	35.0/90.8	192
	nirJ	46 (48)	69 (63)	4×10^{-111} (4×10^{-90})	MA3035 *Methanosarcina acetivorans* C2A (Pden_2494)	2	ND	DAMO_2413	25.0/88.0	ND
	nirF	45 (37)	64 (59)	5×10^{-95} (2×10^{-74})	EDN67735 *Beggiatoa* sp. PS (Pden_2490)	1	19	DAMO_2412	27.2/88.7	29
	fused nirD/G & nirH/L	46 (29)	64 (46)	5×10^{-87} (5×10^{-29})	Rxyl_0505 *Rubrobacter xylanophilus* DSM 9941 (Pden_2491)	1	ND	DAMO_2409	15.4/88.7	ND
Nitric oxide reductase	*norZ1*	65 (29)	77 (47)	0.0 (5×10^{-24})	Gmet_3493 *Geobacter metallireducens* GS-15 (Pden_2483)	1	ND	DAMO_1889	34.1/94.0	ND
	norZ2	34 (26)	52 (46)	2×10^{-121} (6×10^{-29})	Gmet_3493 *Geobacter metallireducens* GS-15 (Pden_2483)	78	ND	DAMO_2434	42.1/91.2	7
	norZ3	34 (26)	53 (46)	4×10^{-121} (2×10^{-27})	Gmet_3493 *Geobacter metallireducens* GS-15 (Pden_2483)	352	4	DAMO_2437	53.5/91.7	67
	norB	Not present in '*M. oxyfera*'								
	norC	Not present in '*M. oxyfera*'								
Nitrous oxide reductase	*nosZ*	Not present in '*M. oxyfera*'								
	nosD	Not present in '*M. oxyfera*'								
	nosF	Not present in '*M. oxyfera*'								
	nosY	Not present in '*M. oxyfera*'								
	nosL	50 (23)	69 (44)	6×10^{-43} (7×10^{-4})	Gsu1258 *Geobacter sulfurreducens* PCA (Pden_4215)	0	ND	DAMO_1959	17.6/88.9	ND

Table 4 | Genes encoding selected carbon metabolism enzymes from '*M. oxyfera*'.

Enzyme	Gene	Best BLASTP hit NCBI (BLASTP against *Methylococcus capsulatus* between brackets)				Transcript-ome coverage	No. of peptides detected	ORF identifier	Enrichment culture "Ooij" sequences	
		% Identity	% Similarity	Expected (E)-value	GenBank identifier				Average coverage /% identity	no. of peptides detected
Methane monooxygenase	*pmoA1*	= (53)	= (73)	= (1×10^{-76})	= (mca2854)	45	1	DAMO_2450	35.3/ 89.9	38
	pmoB1	= (40)	= (60)	= (1×10^{-84})	= (mca2853)	55	8	DAMO_2448	34.0/ 88.4	74
	pmoC1	53 (59)	68 (75)	3×10^{-73} (3×10^{-71})	CAE48351 *Methylocystis* sp. SC2 (mca2855)	36	3	DAMO_2451	63.7/ 90.8	30
	pmoC2	53 (59)	68 (75)	3×10^{-73} (3×10^{-71})	CAE48351 *Methylocystis* sp. SC2 (mca2855)			DAMO_2339		
	mmoX	Not present in '*M. oxyfera*'								
Methanol dehydrogenase	*mxaF1*	= (74)	= (88)	= (0.0)	= (mca0779)	62	69	DAMO_0112	2.4/ 89.7	ND
	mxaF2	68 (40)	80 (59)	0.0 (3×10^{-126})	Msil_3149 *Methylocella silvestris* BL2 (mca0779)	14	108	DAMO_0124	2.4/ 88.6	ND
	mxaF3	62 (49)	74 (63)	0.0 (3×10^{-154})	Minf_0992 *Methylacidiphilum infernorum* V4 (mca0299)	6	38	DAMO_0134	22.3/ 88.5	145
	mxaJ1	54 (61)	72 (76)	2×10^{84} (3×10^{-60})	ABE77337 *Methylomicrobium* sp. HG-1 (mca0780)	29	5	DAMO_0113	1.9/ 89.3	ND
	mxaJ2	46 (29)	62 (52)	2×10^{-118} (7×10^{-30})	Msil_3148 *Methylocella silvestris* BL2 (mca0300)	8	ND	DAMO_0125	3.2/ 89.9	ND
	mxaJ3	50 (35)	67 (53)	4×10^{-76} (8×10^{-39})	AAY96668 uncultured bacterium BAC10-4 (mca0300)	3	ND	DAMO_0136	23.9/ 87.3	5
	mxaG1	59 (52)	67 (66)	5×10^{-49} (6×10^{-43})	Veis_1827 *Verminephrobacter eiseniae* EF01-2 (mca0781)	16	3	DAMO_0114	1.3/ 88.6	ND
	mxaG2	50 (35)	68 (53)	1×10^{-20} (2×10^{7})	Msil_3147 *Methylocella silvestris* BL2 (mca0781)	6	2	DAMO_0127	2.7/ 89.3	ND
	mxaG3	43 (28)	56 (41)	5×10^{-18} (4×10^{-9})	AAY96667 uncultured bacterium BAC10-4 (mca0781)	5	6	DAMO_0138	30.6/ 87.2	13
	mxaI	62 (63)	80 (78)	4×10^{-25} (4×10^{22})	ACB32199 uncultured bacterium 16A2 (mca0782)	27	ND	DAMO_0115	0.8/ 88.2	ND
	mxaR	62 (62)	80 (82)	4×10^{-125} (5×10^{128})	Mfla_1895 *Methylobacillus flagellatus* KT (mca1525)	5	ND	DAMO_0116	1.7/ 89.1	ND
	mxaS	44 (45)	58 (56)	1×10^{-60} (4×10^{-56})	Mfla_1896 *Methylobacillus flagellatus* KT (mca0784)	2	ND	DAMO_0117	1.8/ 89.9	ND
	mxaA	= (33)	= (48)	= (7×10^{-31})	= (mca0785)	1	ND	DAMO_0118	2.4/ 89.4	ND

	mxaC	43 (46)	63 (60)	1×10^{-67} (3×10^{-59})
	mxaK	37 (31)	52 (48)	2×10^{-16} (3×10^{-13})
	mxaL	41 (44)	58 (56)	2×10^{-59} (1×10^{-50})
	mxaD	53 (47)	65 (65)	2×10^{-40} (9×10^{-41})
	mxaE1	42 (-)	66 (-)	4×10^{-75} (-)
	mxaE2	44 (-)	63 (-)	8×10^{-67} (-)
Formaldehyde dehydrogenase	*adhP*	Not present in *'M. oxyfera'*		
Formaldehyde activating enzyme	*fae*	70 (57)	80 (72)	7×10^{-60} (2×10^{-37})
Methylene H_4MPT dehydrogenase	*mtdB*	40 (42)	55 (58)	5×10^{-49} (1×10^{-45})
Methenyl H_4MPT cyclohydrolase	*mch*	53 (37)	68 (57)	5×10^{-84} (3×10^{-60})
Formyltransferase/ hydrolase complex	*fhcA*	55 (48)	70 (65)	6×10^{-162} (8×10^{-148})
	fhcB1	47 (25)	65 (44)	6×10^{-119} (5×10^{-30})
	fhcB2	46 (25)	63 (45)	4×10^{-108} (3×10^{-29})
	fhcC	50 (34)	66 (56)	2×10^{-68} (7×10^{-38})
	fhcD	= (55)	= (71)	= (5×10^{-81})
Methylene H_4F dehydrogenase/ Methenyl H_4F cyclohydrolase	*folD*	58 (-)	74 (-)	1×10^{-87} (-)
5-formyl H_4F cycloligase	*mthfs*	Not present in *'M. oxyfera'*		
Formate H_4F ligase	*fhs*	Not present in *'M. oxyfera'*		
Formyl H_4F deformylase	*purU*	64 (-)	80 (-)	3×10^{-106} (-)
Formate dehydrogenase	*fdhA1*	57 (40)	72 (56)	0.0 (0.0)

MDMS009_1430 *Methylophaga* sp DMS010 (mca1528)	1	ND	DAMO_0119	1.8/ 88.1	ND
Mfla_2036 *Methylobacillus flagellatus* KT (mca1529)	1	ND	DAMO_0120	3.2/ 89.6	ND
Mfla_2035 *Methylobacillus flagellatus* KT (mca1530)	1	2	DAMO_0121‡	1.4/ 89.4	ND
ABS82778 uncultured *Methylophaga* sp. (mca0789)	1	2	DAMO_0121‡	1.4/ 89.4	ND
MDMS009_1396 *Methylophaga* sp DMS010 (-)	1	ND	DAMO_0122	2.8/ 89.1	ND
AAY96670 uncultured bacterium BAC10-4 (-)	4	2	DAMO_0128	3.1/ 90.0	ND
EDL60092 *Planctomyces maris* DSM 8797 (mca2866)	19	13	DAMO_0454	44.6/ 95.2	44
Bphy_6482 *Burkholderia phymatum* STM815 (mca3019)	4	3	DAMO_0455	32.3/ 93.6	40
AAY96682 uncultured bacterium BAC10-4 (mca2863)	3	18	DAMO_0461	21.1/ 87.5	19
AAY96680 uncultured bacterium BAC10-4 (mca2859)	5	2	DAMO_0458	39.3/ 93.6	39
MK0259 *Methanopyrus kandleri* AV19 (mca2860)	4	4	DAMO_0457	39.6/ 92.0	40
AAY96679 uncultured bacterium BAC10-4 (mca2860)	0/0	ND	DAMO_1135/ DAMO_1136	33.7/ 97.1	ND
AAY96681 uncultured bacterium BAC10-4 (mca2857)	5	ND	DAMO_0460	36.0/ 90.8	5
= (mca2858)	4	ND	DAMO_0459	35.1/ 91.6	34
Moth_1516 *Moorella thermoacetica* ATCC 39073 (-)	1	ND	DAMO_1852	31.2/ 88.4	ND
EDT34736 *Geobacillus* sp. WCH70 (-)	1	ND	DAMO_2586	36.3/ 91.1	ND
EEG07387 *Lutiella nitroferrum* 2002 (mca1391)	0	2	DAMO_1138	2.3/ 89.4	ND

	fdhA2	43 (40)	62 (56)	0.0 (0.0)
	fdhB1	54 (43)	68 (59)	5×10^{-125} (1×10^{-67})
	fdhB/C2	40 (36)	59 (54)	2×10^{-107} (2×10^{-79})
	fdhD	Not present in *M. oxyfera*		
Serine glyoxylate aminotransferase	*sga*	53 (34)	71 (53)	5×10^{-113} (7×10^{-53})
Hydroxypyruvate dehydrogenase	*hprA*	Not present in '*M. oxyfera*'		
Serine hydroxymethyltransferase	*glyA*	68 (56)	80 (71)	7×10^{-170} (4×10^{-119})
Glycerate kinase	*gckA*	Not present in '*M. oxyfera*'		
Phosphoenolpyruvate carboxylase	*ppc*	54 (-)	70 (-)	1×10^{-151} (-)
Malyl-CoA lyase	*mcl*	Not present in '*M. oxyfera*'		
Hexulose-6-phosphate synthase	*hspA*	Not present in '*M. oxyfera*'		
Hexulose-6-phosphate isomerise	*sbgU*	Not present in '*M. oxyfera*'		
Ribulose bisphosphate carboxylase	*cbbL*	83 (58)	91 (72)	0.0 (1×10^{-147})
	cbbS	68 (33)	86 (63)	2×10^{-54} (5×10^{-15})
Phosphoribulosekinase	*cbbP1*	67 (55)	78 (69)	9×10^{-112} (5×10^{-93})
	cbbP2	42 (34)	59 (45)	2×10^{-67} (9×10^{-6})
Fructose 1,6-bisphosphatase/ Sedoheptulose 1,7-bisphosphatase	*cbbF-I*	49 (-)	67 (-)	2×10^{-91} (-)
	cbbF-II1	58 (-)	78 (-)	4×10^{-103} (-)
	cbbF-II2	58 (-)	76 (-)	1×10^{-102} (-)
	cbbF-II3	58 (-)	75 (-)	1×10^{-104} (-)

Nther_0100 *Natranaerobius thermophilus* JW/NM-WN-LF (mca2576)	1	27	DAMO_0853	26.8/ 90.3	19
ACD29487 *Ralstonia pickettii* 12J (mca1392)	0	2	DAMO_1137	3.2/ 87.6	ND
Csac_0620 *Caldicellulosiruptor saccharolyticus* DSM 8903 (mca1392)	1	29.4/30	DAMO_0854	21.3/ 89.7	5
EAY55805 *Leptospirillum* sp. Group II UBA (mca1406)	3	13	DAMO_3099	27.1/ 88.1	10
SYN_02367 *Syntrophus aciditrophicus* SB (mca1660)	3	18	DAMO_1077	28.4/ 89.7	8
Daud_0773 *Candidatus* "Desulforudis audaxviator" MP104C (-)	0	ND	DAMO_2168	33.0/ 97.5	12
RC1_4061 *Rhodospirillum centenum* SW (mca2743)	1	12	DAMO_2165	35.1/ 97.5	47
Q09125 *Porphyridium aerugineum* (mca2744)	2	4	DAMO_2166	39.9/ 99.3	5
Tbd_2447 *Thiobacillus denitrificans* ATCC 25259 (mca3051)	1	3	DAMO_2116	12.9/ 93.4	ND
Minf_1261 *Methylacidiphilum infernorum* V4 (mca3051)	3	ND	DAMO_2653	39.4/ 88.1	4
gvip434 *Gloeobacter violaceus* PCC 7421 (-)	1	5	DAMO_2163	28.3/ 89.4	2
Rcas_1213 *Roseiflexus castenholzii* DSM 13941 (-)	2	17	DAMO_2650	29.7/ 90.7	10
MXAN_4455 *Myxococcus xanthus* DK 1622 (-)	2	3	DAMO_2986	34.7/ 89.5	ND
EEB33574 *Desulfovibrio piger* ATCC 29098 (-)	3	22	DAMO_0174	23.7/ 89.6	2

Chapter 5

Integration and outlook

"Halász & Simek (1968) have isolated, but apparently not characterized, strains of a monoflagellated Gram negative organism. These were capable of growth on methane as the sole carbon source and the disappearance of methane during growth was demonstrated. An unusual feature was that the growth of these organisms was accompanied apparently by the production of oxygen."

(Wake et al., 1973)

During the sometimes longer-than-necessary literature research for this thesis it was a slight thrill to find the citation preceding this chapter. It turned out, however, that the reported oxygen production by flagellated methanotrophs was an artifact of translation, and not claimed in the original publication (Halász & Simek, 1968).
This final chapter focuses on some aspects that merit more in-depth discussion than was possible in the previous chapters, or that emerged after publication. Besides more tangible literature findings than the above, it also presents some results from unpublished and ongoing work, together with an outline of promising future research lines.

Role of Archaea

After the disappearance of the archaea initially co-enriched with the NC10 bacteria (Raghoebarsing et al., 2006) from the enrichment culture (chapter 2), and a methane activation mechanism other than reverse methanogenesis had been demonstrated (chapter 4), the question about their role and lifestyle – methanotrophic versus methanogenic – is still not answered. The phylogeny of both the *mcrA* (α-subunit of the Methyl-Coenzyme M Reductase, MCR) and the 16S rRNA gene places this group, termed AOM-associated archaea (AAA) by Knittel & Boetius (2009), in between known methanotrophs (ANME 2) and methanogens of the genera *Methanosarcina* and *Methanosaeta* (Raghoebarsing et al., 2006; Ettwig et al., 2008; Knittel & Boetius, 2009). The habitats where sequences closely related to AAA have been found in nature allow both interpretations (see table 1). Only few studies present hints on the physiological properties of the AAA, but unfortunately these are not coherent. The ZC-a cluster archaea (up to 99.4 % aa identity of the mcrA) enriched from the Tibetan Zoige plateau wetlands (Zhang et al., 2008) are most likely versatile methanogens. In contrast, in Lake Cadagno sediments the AAA, detected by FISH and clone libraries, occur in the sediment zone where porewater chemistry and methane isotopic composition indicated anaerobic methane oxidation coupled to sulfate reduction (Schubert et al., 2007). A methanotrophic lifestyle is also suggested by Hu et al. (2009), who obtained a denitrifying, methane-oxidizing enrichment culture consisting of 40 % archaea (98.2 % identity of rRNA gene) and 30 % NC10 bacteria. As no electron donors other than methane were supplied, and the archaea made up a large part of the enriched community, the authors' conclusion that they should be methanotrophic rather than methanogenic seems plausible, although methanogenesis from leaking intermediates (methanol, formaldehyde) derived from the NC10 bacteria is another possible explanation. As the reaction of the MCR is in principle bidirectional, and it functions in both methanotrophs and methanogens (Zehnder & Brock, 1979; Scheller et al., 2010),

the AAA may well be an intermediate group that can adopt either lifestyle, depending on the prevailing environmental conditions.

Table 1 | Habitats of archaea closely related to the ones found in the original enrichment culture (Raghoebarsing et al., 2006).

habitat	% similarity (gene)[1]	publication	NCBI accession no.
rice roots	99 (*mcrA*)	Conrad et al., 2008	AM746876 +
	97 (*mcrA*)	Lüders et al., 2001	AF313810
minerotrophic fen	99 (16S rRNA)	Cadillo-Quiroz et al., 2008	EU155958 +
peat bog soil	97 (16S rRNA)	Cadillo-Quiroz et al., 2006	DQ301886 +
	99 (*mcrA*)	Zhang et al., 2008	EU275991
river sediments	99 (16S rRNA)	Rastogi et al., 2009	EU247313 +
	98 (16S rRNA)	Liesegang et al., unpublished	EU244163 +
lake sediments	99 (16S rRNA)	Stein et al., 2001	AF293017
	97 (16S rRNA)	Schubert et al., 2007	AM851080
contaminated soil	99 (16S rRNA)	Kasai et al., 2005	AB161352
groundwater	97 (16S rRNA)	Nedelkova et al., unpublished	AJ583383
	97 (16S rRNA)	Giehring et al., unpublished	DQ354743
goldmine borehole water	97 (16S rRNA)	Maclean et al., 2007	EF446788
glacier soil	97 (16S rRNA)	Boyd et al., unpublished	GU122860
submarine permafrost	97 (16S rRNA)	Feige et al., unpublished	FJ982666 +
antarctic cold seeps	98 (16S rRNA)	Niemann et al., unpublished	FN429776
bioreactor with a denitrifying, methane-oxidizing enrichment	98 (16S rRNA)	Hu et al., 2009	FJ907180

[1]compared to sequences of 16S rRNA gene clone D-ARCH (DQ369741) and *mcrA* clone A14 (EU495303).

+ several similar sequences in Genbank.

Methodological aspects

The research presented in this thesis highlights certain known, but nevertheless often ignored shortcomings of established methodology, which will be briefly discussed in the following section.

Molecular detection I The bias introduced by PCR-based techniques is long known and very well documented (von Wintzingerode et al., 1997; Acinas et al., 2005). Quantitative inferences from clone libraries based on DNA extracted from environmental samples are now generally accepted to be very difficult if not impossible. Chapter 3 of this thesis provides an example for the difficulty of even drawing purely qualitative conclusions following this approach. Although NC10 bacteria already dominated the analyzed enrichment culture, their DNA could be readily extracted and, as analyzed in retrospect, their 16S rRNA gene had only one mismatch at the 5' end with one of the general bacterial primers used (1545R from Juretschko et al., 1998), their 16S rRNA gene was not represented in a (rather small: n=31) clone library and thus escaped detection. Yet, even in recent high-profile papers (like e.g. Beal et al., 2009), these type of clone libraries are still used to infer the involvement of phylogenetic groups in specific processes.

Culturing I Another lesson from this and previous work (Strous et al., 1998; Könneke et al., 2005; Raghoebarsing et al., 2006) is that long incubation times and low substrate concentrations may be crucial for the enrichment of slowly-growing microorganisms, and that simple lack of patience might have prevented earlier discovery. Furthermore, some commonly used materials like certain black butyl rubber stoppers for anaerobic incubations and polycarbonate medium bottles (Nalgene) apparently release compounds toxic for '*Candidatus* Methylomirabilis oxyfera'. This was suggested by the loss of enrichment culture 'Twente' after medium supply in the aforementioned bottle type (unpublished and unpublishable) and documented by total inhibition of substrate conversion in batch experiments (chapter 3). This may be among the reasons for the failure of several attempts to obtain '*M. oxyfera*' in pure culture. First, '*M. oxyfera*' planktonic cells were physically enriched by size exclusion of cell aggregates and microorganisms >1.2 µm and <0.7 µm. Subsequently, strictly anaerobic dilution-to-extinction incubations in liquid medium using several electron donors (besides methane also methanol, acetate, formate, succinate and fumarate) were set up and monitored for several months, but yielded growth (of fast-growing heterotrophs) in low dilutions only (together with K. van de Pas-Schoonen, unpublished). Also anaerobic and microaerobic incubations in liquid or gelrite-solidified-medium using alternative stopper material (chapter 3) with methane or methanol as electron donor were not successful (F. van Dooren, unpublished). Possible causes of failure are the slow growth rate of '*M. oxyfera*' under sub-optimal conditions, dependence on cofactors supplied by other community members or nitrite toxicity. With pure cultures, several remaining questions on the metabolism of '*M. oxyfera*' could easily be answered: Could external oxygen be used as an electron acceptor and how fast is it reduced? Could methanol be used as an electron donor? Would '*M. oxyfera*' produce N_2O? In the presently available mixed

cultures, the activity of other microorganisms hampers the interpretation of such experiments. Even small numbers of aerobic organisms may contribute significantly to oxygen consumption of the total biomass. Accordingly, methanol-oxidizers with high specific activity may mask the activity of the generally slow '*M. oxyfera*'. Upon extended incubation (days) with oxygen or methanol, the '*M. oxyfera*'-dominated community changed towards dominance of aerobic methanotrophs or denitrifying methanol oxidizers. Bacteria representing these lifestyles (*Methylocystis* and *Hyphomicrobium* species) could easily be isolated using the aforementioned approaches (F. van Dooren, unpublished). The '*M. oxyfera*'-dominated enrichment culture did produce N_2O (few percent of produced N_2; see figure 6 in chapter 3, and figure 3 in chapter 4), but whether this is attributable to other denitrifying community members or to a metabolic side route (NO-detoxification) of '*M. oxyfera*' remains to be shown. To this end, also physical separation of '*M. oxyfera*' from other microorganisms is an option. For the aforementioned size exclusion filtration, the cell aggregates were left undisturbed and only the small amounts of naturally loose planktonic cells were used because only little biomass was needed for the extinction-to-dilution incubation. For physiological measurements, however, much more cells are required. This can be achieved by optimizing existing methods for gentle cell aggregate disruption, followed by either Percoll density gradient centrifugation (Strous et al., 1999) or flow cytometry (Czechowska et al., 2008). Even more promising would be the growth of '*M. oxyfera*' as suspended single cells in a membrane bioreactor (Van Der Star et al., 2008). In the case of the similarly slowly growing anammox bacteria, this cultivation method yielded a shorter doubling time and higher degree of enrichment (<2 weeks, >97 %; Van der Star et al., 2008; 8 days, B. Kartal & W. Maalcke personal communication). Additionally, diffusion limitation and production of extracellular polymeric substances are reduced.

'Omics' and beyond I Genome analysis can be very fruitful to develop hypotheses about pathways present in an organism (chapter 4), but conclusions should always be based on additional lines of evidence. Inferring physiology from genome information alone can be misleading. For instance, the *pmo* gene cluster of '*M. oxyfera*' found on a contig recovered from an environmental metagenome would have been wrongly interpreted as an indication for the presence of aerobic methane oxidizers. The lack of nitrous oxide reductase may have led to the conclusion that the organism in question is an incomplete denitrifier, producing nitrous oxide instead of dinitrogen as end product. The cultivation of organisms, ideally, but not necessarily in pure culture, is still a prerequisite for the discovery of novel pathways (Leadbetter, 2003; Oremland et al., 2005; Giovannoni & Stingl, 2007). It should not be expected that *in silico* studies alone will advance our understanding of the approximately 50 % unknown proteins encoded in environmental metagenomes (Vieites et al., 2009).

Despite the lack of a pure culture, several predictions based on the analysis of the '*M. oxyfera*' genome, proteome and transcriptome (chapter 4) could be experimentally tested using the enrichment cultures described in chapters 2 and 3. The combination of complementary approaches was necessary to demonstrate a so far unique bacterial metabolism.

The most fascinating question in the biochemistry of '*M. oxyfera*' is the identification and characterization of the enzyme responsible for oxygen production. As a first step towards an answer, the activity should be localized either in the cytosolic or membrane-fraction of lysed cells. Also, determining the transcription and expression levels of candidate genes, e.g. the multi-copper oxidases and NO reductases, under anaerobic vs. microaerobic conditions may be informative. Studies with knock-out mutants, one of the classical ways to investigate enzyme functions, seem unrealistic at present, as '*M. oxyfera*' cannot be grown in pure culture and doubles too slowly to use any of the established screening methods. Alternatively, heterologous expression of candidate genes in suitable hosts and subsequent purification of the expressed proteins may be helpful. In this case, negative results would not be informative, because posttranslational processing and folding may be crucial for proper functioning of the enzyme or enzyme complex. Purification of enzymes directly from the enrichment cultures is possibly the most promising approach, and would be facilitated by the above-mentioned development of a planktonic cell culture. Additionally, a faster and easier oxygen production assay needs to be developed. The stable-isotope method used in chapter 4 to measure oxygen production is very sensitive, but depends on the conversion of nitrite to nitric oxide by the cells. The use of ^{18}O-labeled nitric oxide overcomes the first problem, but is still too time-consuming for the screening of a high number of fractions during purification. To this end, a glucose-oxidase (or another one depending on reactions with oxygen, e.g. luciferase) assay may be used in reverse: providing excess glucose under anaerobic conditions would lead to a color change proportional to the oxygen production.

Environmental detection

Activity I Most studies that quantified denitrification in the environment - "a miserable process to measure" (Groffman et al., 2006) - do not discriminate between different electron acceptors. Dependent on the method used, methane as electron donor would have been either subsumed under bulk denitrification or entirely overlooked. The acetylene-block technique, based on the measurement of N_2O following inhibition of nitrous oxide reductase, is problematic for many reasons (Oremland & Capone, 1988; Groffman et al., 2006), and the inhibition of methane monooxygenase (Prior & Dalton, 1985; chapter 4) adds to the arguments against its use for quantitative inferences. Slurry

experiments may yield potential process rates, but are also inappropriate to infer *in situ* activities, because poorly soluble substrates like methane or hydrogen are very likely to be lost, and the spatial structure of the sediment with its microzonation is disturbed. Methods using undisturbed cores for flow-through experiments, microprofiling, addition of small amounts of labeled substrates and combinations thereof (Laverman et al., 2007; Gao et al., 2010) capture the natural processes much better, but have not yet been combined with measurements of gaseous denitrification substrates.

The assessment of the environmental importance of denitrifying anaerobic methane oxidation was originally one of the aims of the present thesis. Besides the Ooijpolder drainage ditch sampled for the inoculum of the enrichment culture of '*M. oxyfera*' (chapter 3), also other sites have been investigated in the framework of the present PhD project. Several samples from sites with methane production in deeper sediment layers and nitrate supply with overlying or seeping water were tested for anaerobic methane oxidation activity by incubating the sample with nitrate, nitrite and ^{13}C-labeled methane: Ooijpolder and Nederasselt drainage ditch sediments, Horstermeerpolder aquatic sediment and peat soil (Hendriks et al., 2007), Scheldt river tidal freshwater sediments (Laverman et al., 2007) and Brunssumerheide peat soil (van Dijk et al., 2009). Unfortunately, with the exception of the Brunssumerheide samples, none of these showed production of $^{13}C\text{-}CO_2$ from labeled methane under anaerobic conditions. In most cases this can be attributed to the use of inhibiting black butyl rubber stoppers (chapter 3). Additional methodological problems include blurring of the signal due to methanogenic activity accompanied by trace methane oxidation (Zehnder & Brock, 1979) and dilution of the presumably active sediment layer with inactive ones. Because of the steep redox gradients occurring in aquatic sediments, this may only be prevented by using thin slices of sediment cores in which the narrow denitrifying zone has been localized e.g. by microsensor measurements. Also the use of ^{14}C-labeled methane would increase sensitivity, but would involve difficult-to-handle radioactive compounds.

In the groundwater-influenced peat layers of the Brunssumerheide, gradients are less steep than in eutrophic sediments, and a zone of concomitant methane and nitrate depletion had been localized by porewater measurements (van Dijk et al. (2009) and ongoing work). Incubated anaerobically with ^{13}C-labeled methane and ^{15}N-labeled nitrite, this peat layer showed increasing activity after a prolonged period of time (> 2 months). This indicated enrichment, not the *in situ* activity of methane-oxidizing microorganisms. Current studies focus on more detailed *in situ* substrate profiles and the characterization of the microorganisms responsible for methane oxidation in this habitat.

Molecular detection I Comparable to anaerobic ammonium-oxidizing bacteria or ammonia-oxidizing crenarchaeota (reviewed in Francis et al., 2007), '*M. oxyfera*' -like

bacteria may be much more wide-spread in the environment than was known before the development of specific molecular tools for their detection. Excluding the sequences obtained in chapter 3, at present approximately sixty 16S rRNA gene sequences belonging to the NC10 phylum are deposited in Genbank. These were obtained from diverse anaerobic environments, such as lake, river and deep-sea sediments, aquifers, water-logged soils, caves and anaerobic digesters. So far, methane-oxidizing activity can only be attributed to group *a*, which comprises close relatives of '*M. oxyfera*', whereas inferring physiological traits for other species of the NC10 phylum is impossible at present.

The 16S rRNA primers targeting group *a* and *b* (chapter 3) have now also been applied to several other environmental samples, and in most cases sequences of the NC10 phylum, predominantly affiliated with group *a*, could be detected (together with T. van Alen, B. Zhu, F. Luesken, M. Rodriguez Diaz, unpublished). One of these is the Banisveld landfill leachate plume briefly discussed in chapter 1, where occurrence of anaerobic methane oxidation coupled to denitrification was inferred from redox species measurements and the isotopic composition of dissolved methane (van Breukelen & Griffioen, 2004). In February 2009, groundwater samples for DNA extraction were taken and methane and nitrate concentration profiles were determined. Methane concentrations were still comparable to the situation in 2001, but nitrate was not detectable anymore, which is in line with the observations of decreasing nitrate influx at this site already reported by van Breukelen & Griffioen (2004). Although sequences of NC10 bacteria were found using the primers described in chapter 3 (M. Rodriguez Diaz, unpublished), the lack of nitrate and nitrite due to reduced agricultural activity in the surrounding area rendered the site not promising enough to attempt *in situ* or *in vitro* detection of '*M. oxyfera*' activity. The Brunssumerheide field site, where profiles of methane and nitrate pointed to anaerobic methane oxidation coupled to nitrate reduction and where this activity could subsequently be demonstrated (see above), is currently under investigation with respect to its microbial community.

Functional marker genes for methane oxidation and denitrification, e.g. *pmo, smo, nirK* and *nirS*, are often used to assess the diversity and/or abundance of functional guilds in the environment. The comparison of the most commonly used primer pairs for the detection of *pmoA* (Holmes et al., 1995) and *nirS* (Throbäck et al., 2004) with the corresponding genes encoded in the genome of '*M. oxyfera*' showed that the failure of amplification (van der Biezen, 2007) could be attributed to several mismatches. Due to the ever increasing known diversity of these genes the design of truly universal primers is most likely impossible. New primers are currently developed on basis of the two available '*M. oxyfera*' genomes and related environmental sequences (T. van Alen, B. Zhu, F. Luesken, K. Ettwig et al., in prep.).

Lipids and carbon assimilation

Knowledge of the lipid profile of '*M. oxyfera*' would potentially facilitate its environmental detection, in a similar fashion as employed for e.g. aerobic methanotrophs (Evershed et al., 2006; Bodelier et al., 2009) or anaerobic methanotrophic consortia (Pancost et al., 2000; Blumenberg et al., 2004). In contrast to nucleic acids, lipids might persist over geological timescales in many sedimentary environments and allow reconstruction of past biogeochemical regimes (Kuypers et al., 2001; Brocks & Banfield, 2009). In most environments, methane is strongly depleted in the heavy carbon isotope ^{13}C (<-50 ‰ vs. VPDB for most biogenic methane, Whiticar 1999). Therefore, the combination of specific lipids and isotopically light carbon makes a good indicator for organisms that derive their cell carbon from methane. In consortia of methanotrophic archaea and sulfate-reducing bacteria, the archaeal lipids reach $\delta^{13}C$ values clearly below -100, and down to -133 ‰ (Hinrichs et al., 1999; Boetius et al., 2000), the lowest naturally occurring values in organisms (Schidlowski, 2001).

Knowledge of typical lipids also enables stable isotope labeling in laboratory or enclosure experiments that help in identifying active populations (Boschker & Middelburg, 2002; Blumenberg et al., 2005; Crossmann et al., 2005). The phospholipid fatty acid (PLFA) profile of the '*M. oxyfera*'-dominated, nitrite-reducing, methane-oxidizing enrichment culture has been determined several times (Raghoebarsing et al., 2006; S. Schouten, I. Rijpstra, K. Ettwig & F. Luesken, unpublished). Major components (5-40 %) were $C_{16:0}$, 10Me-$C_{16:0}$, 10Me-$C_{16:1\omega10}$, and $C_{18:1}$. Especially the methylated PLFAs could be of diagnostic value because they are generally not very abundant. 10Me-$C_{16:0}$ is a major constituent of the total lipids of sulfate-reducing bacteria of the genera *Desulfobacter* and *Desulfobacula* (6-25 %; Dowling et al., 1986; Kuever et al., 2001; Rütters et al., 2002). It is also sometimes attributed to Actinobacteria (Denef et al., 2009). Among aerobic methanotrophs, it has only been detected in small amounts in one single species (*Methylocystis heyeri*, Bodelier et al., 2009). In few cases its occurrence was correlated with atmospheric aerobic methane oxidation (Crossmann et al., 2005; Knief et al., 2006) or anaerobic methane oxidation coupled to sulfate-reduction (Alain et al., 2006), and in one case with a low isotopic value of -69.4 ‰ (Orphan et al., 2001). The mono-unsaturated fatty acid 10Me-$C_{16:1\omega10}$ is even less common, and to the best of our knowledge not covered in the literature.

The setup to obtain solid evidence for the mentioned PLFAs being synthesized by '*M. oxyfera*' seems straightforward: incubation with ^{13}C-labeled methane for an extended period of time (in the order of days) to allow for sufficient incorporation of carbon into bacterial biomass. These experiments were already performed (Raghoebarsing et al., 2006; K. Ettwig and F. Luesken, unpublished), but the results were always difficult to interpret. Substantial amounts of methane-derived carbon were incorporated in only

few lipids: the $C_{18:1}$, which was initially less depleted in ^{13}C than other abundant ones, the widely distributed $C_{16:0}$ PLFA and diplopterol (both having low diagnostic value), and into some minor lipids ($C_{14:0}$ FA, unmethylated $C_{16:1}$ PLFA). The earlier-mentioned PLFAs that make up the bulk of the biomass remained unlabeled. This might be due to the exclusive growth of microaerophilic methanotrophs or heterotrophic bacteria incorporating methane-derived exudates released by '*M. oxyfera*'. Two explanations are possible for the failure of detecting incorporation of ^{13}C into inferred '*M. oxyfera*'-lipids. First, the batch experiments may not be suitable for growth and carbon incorporation of *M. oxyfera*, although activity in short-term-experiments could be measured accurately as demonstrated in chapters 3 and 4. Second, '*M. oxyfera*' may not be assimilating carbon from methane, but exclusively from CO_2. This is in contrast to the α- and γ-proteobacterial methane-oxidizing bacteria and the anaerobic, archaeal methanotrophs, which derive at least a substantial part of their cell-carbon from methane (Blumenberg et al., 2005; Trotsenko & Murrell, 2008). Instead, '*M. oxyfera*' might be fixing CO_2 via the Calvin-Benson-Bassham cycle, which is also hypothesized to function in the acidophilic, methane-oxidizing Verrucomicrobia (Op den Camp et al., 2009). This cycle, of which the enzyme ribulose-bisphosphate carboxylase catalyzes the initial reaction with CO_2, is typically considered to be associated with an autotrophic lifestyle (Tabita, 1988). '*M. oxyfera*' encodes, transcribes and expresses all genes of this pathway, whereas it lacks several genes of the serine and ribulose-monophosphate cycles present in proteobacterial methanotrophs (see table 3 in chapter 4). A future experimental strategy could be the supply of labeled carbon compounds to continuous cultures to avoid the problems associated with batch incubations. Comparison of labeling quantity and pattern following ^{13}C-methane or ^{13}C-HCO_3 supply could then also contribute to elucidating the pathway of carbon assimilation in '*M. oxyfera*'.

Wider distribution of the oxygen-producing pathway?

Relatives of '*M. oxyfera*' can now be investigated in the environment with the mentioned existing and still-to-be-developed molecular tools. The pathway they employ, however, may also be present in not closely related, nitrite-reducing microorganisms. It would allow them to access substrates like aromatic and aliphatic hydrocarbons, which are difficult to activate under anaerobic conditions.

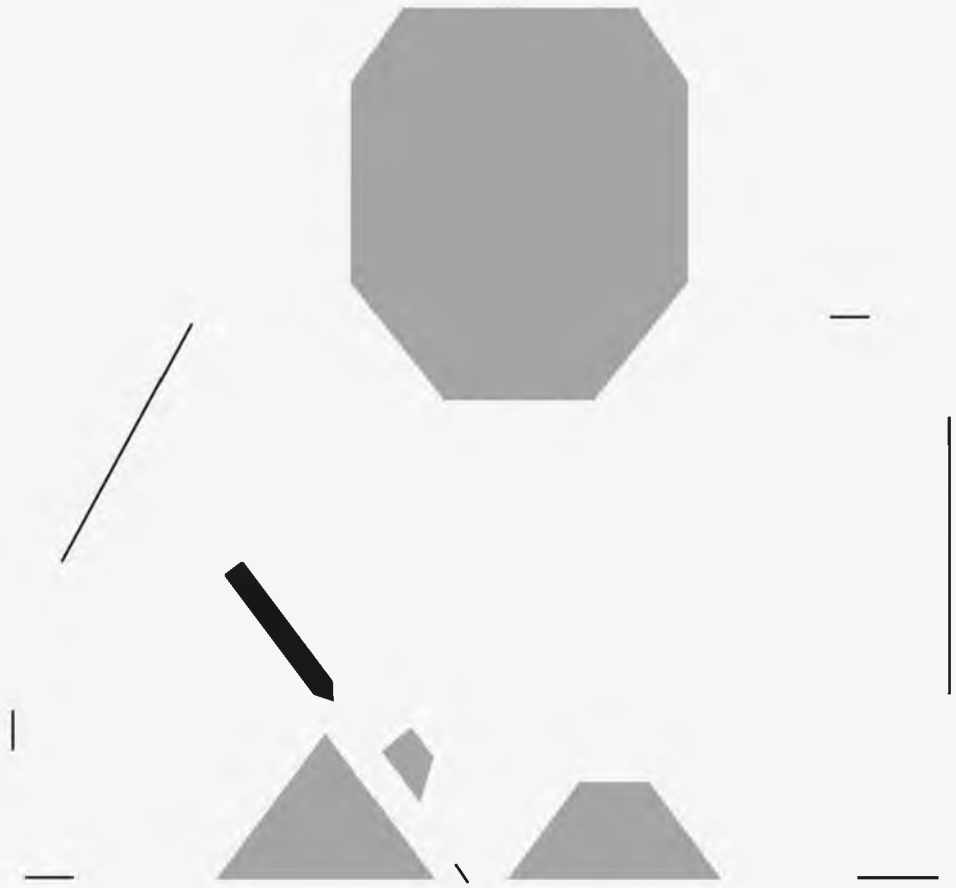
A likely candidate for this is the facultative denitrifying γ–proteobacterium strain HdN1, which grows on a wide variety of substrates, including C6- to C20-alkanes (Ehrenreich et al., 2000; Zedelius et al., 2010). The organism was recently shown to require oxygen, nitrate or nitrite for growth on hexadecane, whereas for growth on the corresponding alcohol and fatty acid nitrous oxide was sufficient as an electron acceptor (Zedelius et al., 2010). Like '*M. oxyfera*', HdN1 did not contain recognizable genes for the glycyl-

radical-catalyzed activation of alkanes, like alkylsuccinate synthase. Instead, two or possibly three monooxygenases were encoded in the genome. These findings suggest that an intermediate, possibly oxygen formed from nitrate or nitrite, was responsible for alkane activation.

Short-chain alkanes (<6 C atoms) serve as a (poor) growth substrate for some sulfate-reducing bacteria (Kniemeyer et al., 2007; Savage et al., 2010). So far, no microorganisms could be enriched or isolated with these compounds under denitrifying conditions (Ehrenreich et al., 2000). '*M. oxyfera*' was shown to oxidize ethane, propane and butane with comparable rates as methane (30, 116 and 27 %, respectively, calculated per mol C; unpublished results), which can be explained by the well known promiscuous activity of pMMO (Leadbetter & Foster, 1958, 1960; Hazeu & De Bruyn, 1980). If this activity would sustain the growth of '*M. oxyfera*' is doubtful, because the resulting alcohols and aldehydes usually cannot be metabolized by methanotrophic bacteria (Hazeu & De Bruyn, 1980; Patel et al., 1980). The attempt to obtain a nitrite-reducing enrichment culture with propane instead of methane as electron donor under the same reactor-operating conditions and with the same inoculum as described in chapter 3 was not successful within 9 months (unpublished results). To this end, however, an inoculum from sites where these alkanes naturally occur (Martini et al., 2003; Hinrichs et al., 2006; Niemann et al., 2006) would be more promising.

Chlorite dismutase, the enzyme that produces oxygen from chlorite (ClO_2^-) in chlorate-reducing bacteria (van Ginkel et al., 1996) has also been found in bacteria and archaea which cannot grow with chlorate as electron acceptor. Its natural function in these organisms, e.g. in *Thermus* (Ebihara et al., 2005), *Nitrospira* (Maixner et al., 2008), *Listeria* and *Nitrobacter* (Füreder, 2009) may thus be other than only respiratory or detoxifying. In *Haloferax volcanii*, chlorite dismutase is hypothesized to produce oxygen for a monooxygenase encoded in the same operon (Bab-Dinitz et al., 2006). *Pseudomonas chloritidismutans* can grow on alkanes with oxygen or chlorate as electron acceptors, but under denitrifying conditions only substrates requiring less activation energy are degraded (Mehboob et al., 2009). These results suggest that oxygen produced through chlorite reduction could be used for alkane activation by a monooxygenase. The ß-proteobacterium *Dechloromonas aromatica* strain RCB can degrade benzene under several reducing conditions: with oxygen, chlorate and also with nitrate (Coates *et al.*, 2001). In the genome of strain RCB, however, only known genes for the aerobic activation of aromatic compounds are encoded, like several mono- and dioxygenases (Salinero *et al.*, 2009). Signature genes of anaerobic hydrocarbon activation (Heider, 2007), like the glycyl-radical enzyme benzyl-succinate synthase cluster, are missing. Physiological experiments under nitrate-reducing conditions strongly suggest the involvement of a hydroxyl radical-mediated activation leading to phenol as primary intermediate (Chakraborty & Coates, 2005). Under chlorate-reducing

conditions this can be explained by the above-mentioned intermediate oxygen production, as it is known from other anaerobic, chlorate-reducing benzene-degraders (Weelink *et al.*, 2008). Salinero et al. (2009) therefore pose the question, whether "oxidation [of benzene] is dependent on intracellularly produced oxygen", and this thesis provides clues how this could also occur under denitrifying conditions. The unknown enzyme that produces oxygen in '*M. oxyfera*' might thus be more widespread in the prokaryotic world. Because nitrate and nitrite are far more abundant and important in the environment than chlorate, they could play an important, still-to-be-explored role in the degradation of recalcitrant hydrocarbons.



References

adapted from ‘De schrijver’ by Bart van der Leck, 1923 (Kröller-Müller Museum, Otterloo)

Acinas, SG, R Sarma-Rupavtarm, V Klepac-Ceraj & MF Polz (2005): PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl. Environ. Microbiol.* **71**: 8966-8969.

Aeckersberg, F, F Bak & F Widdel (1991): Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch. Microbiol.* **156**: 5-14.

Aerts, R & H De Caluwe (1999): Nitrogen deposition effects on carbon dioxide and methane emissions from temperate peatland soils. *Oikos* **84**: 44-54.

Alain, K, T Holler, F Musat *et al.* (2006): Microbiological investigation of methane- and hydrocarbon-discharging mud volcanoes in the Carpathian Mountains, Romania. *Environmental Microbiology* **8**: 574-590.

Allen, MB & CB van Niel (1952): Experiments on bacterial denitrification. *J. Bacteriol.* **64**: 397-412.

Alperin, MJ & WS Reeburgh (1985): Inhibition Experiments on Anaerobic Methane Oxidation. *Appl. Environ. Microbiol.* **50**: 940-945.

Amaral, JA, C Archambault, SR Richards & R Knowles (1995): Denitrification associated with Groups I and II methanotrophs in a gradient enrichment system. *FEMS Microbiology Ecology* **18**: 289-298.

Andersen, K, T Kjær & NP Revsbech (2001): An oxygen insensitive microsensor for nitrous oxide. *Sensors and Actuators B: Chemical* **81**: 42-48.

Bab-Dinitz, E, H Shmuely, J Maupin-Furlow, J Eichler & B Shaanan (2006): *Haloferax volcanii* PitA: an example of functional interaction between the Pfam chlorite dismutase and antibiotic biosynthesis monooxygenase families? *Bioinformatics* **22**: 671-675.

Baker, SC, SJ Ferguson, B Ludwig *et al.* (1998): Molecular genetics of the genus *Paracoccus*: Metabolically versatile bacteria with bioenergetic flexibility. *Microbiol. Mol. Biol. Rev.* **62**: 1046-1078.

Bakermans, C & EL Madsen (2002): Diversity of 16S rDNA and Naphthalene Dioxygenase Genes from Coal-Tar-Waste-Contaminated Aquifer Waters. *Microb. Ecol.* **44**: 95-106.

Balch, WE, GE Fox, LJ Magrum, CR Woese & RS Wolfe (1979): Methanogens - Re-evaluation of a unique biological group. *Microbiol. Rev.* **43**: 260-296.

Barnard, R, X Le Roux, BA Hungate *et al.* (2006): Several components of global change alter nitrifying and denitrifying activities in an annual grassland. *Functional Ecology* **20**: 557-564.

Barnes, RO & ED Goldberg (1976): Methane production and consumption in anoxic marine sediments. *Geology* **4**: 297-300.

Beal, EJ, CH House & VJ Orphan (2009): Manganese- and iron-dependent marine methane oxidation. *Science* **325**: 184-187.

Bédard, C & R Knowles (1997): Some properties of methane oxidation in a thermally stratified lake. *Canadian Journal of Fisheries and Aquatic Sciences* **54**: 1639-1645.

Bedmar, EJ, EF Robles & MJ Delgado (2005): The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium˙japonicum*. *Biochem. Soc. Trans.* **33**: 141-144.

Beijerinck, MW (1904): Über die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. *Zentralblatt für Bakteriologie und Parasitenkunde, II. Abteilung* **25**: 593-599.

Beijerinck, MW (1920): Chemosynthesis at denitrification with sulfur as source of energy. *Koninklijke Nederlandsche Akademie van Wetenschappen Proceedings* **22**: 899-908.

Berks, BC, SJ Ferguson, JWB Moir & DJ Richardson (1995): Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1232**: 97-173.

Berthe-Corti, L & S Fetzner (2002): Bacterial metabolism of n-alkanes and ammonia under oxic, suboxic and anoxic conditions. *Acta Biotechnologica* **22**: 299-336.

Bjerg, PL, K Rugge, JK Pedersen & TH Christensen (1995): Distribution of redox-sensitive groundwater quality parameters downgradient of a landfill (Grindsted, Denmark). *Environmental Science & Technology* **29**: 1387-1394.

Blumenberg, M, R Seifert, K Nauhaus, T Pape & W Michaelis (2005): In vitro study of lipid biosynthesis in an anaerobically methane-oxidizing microbial mat. *Appl. Environ. Microbiol.* **71**: 4345-4351.

Blumenberg, M, R Seifert, J Reitner, T Pape & W Michaelis (2004): Membrane lipid patterns typify distinct anaerobic methanotrophic consortia. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 11111-11116.

Bobbink, R, M Hornung & JGM Roelofs (1998): The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. *Journal of Ecology* **86**: 717-738.

Bodelier, PLE, M-J Bär Gillisen, K Hordijk *et al.* (2009): A reanalysis of phospholipid fatty acids as ecological biomarkers for methanotrophic bacteria. *ISME Journal* **3**: 606-617.

Bodelier, PLE & HJ Laanbroek (2004): Nitrogen as a regulatory factor of methane oxidation in soils and sediments. *FEMS Microbiology Ecology* **47**: 265-277.

Boetius, A, K Ravenschlag, CJ Schubert *et al.* (2000): A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623-626.

Boschker, HTS & JJ Middelburg (2002): Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiology Ecology* **40**: 85-95.

Bowman, JP, LI Sly, PD Nichols & AC Hayward (1993): Revised taxonomy of the methanotrophs - description of *Methylobacter Gen-Nov*, emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group-I methanotrophs. *Int. J. Syst. Bacteriol.* **43**: 735-753.

Bremner, J (1997): Sources of nitrous oxide in soils. *Nutrient Cycling in Agroecosystems* **49**: 7-16.

Brocks, JJ & J Banfield (2009): Unravelling ancient microbial history with community proteogenomics and lipid geochemistry. *Nature Reviews Microbiology* **7**: 601-609.

Broda, E (1975): History of inorganic nitrogen in biosphere. *J. Mol. Evol.* **7**: 87-100.

Broda, E (1977): Two kinds of lithotrophs missing in nature. *Z. Allg. Mikrobiol.* **17**: 491-493.

Brune, A, P Frenzel & H Cypionka (2000): Life at the oxic-anoxic interface: microbial activities and adaptations. *FEMS Microbiol. Rev.* **24**: 691-710.

Burford, JR & JM Bremner (1975): Relationships between denitrification capacities of soils and total, water-soluble and readily decomposable soil organic matter. *Soil Biology & Biochemistry* **7**: 389-394.

Burgin, AJ & SK Hamilton (2007): Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. *Frontiers in Ecology and the Environment* **5**: 89-96.

Cadillo-Quiroz, H, S Brauer, E Yashiro *et al.* (2006): Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA. *Environmental Microbiology* **8**: 1428-1440.

Cadillo-Quiroz, H, E Yashiro, JB Yavitt & SH Zinder (2008): Characterization of the archaeal community in a minerotrophic fen and Terminal Restriction Fragment Length Polymorphism-directed isolation of a novel hydrogenotrophic methanogen. *Appl. Environ. Microbiol.* **74**: 2059-2068.

Cabello, P, MD Roldan & C Moreno-Vivian (2004): Nitrate reduction and the nitrogen cycle in archaea. *Microbiology-SGM* **150**: 3527-3546.

Castresana, J (2004): Evolution and Phylogenetic Analysis of Respiration. In: *Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers.* 1-14. Springer, Dordrecht.

Chakraborty, R & JD Coates (2005): Hydroxylation and carboxylation - two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain RCB. *Appl. Environ. Microbiol.* **71**: 5427-5432.

Chapman, DJ & JW Schopf (1983): *Biological and biochemical effects of the development of an aerobic environment*. Princeton University Press, Princeton.

Chistoserdova, L, MG Kalyuzhnaya & ME Lidstrom (2005): C1-Transfer Modules: from Genomics to Ecology. *ASM News* **71**: 521-528.

Christensen, PB, LP Nielsen, NP Revsbech & J Sorensen (1989): Microzonation of denitrification activity in stream sediments as studied with a combined oxygen and nitrous oxide microsensor. *Appl. Environ. Microbiol.* **55**: 1234-1241.

Coates, JD, R Chakraborty, JG Lack *et al.* (2001): Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas. Nature* **411**: 1039-1043.

Conrad, R (1999): Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiology Ecology* **28**: 193-202.

Conrad, R, M Klose, M Noll, D Kemnitz & PLE Bodelier (2008): Soil type links microbial colonization of rice roots to methane emission. *Global Change Biology* **14**: 657-669.

Costa, C, C Dijkema, M Friedrich *et al.* (2000): Denitrification with methane as electron donor in oxygen-limited bioreactors. *Appl. Microbiol. Biotechnol.* **53**: 754-762.

Crossmann, ZM, P Ineson & RP Evershed (2005): The use of ^{13}C labelling of bacterial lipids in the characterisation of ambient methane-oxidising bacteria in soils. *Organic Geochemistry* **36** 769-778.

Czechowska, K, DR Johnson & JR van der Meer (2008): Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Curr. Opin. Microbiol.* **11**: 205-212.

Daims, H, A Bruhl, R Amann, KH Schleifer & M Wagner (1999): The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **22**: 434-444.

Davies, TR (1975): Isolation of bacteria capable of using methane as hydrogen donor in the process of denitrification. *Water Research* **7**: 575-579.

Dedysh, SN, NS Panikov, W Liesack *et al.* (1998): Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. *Science* **282**: 281-284.

Delwiche, CC (1959): Production and utilization of nitrous oxide by *Pseudomonas denitrificans. J. Bacteriol.* **77**: 55-59.

Demicheli, V, C Quijano, B Alvarez & R Radi (2007): Inactivation and nitration of human superoxide dismutase (SOD) by fluxes of nitric oxide and superoxide. *Free Radic. Biol. Med.* **42**: 1359-1368.

Denef, K, D Roobroeck, MCWM Wadu, P Lootens & P Boeckx (2009): Microbial community composition and rhizodeposit-carbon assimilation in differently managed temperate grassland soils. *Soil Biology & Biochemistry* **41**: 144-153.

Denman, KL, G. Brasseur, A. Chidthaisong, P. Ciais, P.M. Cox, R.E. Dickinson, D. Hauglustaine, C. Heinze, E. Holland, D. Jacob, U. Lohmann, S Ramachandran, P.L. da Silva Dias, S.C. Wofsy and X. Zhang (2007): Couplings between changes in the climate system and biogeochemistry. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.* 499-587. Cambridge University Press, Cambridge.

Devol, AH & SI Ahmed (1981): Are high rates of sulphate reduction associated with anaerobic oxidation of methane? *Nature* **291**: 407-408.

Diekert, G, HH Gilles, R Jaenchen & RK Thauer (1980): Incorporation of 8 Succinate Per Mol Nickel into Factors-F-430 by Methanobacterium-Thermoautotrophicum. *Arch. Microbiol.* **128**: 256-262.

Dowling, NJE, F Widdel & DC White (1986): Phospholipid ester-linked fatty-acid biomarkers of acetate-oxidizing sulfate-reducers and other sulfide-forming bacteria. *J. Gen. Microbiol.* **132**: 1815-1825.

Ducluzeau, AL, R van Lis, S Duval *et al.* (2009): Was nitric oxide the first deep electron sink? *Trends Biochem. Sci.* **34**: 9-15.

Dutilh, BE, MA Huynen & M Strous (2009): Increasing the coverage of a metapopulation consensus genome by iterative read mapping and assembly. *Bioinformatics* **25**: 2878-2881.

Ebihara, A, A Okamoto, Y Kousumi *et al.* (2005): Structure-based functional identification of a novel heme-binding protein from *Thermus thermophilus* HB8. *Journal of Structural and Functional Genomics* **6**: 21-32.

Edgar, RC (2004): MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792-1797.

Ehrenreich, P, A Behrends, J Harder & F Widdel (2000): Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. *Arch. Microbiol.* **173**: 58-64.

Eisentraeger, A, P Klag, B Vansbotter, E Heymann & W Dott (2001): Denitrification of groundwater with methane as sole hydrogen donor. *Water Research* **35**: 2261-2267.

Eller, G, L Känel & M Krüger (2005): Cooccurrence of aerobic and anaerobic methane oxidation in the water column of Lake Plußsee. *Appl. Environ. Microbiol.* **71**: 8925-8928.

Etiope, G & RW Klusman (2002): Geologic emissions of methane to the atmosphere. *Chemosphere* **49**: 777-789.

Evershed, RP, ZM Crossman, ID Bull *et al.* (2006): C-13-Labelling of lipids to investigate microbial communities in the environment. *Curr. Opin. Biotechnol.* **17**: 72-82.

Farhoud, MH, HJCT Wessels, PJM Steenbakkers *et al.* (2005): Protein complexes in the archaeon *Methanothermobacter thermautotrophicus* analyzed by blue native/SDS-PAGE and mass spectrometry. *Molecular & Cellular Proteomics* **4**: 1653-1663.

Farrer, RA, E Kemen, JDG Jones & DJ Studholme (2009): *De novo* assembly of the *Pseudomonas syringae* pv. *syringae* B728a genome using Illumina/Solexa short sequence reads. *FEMS Microbiol. Lett.* **291**: 103-111.

Fenchel, T (2002): *Origin and early evolution of life*. 192. Oxford University Press, Oxford.

Forster, P, V. Ramaswamy, P. Artaxo, T. Berntsen, R. Betts, D.W. Fahey, J. Haywood, J. Lean, D.C. Lowe, G. Myhre, J. Nganga, R. Prinn, G. Raga, M. Schulz and R. Van Dorland (2007): Changes in atmospheric constituents and in radiative forcing. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. 129-234. Cambridge University Press, Cambridge.

Friedman, SH, W Massefski & TC Hollocher (1986): Catalysis of Intermolecular Oxygen Atom Transfer by Nitrite Dehydrogenase of Nitrobacter-Agilis. *J. Biol. Chem.* **261**: 538-543.

Friedmann, HC, A Klein & RK Thauer (1990): Structure and function of the nickel porphinoid, coenzyme F430, and of its enzyme, methyl coenzyme M reductase. *FEMS Microbiol. Lett.* **87**: 339-348.

Fuerst, JA (2007): Semantides and Modern Bacterial Systematics. In: *Encyclopedia of Life Sciences*. John Wiley & Sons, Ltd, Chichester.

Galloway, JN, FJ Dentener, DG Capone *et al.* (2004): Nitrogen cycles: past, present, and future. *Biogeochemistry* **70**: 153-226.

Galloway, JN, AR Townsend, JW Erisman *et al.* (2008): Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* **320**: 889-892.

Gao, H, F Schreiber, G Collins *et al.* (2010): Aerobic denitrification in permeable Wadden Sea sediments. *ISME Journal* **4**: 417-426.

Gayon, U & G Dupetit (1882): Sur la fermentation des nitrates. *Comptes Rendus Hebdomadaires des séances de l'Academie des Sciences* **95**: 644-646.

Gayon, U & G Dupetit (1886): Recherches sur la réduction des nitrates par les infiniments petits. *Mémoires de la societé des sciences physiques et naturelles de Bordeaux* **3e série**: 201-307.

Giovannoni, S & U Stingl (2007): The importance of culturing bacterioplankton in the 'omics' age. *Nature Reviews Microbiology* **5**: 820-826.

Gordon, D, C Abajian & P Green (1998): *Consed*: a graphical tool for sequence finishing. *Genome Res.* **8**: 195-202.

Griffin, BM, J Schott & B Schink (2007): Nitrite, an electron donor for anoxygenic photosynthesis. *Science* **316**: 1870-1870.

Griffioen, J (1999): Comment on `Kinetic modelling of microbially-driven redox chemistry of subsurface environments: Coupling transport, microbial metabolism and geochemistry' by K.S. Hunter, Y. Wang and P. van Cappellen. *Journal of Hydrology* **226**: 121-124.

Groffman, PM, MA Altabet, JK Bohlke *et al.* (2006): Methods for measuring denitrification: diverse approaches to a difficult problem. *Ecological Applications* **16**: 2091-2122.

Guo, JH, XJ Liu, Y Zhang *et al.* (2010): Significant acidification in major chinese croplands. *Science* **327**: 1008-1010.

Hakemian, AS & AC Rosenzweig (2007): The biochemistry of methane oxidation. *Annu. Rev. Biochem.* **76**: 223-241.

Halász, A & F Simek (1968): Gázalakú szénhidrogének mikrobiológiai hasznosítása mikroorganizmusok által. I. Vizsgálati módszerek a metánasszimiláció követésére. (Microbiological utilisation of gaseous hydrocarbons. I. Method for following assimilation of methane.). *Élelmiszertudomány* **2**: 177-184.

Hales, BA, C Edwards, DA Ritchie *et al.* (1996): Isolation and identification of methanogen-specific DNA from blanket bog feat by PCR amplification and sequence analysis. *Appl. Environ. Microbiol.* **62**: 668-675.

Hallam, SJ, PR Girguis, CM Preston, PM Richardson & EF Delong (2003): Identification of methyl coenzyme M reductase A (mcrA) genes associated with methane-oxidizing archaea. *Appl. Environ. Microbiol.* **69**: 5483-5491.

Hallam, SJ, N Putnam, CM Preston *et al.* (2004): Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* **305**: 1457-1462.

Hayes, JM (1983): Geochemical evidence bearing on the origin of aerobiosis, a speculative hypotheis In: *Earth's earliest biosphere*. 291-301. Princeton University Press, Princeton.

Hazeu, W & JC de Bruyn (1980): Ethane oxidation by methane-oxidizing bacteria. *Antonie Van Leeuwenhoek* **46**: 443-455.

Heider, J (2007): Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr. Opin. Chem. Biol.* **11**: 188-194.

Hendriks, DMD, J van Huissteden, AJ Dolman & MK van der Molen (2007): The full greenhouse gas balance of an abandoned peat meadow. *Biogeosciences* **4**: 411-424.

Hinrichs, KU, JM Hayes, SP Sylva, PG Brewer & EF Delong (1999): Methane-consuming archaebacteria in marine sediments. *Nature* **398**: 802-805.

Hinrichs, KU, JM Hayes, W Bach *et al.* (2006): Biological formation of ethane and propane in the deep marine subsurface. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 14684-14689.

Hoehler, TM, MJ Alperin, DB Albert & CS Martens (1994): Field and laboratory studies of methane oxidation in an anoxic marine sediment - evidence for a methanogen-sulfate reducer consortium. *Global Biogeochemical Cycles* **8**: 451-463.

Holland, HD (2006): The oxygenation of the atmosphere and oceans. *Philosophical Transactions of the Royal Society B-Biological Sciences* **361**: 903-915.

Holmes, AJ, C Andria, ME Lidstrom & JC Murrell (1995): Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol. Lett.* **132**: 203-208.

Howard, CS & F Daniels (1958): Stability of nitric oxide over a long time interval. *The Journal of Physical Chemistry* **62**: 360-361.

Hu, S, RJ Zeng, LC Burow *et al.* (2009): Enrichment of denitrifying anaerobic methane oxidizing microorganisms. *Environmental Microbiology Reports* **1**: 377-384.

Intergovernmental Panel on Climate Change (2001): *Climate Change 2001: The scientific basis. Contribution of working group I to the third assessment report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge.

Intergovernmental Panel on Climate Change (2007): *Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. 104. IPCC, Geneva.

Ishihama, Y, J Rappsilber, JS Andersen & M Mann (2002): Microcolumns with self-assembled particle frits for proteomics. *J. Chromatogr. A* **979**: 233-239.

Islas-Lima, S, F Thalasso & J Gomez-Hernandez (2004): Evidence of anoxic methane oxidation coupled to denitrification. *Water Research* **38**: 13-16.

Iversen, N, RS Oremland & MJ Klug (1987): Big Soda Lake (Nevada). 3. Pelagic methanogenesis and anaerobic methane oxidation. *Limnology and Oceanography* **32**: 804-814.

Juretschko, S, G Timmermann, M Schmid *et al.* (1998): Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl. Environ. Microbiol.* **64**: 3042-3051.

Kalyuzhnaya, MG, ME Lidstrom & L Chistoserdova (2008): Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *ISME Journal* **2**: 696-706.

Kalyuzhnaya, MG, W Martens-Habbena, T Wang *et al.* (2009): *Methylophilaceae* link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. *Environmental Microbiology Reports* **1**: 385-392.

Kasai, Y, Y Takahata, T Hoaki & K Watanabe (2005): Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. *Environmental Microbiology* **7**: 806-818.

Käserer, H (1905): Über die Oxydation des Wasserstoffs und des Methans durch Mikroorganismen. *Zeitschrift für das landwirtschaftliche Versuchs- und Untersuchungswesen in Österreich* **8**: 789-792.

Kartal, B, M Koleva, R Arsov *et al.* (2006): Adaptation of a freshwater anammox population to high salinity wastewater. *J. Biotechnol.* **126**: 546-553.

Kartal, B, J Rattray, LA van Niftrik *et al.* (2007): *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst. Appl. Microbiol.* **30**: 39-49.

Klappenbach, JA, JM Dunbar & TM Schmidt (2000): rRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* **66**: 1328-1333.

Kluyver, AJ & HJL Donker (1926): Die Einheit in der Biochemie. *Chem. Zelle u. Gewebe* **13**: 134-190.

Knief, C, S Kolb, P Bodelier, L. E., A Lipski & P Dunfield, F. (2006): The active methanotrophic community in hydromorphic soils changes in response to changing methane concentration. *Environmental Microbiology* **8**: 321-333.

Kniemeyer, O, F Musat, SM Sievert *et al.* (2007): Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. *Nature* **449**: 898-901.

Knittel, K & A Boetius (2009): Anaerobic oxidation of methane: progress with an unknown process. *Annu. Rev. Microbiol.* **63**: 311-334.

Knittel, K, T Lösekann, A Boetius, R Kort & R Amann (2005): Diversity and distribution of methanotrophic archaea at cold seeps. *Appl. Environ. Microbiol.* **71**: 467-479.

Knowles, R (1982): Denitrification. *Microbiol. Rev.* **46**: 43-70.

Knowles, R (2005): Denitrifiers associated with methanotrophs and their potential impact on the nitrogen cycle. *Ecological Engineering* **24**: 441-446.

Kobayashi, M, Y Matsuo, A Takimoto *et al.* (1996): Denitrification, a novel type of respiratory metabolism in fungal mitochondrion. *J. Biol. Chem.* **271**: 16263-16267.

Koizumi, Y, H Kojima & M Fukui (2003): Characterization of depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments. *FEMS Microbiology Ecology* **46**: 147-157.

Könneke, M, AE Bernhard, JR de la Torre *et al.* (2005): Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543-546.

Kool, DM, N Wrage, O Oenema, J Dolfing & JW Van Groenigen (2007): Oxygen exchange between (de) nitrification intermediates and H_2O and its implications for source determination of NO_3^- and N_2O: a review. *Rapid Commun. Mass Spectrom.* **21**: 3569-3578.

Kowalchuk, GA, F. J. de Bruijn, I. M. Head, A. D. Akkermans, J. D. van Elsas (2004): *Molecular Microbial Ecology Manual (MMEM)*. Kluwer Academic Publishing, London.

Krämer, M, M Baumgärtner, M Bender & R Conrad (1990): Consumption of NO by methanotrophic bacteria in pure culture and in soil. *FEMS Microbiol. Lett.* **73**: 345-350.

Krüger, M, A Meyerdierks, FO Glöckner *et al.* (2003): A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878-881.

Kuever, J, M Könneke, A Galushko & O Drzyzga (2001): Reclassification of *Desulfobacterium phenolicum* as *Desulfobacula phenolica comb. nov.* and description of strain Sax(T) as *Desulfotignum balticum gen. nov., sp nov. International Journal of Systematic and Evolutionary Microbiology* **51**: 171-177.

Kulp, TR, SE Hoeft, M Asao *et al.* (2008): Arsenic(III) fuels anoxygenic photosynthesis in hot spring biofilms from Mono Lake, California. *Science* **321**: 967-970.

Kumaraswamy, S, B Ramakrishnan & N Sethunathan (2001): Methane production and oxidation in an anoxic rice soil as influenced by inorganic redox species. *Journal of Environmental Quality* **30**: 2195-2201.

Kuypers, MMM, P Blokker, J Erbacher *et al.* (2001): Massive expansion of marine archaea during a mid-Cretaceous oceanic anoxic event. *Science* **293**: 92-95.

Kuypers, MMM, G Lavik, D Woebken *et al.* (2005): Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proceedings of the National Academy of Sciences* **102**: 6478-6483.

Kuypers, MMM, AO Sliekers, G Lavik *et al.* (2003): Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**: 608-611.

Lam, P, G Lavik, MM Jensen *et al.* (2009): Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proceedings of the National Academy of Sciences* **106**: 4752-4757.

Lane, D. J. (1991). 16S/23S rRNA Sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics*. E. Stackebrandt and M. Goodfellow. New York, John Wiley & Sons: 115-175.

Lane, N (2006): Batteries not included. What can't bacteria do? *Nature* **441**: 274-277.

Larowe, DE, AW Dale & P Regnier (2008): A thermodynamic analysis of the anaerobic oxidation of methane in marine sediments. *Geobiology* **6**: 436-449.

Laverman, AM, C Meile, P Van Cappellen & EBA Wieringa (2007): Vertical distribution of denitrification in an estuarine sediment: Integrating sediment flowthrough reactor experiments and microprofiling via reactive transport modeling. *Appl. Environ. Microbiol.* **73**: 40-47.

Laverman, AM, P Van Cappellen, D van Rotterdam-Los, C Pallud & J Abell (2006): Potential rates and pathways of microbial nitrate reduction in coastal sediments. *FEMS Microbiology Ecology* **58**: 179-192.

Leadbetter, ER & JW Foster (1958): Studies on some methane-utilizing bacteria. *Arch. Mikrobiol.* **30**: 91-118.

Leadbetter, ER & JW Foster (1960): Bacterial oxidation of gaseous alkanes. *Arch. Mikrobiol.* **35**: 92-104.

Leadbetter, JR (2003): Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. *Curr. Opin. Microbiol.* **6**: 274-281.

Lopez-Archilla, AI, D Moreira, S Velasco & P Lopez-Garcia (2007): Archaeal and bacterial community composition of a pristine coastal aquifer in Doñana National Park, Spain. *Aquatic Microbial Ecology* **47**: 123-139.

Lovley, DR & MJ Klug (1983): Sulfate reducers can out-compete methanogens at freshwater sulfate concentrations. *Appl. Environ. Microbiol.* **45**: 187-192.

Lüders, T, KJ Chin, R Conrad & M Friedrich (2001): Molecular analyses of methyl-coenzyme M reductase alpha-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environmental Microbiology* **3**: 194-204.

Luther, GW, B Sundby, BL Lewis, PJ Brendel & N Silverberg (1997): Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen. *Geochimica et Cosmochimica Acta* **61**: 4043-4052.

MacLean, LCW, TJ Pray, TC Onstott *et al.* (2007): Mineralogical, chemical and biological characterization of an anaerobic biofilm collected from a borehole in a deep gold mine in South Africa. *Geomicrobiology Journal* **24**: 491-504.

Mah, RA, DM Ward, L Baresi & TL Glass (1977): Biogenesis of Methane. *Annu. Rev. Microbiol.* **31**: 309-341.

Maixner, F, M Wagner, S Lucker *et al.* (2008): Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium 'Candidatus Nitrospira defluvii'. *Environmental Microbiology* **10**: 3043-3056.

Margulies, M, M Egholm, WE Altman *et al.* (2005): Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376-380.

Martini, AM, LM Walter, TCW Ku *et al.* (2003): Microbial production and modification of gases in sedimentary basins: A geochemical case study from a Devonian shale gas play, Michigan basin. *American Association of Petroleum Geologists Bulletin* **87**: 1355-1375.

Mason, I (1977): Methane as a carbon source in biological denitrification. *Journal Water Pollution Control Federation* **49**: 855-857.

Matějů, V, S Čižinská, J Krejčí & T Janoch (1992): Biological water denitrification - A review. *Enzyme Microb. Technol.* **14**: 170-183.

Matsubara, T & T Mori (1968): Studies on denitrification: IX. Nitrous oxide, its production and reduction to nitrogen. *J. Biochem. (Tokyo).* **64**: 863-871.

McCalley, CK & JP Sparks (2009): Abiotic gas formation drives nitrogen loss from a desert ecosystem. *Science* **326**: 837-840.

McIlvin, MR & MA Altabet (2005): Chemical conversion of nitrate and nitrite to nitrous oxide for nitrogen and oxygen isotopic analysis in freshwater and seawater. *Anal. Chem.* **77**: 5589-5595.

Mehboob, F, H Junca, G Schraa & AJM Stams (2009): Growth of *Pseudomonas chloritidismutans* AW-1T on n-alkanes with chlorate as electron acceptor. *Appl. Microbiol. Biotechnol.* **83**: 739-747.

Meyerdierks, A, M Kube, I Kostadinov *et al.* (2010): Metagenome and mRNA expression analyses of anaerobic methanotrophic archaea of the ANME-1 group. *Environmental Microbiology* **12**: 422-439.

Meyerdierks, A, M Kube, T Lombardot *et al.* (2005): Insights into the genomes of archaea mediating the anaerobic oxidation of methane. *Environmental Microbiology* **7**: 1937-1951.

Michaelis, W, R Seifert, K Nauhaus *et al.* (2002): Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science* **297**: 1013-1015.

Miura, Y, A Watanabe, J Murase & M Kimura (1992): Methane production and its fate in paddy fields. 2. Oxidation of methane and its coupled ferric-oxide reduction in subsoil. *Soil Science and Plant Nutrition* **38**: 673-679.

Modin, O, K Fukushi & K Yamamoto (2007): Denitrification with methane as external carbon source. *Water Research* **41**: 2726-2738.

Moran, JJ, EJ Beal, JM Vrentas *et al.* (2007): Methyl sulfides as intermediates in the anaerobic oxidation of methane. *Environmental Microbiology* **10**: 162–173.

Mulder, A, AA Vandegraaf, LA Robertson & JG Kuenen (1995): Anaerobic ammonium oxidation discovered in a denitrifying fluidized-bed reactor. *FEMS Microbiology Ecology* **16**: 177-183.

Nauhaus, K, A Boetius, M Krüger & F Widdel (2002): In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environmental Microbiology* **4**: 296-305.

Nauhaus, K, T Treude, A Boetius & M Krüger (2005): Environmental regulation of the anaerobic oxidation of methane: a comparison of ANME-I and ANME-II communities. *Environmental Microbiology* **7**: 98-106.

Nauhaus, K, M Albrecht, M Elvert, A Boetius & F Widdel (2007): In vitro cell growth of marine archaeal-bacterial consortia during anaerobic oxidation of methane with sulfate. *Environmental Microbiology* **9**: 187-196.

Nealson, KH (1997): Sediment Bacteria: Who's There, What Are They Doing, and What's New? *Annual Review of Earth and Planetary Sciences* **25**: 403-434.

Niemann, H, J Duarte, C Hensen *et al.* (2006): Microbial methane turnover at mud volcanoes of the Gulf of Cadiz. *Geochimica et Cosmochimica Acta* **70**: 5336-5355.

Niemann, H, T Lösekann, D de Beer *et al.* (2006): Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* **443**: 854-858.

Niemann, H, C Hitz, J Blees *et al.* (2009): Biogeochemical signatures of the anaerobic methane oxidation in a south alpine lake (Lake Lugano). *Geochimica et Cosmochimica Acta, Supplement 1* **73**: A942.

Norland, S, M Heldal & O Tumyr (1987): On the Relation between Dry-Matter and Volume of Bacteria. *Microb. Ecol.* **13**: 95-101.

Nyerges, G, S-K Han & LY Stein (2010): Effects of ammonium and nitrite on growth and competitive fitness of cultivated methanotrophic bacteria. *Appl. Environ. Microbiol.*: doi:10.1128/AEM.00747-10.

Nyerges, G & LY Stein (2009): Ammonia cometabolism and product inhibition vary considerably among species of methanotrophic bacteria. *FEMS Microbiol. Lett.* **297**: 131-136.

Op den Camp, HJM, T Islam, MB Stott *et al.* (2009): Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia. Environmental Microbiology Reports* **1**: 293-306.

Orcutt, B, A Boetius, M Elvert, V Samarkin & SB Joye (2005): Molecular biogeochemistry of sulfate reduction, methanogenesis and the anaerobic oxidation of methane at Gulf of Mexico cold seeps. *Geochimica et Cosmochimica Acta* **69**: 4267-4281.

Oremland, RS & DG Capone (1988): Use of "specific" inhibitors in biogeochemistry and microbial ecology. *Advances in Microbial Ecology* **10**: 285-383.

Oremland, RS, DG Capone, JF Stolz & J Fuhrman (2005): Whither or wither geomicrobiology in the era of 'community metagenomics'. *Nature Reviews Microbiology* **3**: 572-578.

Orphan, VJ, CH House, KU Hinrichs, KD McKeegan & EF Delong (2001): Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**: 484-487.

Orphan, VJ, CH House, KU Hinrichs, KD McKeegan & EF Delong (2002): Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc. Natl. Acad. Sci. U. S. A.* **99**: 7663-7668.

Osaka, T, Y Ebie, S Tsuneda & Y Inamori (2008): Identification of the bacterial community involved in methane-dependent denitrification in activated sludge using DNA stable-isotope probing. *FEMS Microbiology Ecology* **64**: 494-506.

Pancost, RD, JS Sinninghe Damsté, S de Lint, MJEC van der Maarel & JC Gottschal (2000): Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic archaea and bacteria. *Appl. Environ. Microbiol.* **66**: 1126-1132.

Panganiban, AT, TE Patt, W Hart & RS Hanson (1979): Oxidation of methane in the absence of oxygen in lake water samples. *Appl. Environ. Microbiol.* **37**: 303-309.

Parvulescu, VI, P Grange & B Delmon (1998): Catalytic removal of NO. *Catalysis Today* **46**: 233-316.

Patel, RN, CT Hou, AI Laskin, A Felix & P Derelanko (1980): Microbial oxidation of gaseous hydrocarbons - production of secondary alcohols from corresponding normal-alkanes by methane-utilizing bacteria. *Appl. Environ. Microbiol.* **39**: 720-726.

Pavlov, AA, JF Kasting, LL Brown, KA Rages & R Freedman (2000): Greenhouse warming by CH_4 in the atmosphere of early Earth. *Journal of Geophysical Research-Planets* **105**: 11981-11990.

Payne, WJ (1973): Reduction of nitrogenous oxides by microorganisms. *Bacteriol. Rev.* **37**: 409-452.

Payne, WJ, PS Riley & CD Cox, Jr. (1971): Separate nitrite, nitric oxide, and nitrous oxide reducing fractions from *Pseudomonas perfectomarinus. J. Bacteriol.* **106**: 356-361.

Pfaltz, A, Jaun, B., Faessler, A., Eschenmoser, A., Jaenchen, R., Gilles, H.H., Diekert, G., Thauer, R.K. (1982): Factor F430 from methanogenic bacteria: Structure of the porphinoid ligand system. . *Helvetica Chimica Acta* **65**: 828-866.

Philippot, L (2002): Denitrifying genes in bacterial and archaeal genomes. *Biochimica et Biophysica Acta-Gene Structure and Expression* **1577**: 355-376.

Philippot, L, P Mirleau, S Mazurier *et al.* (2001): Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar, nir, nor* and *nos* genes. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1517**: 436-440.

Pimenov, NV, II Rusanov, MN Poglazova *et al.* (1997): Bacterial mats on coral-like structures at methane seeps in the Black Sea. *Microbiology* **66**: 354-360.

Polz, MF & CM Cavanaugh (1998): Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* **64**: 3724-3730.

Prior, SD & H Dalton (1985): Acetylene as a suicide substrate and active-site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS Microbiol. Lett.* **29**: 105-109.

Rabalais, NN, RE Turner & WJ Wiseman (2002): Gulf of Mexico hypoxia, aka "the dead zone". *Annual Review of Ecology and Systematics* **33**: 235-263.

Rabus, R & F Widdel (1995): Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch. Microbiol.* **163**: 96-103.

Rabus, R, H Wilkes, A Behrends *et al.* (2001): Anaerobic initial reaction of n-alkanes in a denitrifying bacterium: evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in n-hexane metabolism. *J. Bacteriol.* **183**: 1707-1715.

Raghoebarsing, AA, A Pol, KT van de Pas-Schoonen *et al.* (2006): A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918-921.

Raghoebarsing, A (2006): *New directions in microbial methane oxidation* (thesis). Radboud University Nijmegen (http://webdoc.ubn.ru.nl/ mono/r/raghoebarsing_a/new_diinm.pdf).

Rappé, MS & SJ Giovannoni (2003): The uncultured microbial majority. *Annu. Rev. Microbiol.* **57**: 369-394.

Rappsilber, J, Y Ishihama & M Mann (2003): Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **75**: 663-670.

Rastogi, G, RK Sani, BM Peyton, JG Moberly & TR Ginn (2009): Molecular studies on the microbial diversity associated with mining-impacted Coeur d'Alene river sediments. *Microb. Ecol.* **58**: 129-139.

Reeburgh, WS (1976): Methane consumption in Cariaco Trench waters and sediments. *Earth and Planetary Science Letters* **28**: 337-344.

Reeburgh, WS (2007): Oceanic methane biogeochemistry. *Chem. Rev.* **107**: 486-513.

Ren, T, R Roy & R Knowles (2000): Production and consumption of nitric oxide by three methanotrophic bacteria. *Appl. Environ. Microbiol.* **66**: 3891-3897.

Revsbech, NP (1989): An oxygen microsensor with a guard cathode. *Limnology and Oceanography* **34**: 474-478.

Rigby, M, RG Prinn, PJ Fraser *et al.* (2008): Renewed growth of atmospheric methane. *Geophysical Research Letters* **35** (22): L22805.

Risgaard-Petersen, N, AM Langezaal, S Ingvardsen *et al.* (2006): Evidence for complete denitrification in a benthic foraminifer. *Nature* **443**: 93-96.

Robertson, GP & PM Vitousek (2009): Nitrogen in agriculture: balancing the cost of an essential resource. *Annual Review of Environment and Resources* **34**: 97-125.

Rouvière, PE & RS Wolfe (1987): Use of Subunits of the Methylreductase Protein for Taxonomy of Methanogenic Bacteria. *Arch. Microbiol.* **148**: 253-259.

Rudd, JWM, A Furutani, RJ Flett & RD Hamilton (1976): Factors controlling methane oxidation in shield lakes - role of nitrogen fixation and oxygen concentration. *Limnology and Oceanography* **21**: 357-364.

Rudd, JWM & JTG Hamilton (1975): Methane oxidation in a eutrophic Canadian shield lake. *Verhandlungen der Internationalen Vereinigung für Limnologie* **19**: 2669-2673.

Rueter, P, R Rabus, H Wilkes *et al.* (1994): Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature* **372**: 455-458.

Rütters, H, H Sass, H Cypionka & J Rullkötter (2002): Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *J. Microbiol. Methods* **48**: 149-160.

Sacks, LE & HA Barker (1952): Substrate oxidation and nitrous oxide utilization in denitrification. *J. Bacteriol.* **64**: 247-252.

Salinero, KK, K Keller, W Feil *et al.* (2009): Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. *BMC Genomics* **10**: 351.

Savage, KN, LR Krumholz, LM Gieg *et al.* (2010): Biodegradation of low-molecular-weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates and community patterns. *FEMS Microbiology Ecology* **72**: 485-495.

Scheller, S, M Goenrich, R Boecher, RK Thauer & B Jaun (2010): The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane. *Nature* **465**: 606-U97.

Schidlowski, M (2001): Carbon isotopes as biogeochemical recorders of life over 3.8 Ga of Earth history: evolution of a concept. *Precambrian Research* **106**: 117-134.

Schink, B (1997): Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev.* **61**: 262-280.

Schloesing, T & A Müntz (1877): Sur la nitrification des ferments organisés. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **84**: 301-303.

Schreiber, F, L Polerecky & D de Beer (2008): Nitric oxide microsensor for high spatial resolution measurements in biofilms and sediments. *Anal. Chem.* **80**: 1152-1158.

Schubert, CJ, T Loesekann, K Knittel & A Boetius (2007): Anaerobic oxidation of methane in sediments of a high alpine lake. *Geophysical Research Abstracts* **9**: 10229.

Selmer, T, A Pierik, J. & J Heider (2005): New glycyl radical enzymes catalysing key metabolic steps in anaerobic bacteria. *Biol. Chem.* **386**: 981-988.

Shapleigh, J (2006): The Denitrifying Prokaryotes. In: *The Prokaryotes*. 769-792. Springer, New York.

Shima, S & RK Thauer (2005): Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic Archaea. *Curr. Opin. Microbiol.* **8**: 643-648.

Shindell, DT, G Faluvegi, DM Koch *et al.* (2009): Improved Attribution of Climate Forcing to Emissions. *Science* **326**: 716-718.

Smemo, KA & JB Yavitt (2007): Evidence for anaerobic CH_4 oxidation in freshwater peatlands. *Geomicrobiology Journal* **24**: 583-597.

Smith, CJ, DB Nedwell, LF Dong & AM Osborn (2006): Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology* **8**: 804-815.

Smith, RL, BL Howes & SP Garabedian (1991): *In situ* Measurement of Methane Oxidation in Groundwater by Using Natural-Gradient Tracer Tests. *Appl. Environ. Microbiol.* **57**: 1997-2004.

So, CM, CD Phelps & LY Young (2003): Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. *Appl. Environ. Microbiol.* **69**: 3892-3900.

So, CM & LY Young (2001): Anaerobic biodegradation of alkanes by enriched consortia under four different reducing conditions. *Environmental toxicology and chemistry* **20**: 473-478.

Söhngen, NL (1906): Über Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. *Zeitschrift für Bakteriologie, Parasitenkunde und Infektionskrankheiten, II. Abteilung* **15**: 513-517.

Sollo, FW, HF Mueller & TE Larson (1976): Denitrification of wastewater effluents with methane *Journal Water Pollution Control Federation* **48**: 1840-1842.

Sperl, GT & DS Hoare (1971): Denitrification with methanol: a selective enrichment for *Hyphomicrobium* species. *J. Bacteriol.* **108**: 733-736.

St. John, RT & TC Hollocher (1977): Nitrogen-15 tracer studies on pathway of denitrification in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **252**: 212-218.

Stahl, DA & R Amann (1991): Development and application of nucleic acid probes. In: *Nucleic acid techniques in bacterial systematics*. 205-248. John Wiley, Chichester.

Stein, LY, MT La Duc, TJ Grundl & KH Nealson (2001): Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environmental Microbiology* **3**: 10-18.

Stoecker, K, B Bendinger, B Schöning *et al.* (2006): Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 2363-2367.

Straub, KL, M Benz, B Schink & F Widdel (1996): Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl. Environ. Microbiol.* **62**: 1458-1460.

Strous, M, JA Fuerst, EHM Kramer *et al.* (1999): Missing lithotroph identified as new planctomycete. *Nature* **400**: 446-449.

Strous, M, JJ Heijnen, JG Kuenen & MSM Jetten (1998): The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl. Microbiol. Biotechnol.* **50**: 589-596.

Strous, M & MSM Jetten (2004): Anaerobic oxidation of methane and ammonium. *Annu. Rev. Microbiol.* **58**: 99-117.

Stüven, R & E Bock (2001): Nitrification and denitrification as a source for NO and NO_2 production in high-strength wastewater. *Water Research* **35**: 1905-1914.

Sun, WJ, R Sierra & JA Field (2008): Anoxic oxidation of arsenite linked to denitrification in sludges and sediments. *Water Research* **42**: 4569-4577.

Sweerts, JPRA & D De Beer (1989): Microelectrode Measurements of Nitrate Gradients in the Littoral and Profundal Sediments of a Meso-Eutrophic Lake (Lake Vechten, the Netherlands). *Appl. Environ. Microbiol.* **55**: 754-757.

Tabita, FR (1988): Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol. Rev.* **52**: 155-189.

Tamura, K, J Dudley, M Nei & S Kumar (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596-1599.

Thalasso, F, A Vallecillo, P GarciaEncina & F FdzPolanco (1997): The use of methane as a sole carbon source for wastewater denitrification. *Water Research* **31**: 55-60.

Thauer, RK (1998): Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology-SGM* **144**: 2377-2406.

Thauer, RK & S Shima (2006): Biogeochemistry - Methane and microbes. *Nature* **440**: 878-879.

Thauer, RK & S Shima (2008): Methane as fuel for anaerobic microorganisms. *Ann. N. Y. Acad. Sci.* **1125**: 158-170.

Throbäck, IN, K Enwall, A Jarvis & S Hallin (2004): Reassessing PCR primers targeting *nirS, nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology* **49**: 401-417.

Tomassen, HBM, AJP Smolders, LPM Lamers & JGM Roelofs (2003): Stimulated growth of *Betula pubescens* and *Molinia caerulea* on ombrotrophic bogs: role of high levels of atmospheric nitrogen deposition. *Journal of Ecology* **91**: 357-370.

Treude, T, M Krüger, A Boetius & BB Jørgensen (2005): Environmental control on anaerobic oxidation of methane in the gassy sediments of Eckernförde Bay (German Baltic). *Limnology and Oceanography* **50**: 1771-1786.

Trotsenko , YA & JC Murrell (2008): Metabolic aspects of aerobic obligate methanotrophy. *Adv. Appl. Microbiol.* **63**: 183-229.

Valentine, DL & WS Reeburgh (2000): New perspectives on anaerobic methane oxidation. *Environmental Microbiology* **2**: 477-484.

Valentine, DL (2002): Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 271-282.

Vallenet, D, L Labarre, Z Rouy *et al.* (2006): MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Res.* **34**: 53-65.

van Beilen, J & E Funhoff (2007): Alkane hydroxylases involved in microbial alkane degradation. *Appl. Microbiol. Biotechnol.* **74**: 13-21.

van Breukelen, BM & J Griffioen (2004): Biogeochemical processes at the fringe of a landfill leachate pollution plume: potential for dissolved organic carbon, Fe(II), Mn(II), NH_4, and CH_4 oxidation. *Journal of Contaminant Hydrology* **73**: 181-205.

van Breukelen, BM, WFM Röling, J Groen, J Griffioen & HW van Verseveld (2003): Biogeochemistry and isotope geochemistry of a landfill leachate plume. *Journal of Contaminant Hydrology* **65**: 245-268.

van Cleemput, O (1998): Subsoils: Chemo- and biological denitrification, N_2O and N_2 emissions. *Nutrient Cycling in Agroecosystems* **52**: 187-194.

van der Biezen, E (2007): *The nitrite reductase gene involved in anaerobic methane oxidation* (Bachelor thesis). 34. Hogeschool van Arnhem en Nijmegen, Nijmegen.

van der Star, WRL, AI Miclea, UGJM van Dongen *et al.* (2008): The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnol. Bioeng.* **101**: 286-294.

van de Vossenberg, J, JE Rattray, W Geerts *et al.* (2008): Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production. *Environmental Microbiology* **10**: 3120-3129.

van Dijk, G, C Fritz, AJP Smolders *et al.* (2009): De Brunssummerheide, een uniek maar bedreigd stukje Nederland. *Natuurhistorisch Maandblad* **98**: 233-238.

van Ginkel, CG, GB Rikken, AGM Kroon & SWM Kengen (1996): Purification and characterization of chlorite dismutase: A novel oxygen-generating enzyme. *Arch. Microbiol.* **166**: 321-326.

Vieites, JM, M-E Guazzaroni, A Beloqui, PN Golyshin & M Ferrer (2009): Metagenomics approaches in systems microbiology. *FEMS Microbiol. Rev.* **33**: 236-255.

Vitousek, PM, JD Aber, RW Howarth *et al.* (1997): Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* **7**: 737-750.

Vitousek, PM & RW Howarth (1991): Nitrogen limitation on land and in the sea: How can it occur? *Biogeochemistry* **13**: 87-115.

von Wintzingerode, F, UB Göbel & E Stackebrandt (1997): Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21**: 213-229.

Wake, LV, P Rickard & BJ Ralph (1973): Isolation of Methane Utilizing Microorganisms - Review. *J. Appl. Bacteriol.* **36**: 93-99.

Walsh, DA, E Zaikova, CG Howes *et al.* (2009): Metagenome of a versatile chemolithoautotroph from expanding oceanic dead zones. *Science* **326**: 578-582.

Ward, BB, AH Devol, JJ Rich *et al.* (2009): Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature* **461**: 78-81.

Weatherly, DB, JA Atwood, TA Minning *et al.* (2005): A heuristic method for assigning a false-discovery rate for protein identifications from Mascot database search results. *Molecular and Cellular Proteomics* **4**: 762-772.

Weelink, SAB, NCG Tan, H ten Broeke *et al.* (2008): Isolation and characterization of *Alicycliphilus denitrificans* strain BC, which grows on benzene with chlorate as the electron acceptor. *Appl. Environ. Microbiol.* **74**: 6672-6681.

Whiticar, MJ (1999): Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chemical Geology* **161**: 291-314.

Widdel, F & F Musat (2010): Energetic and other quantitative aspects of microbial hydrocarbon utilization. In: *Handbook of Hydrocarbon and Lipid Microbiology.* Springer, Berlin, Heidelberg.

Wilhelm, E, R Battino & RJ Wilcock (1977): Low-Pressure Solubility of Gases in Liquid Water. *Chem. Rev.* **77**: 219-262.

Wilm, M, A Shevchenko, T Houthaeve *et al.* (1996): Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**: 466-469.

Wilmes, P, SL Simmons, VJ Denef & JF Banfield (2009): The dynamic genetic repertoire of microbial communities. *FEMS Microbiol. Rev.* **33**: 109-132.

Winfrey, MR & JG Zeikus (1977): Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. *Appl. Environ. Microbiol.* **33**: 275-281.

Winogradsky, S & V Omeliansky (1899): Über den Einfluss der Organischen Substanzen auf die Arbeit der nitrifizierenden Mikroben. *Zentralblatt für Bakteriologie, Parisitenkunde und Infektionskrankheiten, 2. Abteilung: Allgemeine landwirschaftlich-technologische Bakteriologie, Gärungsphysiologie, Pflanzenpathologie und Pflanzenschutz* **5**: 329-440.

Wrage, N, GL Velthof, ML van Beusichem & O Oenema (2001): Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* **33**: 1723-1732.

Wullstein, LH & CM Gilmour (1966): Non-enzymatic formation of nitrogen gas. *Nature* **210**: 1150-1151.

Zedelius, J, R Rabus, O Grundmann *et al.* (2010): Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environmental Microbiology Reports*: in press, DOI: 10.1111/j.1758-2229.2010.00198.x.

Zehnder, AJB & TD Brock (1979): Methane formation and methane oxidation by methanogenic bacteria. *J. Bacteriol.* **137**: 420-432.

Zehnder, AJB & TD Brock (1980): Anaerobic methane oxidation - occurrence and ecology. *Appl. Environ. Microbiol.* **39**: 194-204.

Zengler, K, HH Richnow, R Rossello-Mora, W Michaelis & F Widdel (1999): Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**: 266-269.

Zhang, G, J Tian, N Jiang *et al.* (2008): Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I. *Environmental Microbiology* **10**: 1850-1860.

Zhou, J, MA Bruns & JM Tiedje (1996): DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* **62**: 316-322.

Zumft, WG (1993): The biological role of nitric oxide in bacteria. *Arch. Microbiol.* **160**: 253-264.

Zumft, WG (1997): Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**: 533-616.

Zumft, WG (2005): Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J. Inorg. Biochem.* **99**: 194-215.

Zumft, WG & PMH Kroneck (2007): Respiratory transformation of nitrous oxide (N_2O) to Dinitrogen by Bacteria and Archaea In: *Advances in Microbial Physiology*. 107-227. Academic Press.

Summary

Samenvatting

Zusammenfassung

English summary

The anaerobic oxidation of methane coupled to nitrite reduction of is one of the latest discoveries linking the carbon and nitrogen cycles. In the introduction of this thesis (chapter 1), denitrification and methane oxidation are reviewed in the context of these cycles, and the current (sparse) knowledge on the coupling of these two processes is summarized. Many microorganisms respire anaerobically using nitrogen oxides as terminal electron acceptors, and various organic and inorganic electron donors. Although methane is an abundant electron donor in many anaerobic environments, its oxidation by denitrifying microorganisms had never been described. In several cases, anaerobic methane oxidation in presence of nitrate (NO_3^-) or nitrite (NO_2^-) was reported, but proof for its direct coupling to denitrification and the identification of the responsible microorganisms was entirely missing. For the first time in 2006, an enrichment culture was obtained and characterized that could use methane as an electron donor for denitrification (Raghoebarsing et al., 2006). Based on the community composition – about 10 % archaea of the order *Methanosarcinales* and 80 % bacteria of the uncultured NC10 phylum – a reverse methanogenesis mechanism was hypothesized: taking advantage of the same enzyme system as for methanogenesis, the archaeon would oxidize methane and shuttle the reducing equivalents to nitrite-reducing bacteria. The aim of this thesis was to further characterize these nitrite-reducing methanotrophic microorganisms, and to study the underlying biochemical process.

Chapter 2 addresses the role of the archaea in the process. The previously described enrichment culture ("Twente") was scaled up to increase total activity and microbial biomass. The gene encoding the archaeal methyl-coenzyme M reductase (MCR), responsible for methane production by methanogens and methane activation by anaerobic methanotrophs, was sequenced and the associated cofactor F_{430} identified by MALDI-TOF analysis. F_{430} was identical to the one of common methanogens. By combining the sensitive detection of F_{430} and fluorescence in situ hybridization (FISH) with specific probes, the decline and finally disappearance of the archaeal population in the enrichment culture was documented. Moreover, the potent MCR inhibitor bromoethane sulfonate did not affect the methane-oxidizing activity, which remained coupled to nitrite reduction to nitrogen gas in the expected 3:8 (CH_4:NO_2^-) stoichiometry. Taken together, these results prove that archaea were not crucial for the process, which could be carried out by bacteria alone.

The following chapter (3) focuses on the NC10 bacteria implicated in anaerobic methane oxidation and introduces methods for their specific detection and quantification. A freshwater sediment (from the Ooijpolder near Nijmegen) harboring a wide diversity of NC10 bacteria was chosen as the inoculum for a second enrichment culture (designated "Ooij"). The culture was continuously supplied with nitrite-containing mineral medium

and methane. After four months, nitrite-reducing activity became measurable, and the further increase in nitrite consumption closely matched the change in abundance of a single group of NC10 bacteria. FISH analysis of the enriched biomass verified the dominance of NC10 phylum bacteria very similar to those enriched in the previous culture ("Twente"). Hereby, the identity of NC10 bacteria as nitrite-reducing anaerobic methane oxidizers was established.

The enrichment culture from chapter 3 also served the physiological experiments described in chapter 4. At this point, the reverse-methanogenesis mechanism had been ruled out and the NC10 bacteria had been identified as the key players, questioning the nature of their metabolism. To address this question, the metagenomes of the above enrichment cultures were sequenced. From the "Twente" metagenome, the complete genome sequence (2.75 Mb) of the dominant NC10 bacterium was assembled, and complemented by the partial genome (~90 %) of culture "Ooij". Curiously, functional gene annotation showed that both genomes contained the entire aerobic methane oxidation pathway. Furthermore, all genes were also represented in the transcriptome and proteome. In contrast, the denitrification pathway was not complete: the gene coding for nitrous oxide reductase, which catalyzes the production of nitrogen gas (N_2) from nitrous oxide (N_2O), was missing. Based on these contradictory findings, a new biochemical mechanism was proposed: N_2 and oxygen (O_2) would be formed from two molecules of nitric oxide (NO) by a novel enzyme, oxygen being consumed in conventional aerobic methane oxidation.

To test this hypothesis, we performed a series of physiological experiments with the enrichment culture "Ooij". Using stable isotopes of nitrogen (^{15}N), it was shown that nitrous oxide was indeed not an intermediate in the production of N_2 from NO_2^-. Next, the activity of the key enzyme of aerobic methane oxidation, particulate methane mono-oxygenase (pMMO), was demonstrated. pMMO catalyzes the first step in methane oxidation, notably the incorporation of free oxygen into methane to yield methanol; the enzyme uses propylene as an artificial substrate, producing propylene oxide. Here, pMMO was active both under aerobic as well as anaerobic, NO_2^--reducing conditions. The use of ^{18}O-labeled NO_2^- confirmed that the oxygen atom in propylene oxide was derived from NO_2^-, as proposed. In a similar setup, O_2 could be measured as a free intermediate by inhibiting the pMMO, thus enabling the extracellular accumulation of ^{18}O-labeled O_2 from ^{18}O-NO_2^-. The now genetically and physiologically better characterized NC10 bacterium was tentatively named '*Candidatus* Methylomirabilis oxyfera', alluding to its peculiar transient O_2 production.

The final outlook chapter (5) discusses some likely implications of the new catabolic pathway in '*M. oxyfera*' and points out important unanswered questions about its metabolism. Future research will reveal how widespread both the oxygen-producing

metabolism and '*M. oxyfera*'-like bacteria are in nature, and determine their significance for the biological cycling of carbon and nitrogen.

Samenvatting

De anaërobe (zuurstofloze) oxidatie van methaan gekoppeld aan de reductie van nitriet is een van de meest recente ontdekkingen in de koolstof- en stikstofkringlopen. In de inleiding van dit proefschrift (hoofdstuk 1) plaatsen we de denitrificatie en methaanoxidatie door micro-organismen binnen het geheel van de methaan- en stikstofcycli en geven we een overzicht van de schaarse kennis, hoe beide processen aan elkaar gekoppeld zijn. Veel micro-organismen verademen geen zuurstof, maar gebruiken stikstofoxiden, bij voorbeeld nitraat (NO_3^-) of nitriet (NO_2^-) als elektronacceptor. Als elektrondonor (en meestal ook koolstofbron) gebruiken zij een scala van organische en anorganische verbindingen. Hoewel methaan veel voorkomt in zuurstofloze omgevingen, zoals natte sedimenten, is de afbraak ervan door denitrificerende bacteriën, die ook op deze plekken voorkomen, heel lang onopgemerkt gebleven. Sommige onderzoeken maken weliswaar melding van een anaërobe afbraak van methaan in aanwezigheid van nitraat en nitriet, maar bewijs voor de directe koppeling tussen beide processen was nog niet eerder geleverd; de micro-organismen die dit zouden kunnen uitvoeren bleven onbekend. Pas in 2006 is voor het eerst een ophoping (uit Twentekanaal-sediment) verkregen en beschreven, die de oxidatie van methaan kon koppelen aan denitrificatie (Raghoebarsing et al., 2006). Op grond van de samenstelling van de microbiële gemeenschap – ongeveer 10 % archaea uit de orde van de *Methanosarcinales* en 80 % bacteriën behorend tot het niet eerder gekweekte NC10 phylum – is toen aangenomen, dat het onderliggende biochemische proces een omgekeerde methanogenese zou zijn: de archaea zouden met behulp van hetzelfde enzymsysteem voor de vorming ervan, methaan oxideren en de hierbij vrijkomende elektronen overdragen op de denitrificerende bacteriën.

Het doel van dit proefschrift was om deze nitrietreducerende en methaanafbrekende microben verder te karakteriseren en het biochemische mechanisme te ontrafelen dat dit proces mogelijk maakt.

In Hoofdstuk 2 richten we ons op de rol van de archaea. Om meer activiteit en biomassa te verkrijgen hebben we daartoe eerst de bovengenoemde ophoping ("Twente") opgeschaald. We hebben de sequentie bepaald van het gen dat codeert voor het methyl-coenzym M reductase (MCR) en met behulp van MALDI-TOF-analyse de aard vastgesteld van F_{430}, de cofactor van het MCR eiwit. MCR katalyseert bij methanogenen de laatste stap in de methaanproductie en bij de nauw verwante methaanafbrekende archaea de eerste stap van dit proces, de activering van methaan. Door een gevoelige meetmethode voor de hoeveelheid F_{430} te combineren met fluorescentie *in situ*

hybridisatie (FISH) konden we aantonen, dat de archaea in de biomassa in de loop van de tijd in aantal afnamen en tenslotte helemaal verdwenen. Ook bleek dat broomethaansulfonaat, een specifieke remmer van MCR, geen effect had op de activiteit van de cultuur: de oxidatie van methaan ging door en bleek nog steeds gekoppeld te zijn aan de reductie van nitriet tot stikstofgas (N_2), en wel in de verwachte 3:8 (CH_4:NO_2^-) stoichiometrie. Deze resultaten lieten zien, dat de archaea niet nodig waren en dat het proces blijkbaar door de NC10 bacteriën alleen kon worden uitgevoerd.

In hoofdstuk 3 staan de NC10 bacteriën centraal die bij de anaërobe methaanoxidatie betrokken zijn. Allereerst beschrijven we methodes om deze micro-organismen specifiek te kunnen detecteren en kwantificeren. We hebben een tweede ophoping ("Ooij") ingezet en deze aangeënt met een zoetwater sediment uit een sloot in de Ooijpolder bij Nijmegen, dat een grote diversiteit aan NC10 bacteriën bevatte. De ophoping vond plaats in een continu cultuur met zuiver mineraal nitriethoudend medium en begassing met methaan. Vier maanden na aanenten was de nitrietreductie zodanig toegenomen dat die meetbaar werd en de verdere toename in het nitrietverbruik bleek nauwkeurig gecorreleerd te zijn aan de toename van het aantal bacteriën van één bepaalde groep binnen het NC10 phylum. Met behulp van FISH konden we ook aantonen dat de biomassa vooral bestond uit NC10 bacteriën die nauw verwant waren aan die uit de eerdere ophoping ("Twente"). De betrokkenheid van NC10 bacteriën bij de methaanoxidatie gekoppeld aan de reductie van nitriet kon zo worden bevestigd.

De ophoping ("Ooij") uit hoofdstuk 3 is daarna gebruikt voor fysiologische experimenten die beschreven staan in hoofdstuk 4. De hypothese van de omgekeerde methanogenese was nu terzijde geschoven en de NC10 bacteriën bleken de enige hoofdrolspelers, wat de onmiddellijke vraag opriep naar het metabolisme van deze organismen. Om een antwoord te vinden op deze vraag zijn de metagenomen van de twee ophopingen gesequenceerd. Het "Twente"-metagenoom verschafte alle gegevens om het volledige genoom (2.75 Mb) van de dominante NC10 bacterie te kunnen samenstellen. Deze informatie konden we aanvullen met het bijna gehele genoom (ongeveer 90 %) van de NC10 bacteriën uit ophoping "Ooij". Uit de vergelijking met bekende genen van andere organismen bleek heel verrassend dat beide genomen het hele repertoire aan genen bevatten voor de aërobe (zuurstofgebruikende) afbraak van methaan. De betreffende genen werden allemaal ook overgeschreven in mRNA en alle door die genen gecodeerde eiwitten waren terug te vinden in het proteoom. De metabole route voor denitrificatie bleek daarentegen onvolledig. Het enzym dat de vorming van stikstof uit lachgas (N_2O) katalyseert, het N_2O-reductase, was afwezig. Op het eerste gezicht waren de resultaten verwarrend en tegenstrijdig: een aërobe afbraakroute voor methaan in een anaëroob levende bacterie, die daarnaast stikstof maakt zonder de bekende genen te hebben voor de vorming ervan. Om deze tegenstrijdigheid op te lossen hebben we

een nieuw biochemisch mechanisme voorgesteld. Hierbij zouden stikstof (N_2) en zuurstof (O_2) gevormd worden uit twee moleculen stikstofmonoxide (NO) door een nieuw, nog onbekend enzym. De zuurstof kon dan worden gebruikt voor de gangbare aërobe afbraak van methaan.

Om deze hypothese te toetsen hebben we een serie fysiologische proeven uitgevoerd met de ophoping "Ooij". Experimenten met stabiele stikstofisotopen (^{15}N) toonden aan dat lachgas inderdaad geen tussenproduct was bij het maken van stikstof. Daarnaast konden we de activiteit vaststellen van het sleutelenzym van de aërobe methaanafbraakroute, het methaanmonooxygenase (MMO). MMO katalyseert de inbouw van vrije zuurstof in methaan onder de vorming van methanol; het enzym kan propyleen als kunstmatig substraat gebruiken dat hierbij wordt omgezet in het goed meetbare propyleenoxide. De MMO activiteit met propyleen vond zowel onder aërobe als anaërobe, nitriet-reducerende omstandigheden plaats. Door de zuurstofatomen in nitriet (NO_2^-) nu te labelen met het stabiele zuurstofisotoop ^{18}O, konden we bewijzen dat het zuurstofatoom in propyleenoxide afkomstig was van zuurstof uit nitriet. Op een vergelijkbare manier kon worden vastgesteld dat zuurstof een vrij tussenproduct was in het proces: gelabelde zuurstof afkomstig van nitriet hoopte extracellulair duidelijk meetbaar op als het MMO gelijktijdig geremd werd. Op basis van alle nieuwe gegevens is de NC10-bacterie nu genetisch en fysiologisch veel beter begrepen en we hebben het organisme '*Candidatus* Methylomirabilis oxyfera' gedoopt als toespeling op de wonderlijke zuurstofproductie.

In het laatste hoofdstuk (5) bediscussiëren we de betekenis van onze bevindingen en stippen we de belangrijkste openliggende vragen aan met betrekking tot het metabolisme van '*Methylomirabilis oxyfera*'. Toekomstig onderzoek zal bovendien moeten uitwijzen hoe wijdverspreid nauw verwanten van '*M. oxyfera*' zijn in de natuur, wat de precieze rol is in het milieu en welke bijdrage deze micro-organismen en mogelijk vergelijkbare zuurstofvormende processen leveren aan de koolstof- en stikstofkringlopen.

Zusammenfassung

Anaerobe Methanoxidation in Verbindung mit Denitrifizierung ist eine der neuesten Entdeckungen im Kohlenstoff- und Stickstoffkreislauf der Erde. In der Einleitung zu dieser Doktorarbeit (Kapitel 1) beschreiben wir mikrobielle Denitrifikation und Methanoxidation im Kontext dieser globalen Stoffkreisläufe und fassen das bisher spärliche Wissen über die Kopplung beider Prozesse zusammen. Viele Mikroorganismen veratmen keinen Sauerstoff, sondern benutzen stattdessen Stickstoffoxide, wie zum Beispiel Nitrat (NO_3^-) oder Nitrit (NO_2^-), als Elektronenakzeptoren. Als Elektronendonoren (und meistens auch als Kohlenstoffquelle) verwenden sie eine ganze

Reihe verschiedener organischer und anorganischer Verbindungen. Obwohl sauerstofffreie Milieus wie z.B. nasse Sedimente viel Methan enthalten, ist dessen Abbau durch dort lebende denitrifizierende Bakterien sehr lange unentdeckt geblieben. In einigen Untersuchungen wurde zwar anaerober Abbau von Methan bei gleichzeitiger Anwesenheit von Nitrat und Nitirit beobachtet, aber die direkte Kopplung an Denitrifikation war noch nicht eher bewiesen worden. Auch waren keine Mikroorganismen bekannt, die diesen Prozess ausführen könnten. Erst im Jahr 2006 gelang es, eine Kultur aus Sedimenten des niederländischen Twentekanals anzureichern, die Methanoxidation und Denitrifizierung aneinander koppelte. Wegen der Zusammenstellung der mikrobiellen Flora – ungefähr 10 % waren Archaeen aus der Ordnung der Methanosarcinales und 80 % gehörten zu dem nicht kultivierten Bakterienstamm „NC10" – wurde damals angenommen, dass der Prozess durch 'umgekehrte Methanogenese' zu erklären sei: Die Archaeen würden mit den gleichen Enzymen, welche auch für die Methanproduktion genutzt werden, Methan oxidieren, und die dabei freiwerdenden Elektronen auf die denitrifizierenden Bakterien übertragen.

Ziel dieser Arbeit war es, diese denitrifizierenden und methanabbauenden Mikroorganismen näher zu charakterisieren und herauszufinden, welcher biochemische Stoffwechselweg diese Reaktionen ermöglicht.

In Kapitel 2 konzentrieren wir uns auf die Rolle der Archaeen. Dazu wurde zunächst die schon zuvor beschriebene Anreicherungskultur („Twente") im Umfang vergrößert, um mehr Biomasse und höhere mikrobielle Aktivität zu erhalten. Wir haben das Gen der archaealen Methyl-Coenzym M-Reduktase (MCR) sequenziert und mithilfe von MALDI-TOF-Analyse den Kofaktor F_{430} dieses Enzyms identifiziert. MCR katalysiert bei Methanogenen den letzten Schritt der Methanproduktion und bei den nah verwandten methanoxidierenden Archaeen den ersten Schritt, die Aktivierung von Methan. F_{430} war identisch mit dem gewöhnlicher Methanogene. Durch die Kombination einer sensitiven Messmethode zur Quantifizierung des Kofaktors F430 mit Fluoreszenz-*in-situ*-Hybridisierung (FISH) konnten wir zeigen, dass die Archaeen im Laufe der Zeit weniger wurden und schließlich vollständig verschwanden. Zudem hatte Bromethansulfonat, ein spezifischer Hemmstoff der MCR, keinen Effekt auf die Aktivität der Anreicherungskultur: Methanoxidierung dauerte an, und war noch immer an die Reduktion von Nitrit zu Stickstoff (N_2) in der zu erwartenden 3:8 (CH_4:NO_2^-) Stöchiometrie gekoppelt. Diese Resultate zeigten, dass die Archaeen verzichtbar waren, und die NC10-Bakterien den Prozess anscheinend alleine ausführen konnten.

In Kapitel 3 liegt der Fokus auf den NC10-Bakterien, die mit der anaeroben Methanoxidation assoziiert sind. Zunächst beschreiben wir molekulare Methoden um diese Bakterien spezifisch zu detektieren und zu quantifizieren. Als Ausgangsmaterial für eine weitere Anreicherungskultur haben wir eine Sedimentprobe aus einem Süßwasser-

Entwässerungsgraben im Ooijpolder bei Nimwegen verwendet, welche eine große Diversität von NC10-Bakterien aufwies. Diese wurde kontinuierlich mit Methan begast und mit rein mineralischem nitrithaltigem Medium versorgt. Nach vier Monaten hatte die Nitritreduktion der Bakterienkultur ein messbares Niveau erreicht, und ihre weitere Zunahme korrelierte mit der Vermehrung einer bestimmten Gruppe von Bakterien innerhalb des NC10-Stammes, die auch schon in der „Twente"-Kultur dominiert hatte. Mithilfe von FISH konnten wir zudem zeigen, dass diese Gruppe den Großteil der Biomasse in der „Ooij"-Anreicherungskultur ausmachte. Damit war deutlich, dass sie eine zentrale Rolle bei der anaeroben Methanoxidation gekoppelt an Nitritreduktion spielten.

Die Anreicherungskultur „Ooij" aus Kapitel 3 wurde daneben auch für die physiologischen Experimente von Kapitel 4 verwendet. Nachdem die Hypothese der 'umgekehrten Methanogenese' widerlegt war, stellte sich die Frage, welchen biochemischen Mechanismus die verbliebenen NC10-Bakterien verwendeten. Um einer Antwort näher zu kommen, wurden die Metagenome der beiden Anreicherungskulturen sequenziert. Aus dem „Twente"-Metagenom konnte das vollständige Genom (2.75 Mb) der dominanten NC10-Bakterien rekonstruiert werden. Ergänzt wurde diese Information durch das zu 90 % vollständige Genom der NC10-Bakterien in der „Ooij"-Anreicherung. Überraschenderweise ergab ein Vergleich mit von anderen Organismen bekannten Genen, dass beide Genome das komplette Repertoire für den aeroben Abbau von Methan enthielten. Die entsprechenden Gene konnten alle auch im Transkriptom (mRNA) und Proteom detektiert werden. Im Gegensatz dazu war der Stoffwechselweg der Denitrifizierung unvollständig – die N_2O-Reduktase fehlte, welche den letzten Schritt, die Produktion von Stickstoff aus Distickstoffoxid (Lachgas) katalysiert. Auf den ersten Blick waren diese Ergebnisse sehr widersprüchlich: Ein aerober Stoffwechselweg für Methan in einem anaerob lebenden Bakterium, welches Stickstoff produziert ohne über die dazu notwendigen Gene und Enzyme zu verfügen. Um diesen Widerspruch erklären zu können, haben wir einen neuen biochemischen Mechanismus vorgestellt: Ein noch unbekanntes Enzym formt aus zwei Molekülen Stickstoffmonoxid (NO) molekularen Sauerstoff (O_2) und Stickstoff (N_2). Der Sauerstoff kann dann für die bekannte aerobe Abbauroute verwendet werden.

Um diese Hypothese zu testen haben wir eine Reihe physiologischer Experimente mit der „Ooij"-Kultur durchgeführt. Mithilfe stabiler Stickstoffisotope (^{15}N) konnten wir beweisen, dass N_2O tatsächlich nicht als Zwischenprodukt bei der Stickstoffbildung auftritt. Weiterhin konnten wir die Aktivität der Methanmonooxygenase (MMO) zeigen, dem Schlüsselenzym aerober Methanoxidierer. MMO katalysiert den Einbau von Sauerstoff in Methan, woraufhin Methanol entsteht. Anstelle von Methan setzt das Enzym auch das künstliche Substrat Propylen um, das zu dem gut messbaren Propylenoxid oxidiert wird. Diese MMO-Aktivität mit Propylen konnten wir sowohl

aerob als auch anaerob mit Nitrit nachweisen. Indem wir die Sauerstoffatome im Nitrit (NO_2^-) mit dem stabilen Sauerstoffisotop ^{18}O markiert haben, konnten wir beweisen, dass das Sauerstoffatom im Propylenoxid tatsächlich aus dem Nitrit stammte. Auf ähnliche Weise gelang auch der Nachweis freien, molekularen Sauerstoffs als Zwischenprodukt: Bei Zuabe von ^{18}O-markiertem Nitrit und gleichzeitiger Hemmung der MMO akkumulierte ^{18}O-O_2 extrazellulär und konnte so gemessen werden. In Anspielung auf diese überraschende Sauerstoffproduktion haben wir die nun auf genetischer und physiologischer Ebene wesentlich besser charakterisierten NC10-Bakterien '*Candidatus* Methylomirabilis oxyfera' genannt.

Im Schlusskapitel (5) diskutieren wir die Bedeutung dieses neuen Stoffwechselweges und reißen die wichtigsten der noch immer vielen offenen Fragen über '*Methylomirabilis oxyfera*' kurz an. Weitergehende Forschung sollte untersuchen, wie weit verbreitet '*M. oxyfera*' und seine nahen Verwandten in der Natur sind, und welche Rolle der hier beschriebene und möglicherweise weitere sauerstoffproduzierende Prozesse im Methan- und Stickstoffkreislauf der Erde spielen.

hello katharina;

Dankwoord

Acknowledgements

Danksagung

A work that goes along with reactor catastrophes, gas explosions and the ruin of entire cultures, all this in an outlandish country, obviously requires good friends and colleagues to cope with it. I am blessed in this respect and why I liked and like it in Nijmegen hopefully becomes clear from these acknowledgements.

Marc, jouw projectbeschrijving heeft me onmiddelijk gegrepen, en na ons eerste telefoongesprek wist ik dat ik graag in Nijmegen met jou zou willen werken. Ik heb er geen seconde spijt van gehad, en we kunnen het er waarschijnlijk overeens worden dat mijn traject weliswaar heel anders, maar zeker interessanter is gelopen dan we ons allebei hadden voorgesteld. Bedankt voor alle enthousiaste en inspirerende discussies - bij hoofdstuk 4 was tenslotte zelfs het schrijven een plezier. Mike, ook van jou heb ik veel geleerd, maar ben nog ver verwijderd van een verglijkbaar goed tijdmanagement. Ongelofelijk hoe je altijd nog op de ongunstigste momenten tijd en geduld kon vinden voor een gesprek, email, manuscriptcorrectie of een onmogelijke foto (waarvan je hopelijk ook in de toekomst niet te veel misbruik maakt). Jullie als elkaar aanvullende begeleiders lieten mij aangenaam veel vrijheid, maar wisten me op de goede momenten rust en steun te geven om het weer aan te kunnen.

Ashna, nu aan het eind van mijn promotie waardeer ik nog veel meer, hoe je mij toen in diezelfde situatie verwelkomt en ingewerkt hebt, hoewel ik toch met je kindje doorging. Verder ook bedankt voor de manier waarop je met mijn eerste zinnen in het Nederlands omging, onze excursies (Sinterklaas!) en etentjes. In deze vroege tijd in Nijmegen hebben me nog veel meer mensen geholpen me snel thuis te voelen. Maria, meine erste Nacht in Nijmegen hab ich bei Dir verbracht - demnächst müssen wir endlich mal eine tanzen gehen! Markus, selbst beim Anstreichen war es hartstikke gezellig mit Dir, und Du wurdest auch nicht müde bei allen möglichen molekularbiologischen Fragen zu helfen. Jack, ik mis je "Spätzchen" ondanks alle Fettnäpfchen! Bedankt ook Marcel, Peter en Nico (ja, je had al heel vroeg het zuurstofidee geopperd!), dat ik soms samen met de Black T-Shirt Club de culinaire geheimen van de kantine mocht ontdekken. Met jullie was het leven vaak toch voor even een krentenbol. Wouter W., mijn eerste bureaubuurman, bedankt voor de gezegdes en de HPLC-hulp. Arjan, mijn Co-RP, niets is voor de planning van een experiment nuttiger dan een stevige discussie met jou. En niet veel is lekkerder dan wat jij kookt! Wim, bij alle kleine en grote catastrofen op het lab kon ik op je hulp rekenen, en je opwekkende lach is met niks te betalen. Marjan, gedeeld leed (dear oh dear) is half leed, gedeelde vreugde (tree-hugging and whale-watching) is dubbele vreugde! Suzanne, ook met jou was het een plezier de PhD area (en je beroemde lab-la!) te delen, vooral na 18:00. Huub, ohne Aufschmuck een groot bedankt voor je begeleiding. Verder zal ik het tweede, inofficiële Vorstellungsgespräch nooit vergeten, wie weet of ik anders goed was terecht gekomen...

Now for the DAMO group - long time since the last meeting and I start missing it! Katinka, van reactoren over FISH tot een dekbedovertrek gaat de schaal aan hulp die ik

van je heb gekregen. Aan jou en Francisca (Luesken, that's cool!) hartelijk dank voor alle experimenten samen en de vakantiediensten. Ming, I enjoyed our NO-experiment and discussions a lot and now expect a lot of discoveries to come. Baoli, I'm very glad that you took over the Brunssum project so well, and I'm looking forward to many more projects in the future. Margaret, I enjoyed our joint manuscript-writing and name-giving. Since you left, I used your reports and tables a lot, although the previous situation of just walking by and ask you was much more enjoyable. Theo, ik bewonder je kennis en geduld met PCR-technieken, laat ons gauw ook primers voor een nog interessanter enzym ontwerpen!

Marnix, Erwin en Faas, jullie hebben allemaal uitdagende projecten gekozen, en hebben die zeer zelfstandig uitgevoerd. Hopelijk vonden jullie deze begeleiding met wat afstand ook zo prettig. Daan, we hebben veel geestdrift en teleurstelling tijdens je stage gedeeld, en ik ben dankbaar dat we dat nog steeds met mooie en trieste momenten doen. Het samen drinken zal er ook nog van komen! Uit jouw practicumjaar, mijn eerste, ken ik ook Ahmad, en hoewel je geen lievelingen onder studenten mag hebben had ik die stiekem toch. Hopelijk klingt dit *not that bad at all*, en niet Duits-vies. Nardy, GC-, area- en kamergenote, met 100 keer zo veel flessen te hebben gemeten ben je nog bijna sneller klaar dan ik. Ronald, jij hebt me al gepasseerd, lopend sowieso, maar toch mocht ik je paranimf zijn. Adam, Alex, Baolan, Bart, Bas, Carmen, Daniel, Fabio, Frans, Greg, Harry, Jaime, Jan, Jayne, Jelle, Jennifer, John, Ke, Klaas, Laura, Lina, Maartje, Marianne, Marina, Mathilde, Monica, Nathalie, Pieter, Rob, Roland, Sarah, Titti, Wang and Wouter - all of you, and I'm afraid I still forgot some people, made life and work much more pleasant.

Our corridor neighbours from the fish and plant departments, thanks for all the evening activities! IWWR seminar series girls & boys, it was nice planning with you and of course going out with our speakers. Fons, Christian, Gijs en Leon, hartelijk bedankt voor de monsternamen samen en de discussies over de ecologische relevantie van onze beestjes. Marcel, Johannes, Frank und Dirk, herzlichen Dank für die heitere und produktive Zusammenarbeit, den geduldigen email-Verkehr, und nicht zu vergessen auch die Abende in Bremen, Hannover en Davos. Eric Pelletier and the Genoscope team, I know you only via mail, but I would really like to once share real and not just virtual champagne with you. Seigo Shima and Antonio Pierik, thanks for hosting me in Marburg - not finding what we were looking for turned out to be just as interesting after all. Thilo Behrends, Pieter Kleingeld, Dimmie Hendriks, Boris van Breukelen, Wilfred Röling, Frank Maixner and Stephanie Füreder -our collaborations unfortunately did not (yet) become visible, but still it was worth trying. Stefan Schouten en Jaap Sinninghe Damsté, mooi dat er nu een grote kans ligt om met jullie uitgebreid over de lipiden samen te werken.

Naomi, Du warst belangreich für mein leibliches (vorzügliches Essen, körperliche Ertüchtigung) und seelisches (nächtliche Gespräche im Labor und auf der Strasse) Wohl

im abgelaufenen Jahr, und hast mein Deutsch auf Peil gehalten. Pilar, Sara, Sonja, Veronica and Shaun, I would still like to thank you and my other housemates from the Villa in Plön. With you, besides having a very good and special time together, I was for the first time immersed in a scientific environment, and realized that this was what I liked to do. I'm happy to be still in contact with many of you. Ook Wouter, Pieter en mijn verdere recente buren, voisins, Nachbarn in Hatert worden bedankt voor het prettige samen leven, samen eten en het geruste gevoel bij aan- en afwezigheid.
Zita, mit Dir habe ich meine ersten wissenschaftlichen Erfahrungen und viele Corbiculas gesammelt. Und mit Stickstoff haben wir irgendwie auch schon gearbeitet, oder er gegen uns. Aber wofür ich Dir und Bernd vor allem danken will, ist Eure Freundschaft, ob die Sterne nun gut stehen oder nicht. Niels, bei unseren schon traditionell gewordenen Vor-Weihnachtstreffen musstest Du immer 'ne Menge Stress auffangen und Pfannkuchen essen, danke dafür und für alle langen Telefonate! Stefan, Hasi, ähnliches gilt für Dich, besonderen Dank für die Telefonseelsorge zu jeder (Un-)Zeit!
Jan, je wilt geen dank, maar krijgt hem toch.
B-to-the-R-to-the-O, from the light times in the dark PhD area we finally came to see the sunlight in dark times. We shared chocolate, Chouffe and coffee. We fought blood, sweets and tears with an immeasurable international army of the Norwegian Green, the Bostancı, Quincy, *Parus caeruleus*, Tacotaco, a certain anthem, modified pictures, juicy oranges. Not only did you share a glimpse of the immense wisdom of Turkish sayings with me, but also let me profit from your own. It's good to know about VVD muziek, cravats (that's really not obvieus), and the third possibility. Dankjewel, hoor! Although I know that ignorance is not bliss, I still wonder whether I borrowed or lent you Paris. Probably none of them - you've acquired her by prescription in the mean time.
Mama und Papa, Hannes und Dorothee, Willi und Kagi, ich weiß was ich an Euch habe. Es war in all den Jahren immer gut nach Hause zu kommen, und sei es nur am Telephon. Ihr habt schon meinen Heuaufguß geduldig ertragen, und meinen Aufenthalt an gefährlich geachteten Orten - da waren Reaktoren in den Niederlanden zum Glück kein Problem mehr. Viel mehr habt Ihr wahrscheinlich unter meiner notorisch schlechten Zeiteinteilung gelitten, und natürlich schreibe ich diese Zeilen in letzter Minute. Aber ich freue mich auf gemeinsame Wochenenden, Ausflüge und den Urlaub mit meiner kleinen Paranymphe!
György, a legnehezebb utoljára. Te játszottad a legfontosabb szerepet az életemben az utolsó években. Ki tudja, hogy vészeltem volna át a frusztráló első éveket anélkül, hogy minden este megosztottam volna veled a nap esemenyeit és hétvégén mindent. Sokkal többért tartozom neked köszönettel, mint az eszmecserékért (emlékszel, hogy már Plönben ^{18}O-ról beszéltünk?), magyar nyelvű szakirodalom kereséséért és a doktori írása alatti rendszeres érdeklődésért, hogy hogyan haladok. De ez egy más lapra tartozik.

Katharina Friederike Ettwig was born on April 17 1978 in Duisburg-Rheinhausen, Germany. After obtaining her German high school diploma from Amplonius-Gymnasium Rheinberg in 1997, she moved to Essen to study Environmental Sciences, supported by a fellowship of the German National Academic Foundation. She gained practical experience as an intern in nature reserves, bird ringing stations and as research assistant in the Departments of Hydrobiology and Botany at the University of Essen. Next to her interest in geo- and life sciences, she was politically active as elected vice-chairperson of the student's union, member of the faculty's council, and member of the academic senate of the University of Essen. In her first research internship in 2002-2003 she investigated trace metal accumulation in benthic organisms at the Research Center Jülich. Her interest in the role of microorganisms in the cycling of greenhouse gasses brought her to do her master thesis at the Max Planck Institute of Limnology in Plön, where she compared methane production and methanogen communities in lakes. After her Diploma in Environmental Sciences in 2005, she pursued her interest in environmentally relevant microorganisms as a PhD student in the Department of Microbiology, Radboud University Nijmegen. As part of this, she attended the Marine Biological Laboratory's summer course on Microbial Diversity in Woods Hole. Her work on anaerobic methane oxidation coupled to nitrite reduction, supervised by Marc Strous and Mike Jetten, led to the present thesis. Since January 2010 she is employed by the same department to study the biogeology of nitrite-dependant anaerobic methane oxidation.

Picture by Jan Rothstein

Ettwig KF, MK Butler, D Le Paslier, E Pelletier, S Mangenot, MMM Kuypers, F Schreiber, BE Dutilh, J Zedelius, D de Beer, J Gloerich, HJCT Wessels, TA van Alen, F Luesken, ML Wu, KT van de Pas-Schoonen, HJM Op den Camp, EM Janssen-Megens, K-J Francoijs, H Stunnenberg, J Weissenbach, MSM Jetten & M Strous (2010): Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543-548.

Ettwig KF, T van Alen, KT van de Pas-Schoonen, MSM Jetten, & M Strous (2009): Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. *Applied and Environmental Microbiology* **75** (11): 3656-3662.

Ettwig KF, A Pol, MK Butler, D Le Paslier, MMM Kuypers, HJM op den Camp, MSM Jetten, M Strous (2009) What is extreme? Methanotrophy at pH 1 or without oxygen? *Geochimica et cosmochimica acta* **73**: 342A.

Luesken FA, **KF Ettwig**, MK Butler, ML Wu, B Dutilh, MMM Kuypers, F Schreiber, KT van de Pas-Schoonen, T van Alen, M Rodriguez, E Pelletier, D Le Paslier, MSM Jetten, M Strous (2009): Anaerobic methane-oxidizing bacteria: surprising players in the methane and nitrogen cycle. *Antonie van Leeuwenhoek* **95**: 73.

Ettwig KF, S Shima, KT van de Pas-Schoonen, J Kahnt, MH Medema, HJM Op den Camp, MSM Jetten, M Strous (2008): Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environmental Microbiology* **10**: 3164-3173.

Raghoebarsing AA, A Pol, KT van de Pas-Schoonen, AJP Smolders, **KF Ettwig**, WIC Rijpstra, S Schouten, JS Sinninghe Damsté, HJM Op den Camp, MSM Jetten, & M Strous (2006): A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* 440: 918-921.

Sebesvári Z, **KF Ettwig** & H Emons (2005): Biomonitoring of tin and arsenic in different compartments of a limnic ecosystem with emphasis on *Corbicula fluminea* and *Dikerogammarus villosus*. *Journal of Environmental Monitoring* **7** (3): 203-207.