Nitrate- and nitrite-dependent anaerobic oxidation of methane

Cornelia U. Welte*[1,2], Olivia Rasigraf[1,3], Annika Vaksmaa[1], Wouter Versantvoort[1], Arslan Arshad[1], Huub J.M. Op den Camp[1], Mike S.M. Jetten[1,2,3], Claudia Lüke[1], Joachim Reimann*[1]

1 Department of Microbiology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525AJ Nijmegen, The Netherlands

2 Soehngen Institute of Anaerobic Microbiology, Heyendaalseweg 135, 6525AJ Nijmegen, The Netherlands

3 Netherlands Earth Systems Science Center, Heyendaalseweg 135, 6525AJ Nijmegen, The Netherlands

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* Address correspondence to Cornelia U. Welte, c.welte@science.ru.nl and Joachim Reimann, j.reimann@science.ru.nl

Summary

Microbial methane oxidation is an important process to reduce the emission of the greenhouse gas methane. Anaerobic microorganisms couple the oxidation of methane to the reduction of sulfate, nitrate and nitrite, and possibly oxidized iron and manganese minerals. In this article, we review the recent finding of the intriguing nitrate- and nitrite-dependent anaerobic oxidation of methane (AOM). Nitrate-dependent AOM is catalyzed by anaerobic archaea belonging to the ANME-2d clade closely related to *Methanosarcina* methanogens. They were named '*Candidatus* Methanoperedens nitroreducens' and use reverse methanogenesis with the key enzyme methyl-coenzyme M (methyl-CoM) reductase for methane activation. Their major end product is nitrite which can be taken up by nitrite-dependent methanotrophs. Nitrite-dependent AOM is performed by the NC10 bacterium '*Candidatus* Methylomirabilis oxyfera' that probably utilizes an intra-aerobic pathway through the dismutation of NO to N_2 and O_2 for aerobic methane activation by methane monooxygenase, yet being a strictly anaerobic microbe. Environmental distribution, physiological and biochemical aspects are discussed in this article as well as the cooperation of the microorganisms involved.

Key words: denitrification, methanotrophy, ANME, NC10, Mcr, archaea, anoxic, n-DAMO

36 # Introduction

37 Methane is an important greenhouse gas contributing substantially to the greenhouse effect and
38 global warming. It is produced in anoxic ecosystems by methanogenic archaea (Thauer *et al.*,
39 2008) as well as in the oxic ocean by phosphate-starved bacterioplankton through the
40 demethylation of methyl-phosphonates produced by ammonia oxidizing archaea (Karl *et al.*,
41 2008; Metcalf *et al.*, 2012). The major part of the produced methane (50-80 %) is oxidized by
42 aerobic and anaerobic methanotrophic microorganisms (Thauer *et al.*, 2008; Conrad, 2009).
43
44 In oxic environments, methane is consumed by aerobic bacterial methanotrophs with
45 representatives from alpha- and gammaproteobacteria (Semrau *et al.*, 2010) as well as from the
46 Verrucomicrobia (Op den Camp *et al.*, 2009; van Teeseling *et al.*, 2014). In anoxic
47 environments, however, these bacterial groups are probably not involved in methane oxidation.
48 In 2000, ANaerobic MEthanotrophic (ANME) archaea closely related to methanogenic archaea
49 were discovered and proven to be involved in methane oxidation in ecosystems devoid of oxygen
50 (Boetius *et al.*, 2000; Raghoebarsing *et al.*, 2006; Orphan *et al.*, 2001; Haroon *et al.*, 2013).
51 ANME archaea belonging to the clades ANME-1, ANME-2a/b, ANME-2c and ANME-3 have
52 been associated with anaerobic oxidation of methane coupled to sulfate reduction in consortia
53 with deltaproteobacterial sulfate reducers (Knittel and Boetius, 2009). Sulfate is abundant in
54 marine ecosystems but generally quite low in freshwater systems (Reeburgh and Heggie, 1977),
55 for which nitrate – and to some extent nitrite – are more relevant electron acceptors. In 2006, an
56 archaeal-bacterial enrichment culture was obtained that coupled the oxidation of methane to
57 denitrification (Raghoebarsing *et al.*, 2006). The archaeal partner couples anaerobic methane
58 oxidation to the reduction of nitrate to nitrite (Raghoebarsing *et al.*, 2006; Haroon *et al.*, 2013;

59 Arshad *et al.*, 2015). The archaea belong to the ANME-2d clade and the investigated
60 representative was named '*Candidatus* Methanoperedens nitroreducens'. Its metabolism involves
61 a complete reverse methanogenesis pathway with methyl-CoM reductase as the methane
62 activating enzyme. Cytoplasmic oxidation of methane to CO_2 is linked to an elaborate branched
63 membrane-bound respiratory chain involving many unusual protein complexes (Haroon *et al.*,
64 2013; Arshad *et al.*, 2015) and a high number of *c*-type cytochromes (Haroon *et al.*, 2013;
65 Arshad *et al.*, 2015; Kletzin *et al.*, 2015). The bacterial partner belongs to the NC10 clade and
66 perform nitrite-dependent methane oxidation (Ettwig *et al.*, 2008). In the previously mentioned
67 consortia, nitrite is provided by *'M. nitroreducens'* which benefits from the removal of its toxic
68 end product. Subsequent metagenome sequencing and physiological experiments of the NC10
69 bacteria provided strong indication for an intra-aerobic methane oxidation metabolism in which
70 nitrite is first reduced to nitric oxide (NO) which is then putatively dismutated to molecular
71 nitrogen and oxygen (Ettwig *et al.*, 2010). The NC10 bacterium responsible for this process,
72 '*Candidatus* Methylomirabilis oxyfera', uses particulate methane monooxygenase (pMMO) for
73 methane oxidation via the aerobic pathway similar to aerobic methanotrophic bacteria.
74
75 Here we give an overview of the current knowledge on nitrate- and nitrite-dependent anaerobic
76 methanotrophs that were first identified as denitrifying consortia: their ecology, distribution and
77 the proposed underlying biochemical pathways.
78
79 **The microbial ecology and diversity of nitrate- and nitrite-AOM**
80 Nitrate and nitrite are common constituents at oxic/anoxic interfaces where ammonium, diffusing
81 from deeper anoxic layers, is oxidized with oxygen diffusing from overlaying oxic zones (Brune

82 *et al.*, 2000). Here, methane can be used as electron donor to sustain populations of
83 nitrate/nitrite-dependent anaerobic methane oxidizing (N-AOM) microorganisms. In view of
84 expanding eutrophication around the globe (Galloway *et al.*, 2008), hypoxic zones with elevated
85 reactive nitrogen and methane concentrations as potential habitats for N-AOM microorganisms
86 will increase.

87

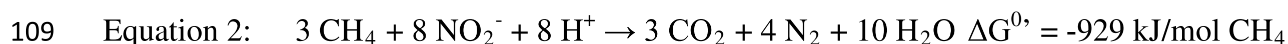
88 Although the potential of methane as electron donor for denitrification was recognized already
89 more than a decade ago (Sollo *et al.*, 1976; Mason, 1977; Panganiban *et al.*, 1979; Smith *et al.*,
90 1991; Thalasso *et al.*, 1997; Costa *et al.*, 2000; Eisentraeger *et al.*, 2001; Waki *et al.*, 2002; Islas-
91 Lima *et al.*, 2004) the first microbiological evidence for N-AOM came from an enrichment
92 culture originating from a highly eutrophic freshwater sediment of Twentekanaal in the
93 Netherlands (Raghoebarsing *et al.*, 2006). The enrichment culture oxidized methane to CO_2
94 while performing full denitrification to N_2. 16S rRNA gene sequencing enabled the design of
95 specific fluorescence *in situ* hybridization (FISH) probes, DARCH-872 and DARCH-641
96 (Raghoebarsing *et al.*, 2006; Schubert *et al.*, 2011; Ettwig *et al.*, 2016) for archaea and DBACT-
97 193 for bacteria (Raghoebarsing *et al.*, 2006), which were used for visualization of the culture
98 and are still commonly used to date (Figure 1). Molecular characterization revealed that this first
99 enrichment culture was mainly composed of anaerobic methane-oxidizing archaea (affiliated to
100 the GOM-ArcI/ANME-2D clade, Figure 2A) related to known methanogens and bacteria without
101 any cultured members and belonging to a new clade.

102

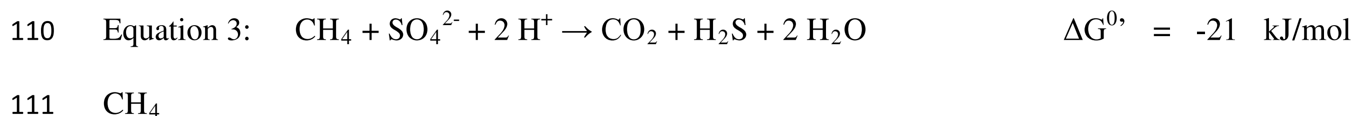
103 Today we know that the two dominant microorganisms, '*M. nitroreducens*' and '*M. oxyfera*', are
104 both capable of methane oxidation independent of each other (Ettwig *et al.*, 2008; Ettwig *et al.*,

105 2010; Haroon *et al.*, 2013). '*M. nitroreducens*' and other '*Methanoperedens*'-like archaea couple
106 AOM to nitrate reduction to nitrite (Equation 1) while '*M. oxyfera*'-like bacteria reduce nitrite to
107 dinitrogen gas (Equation 2).

108 Equation 1: $CH_4 + 4\ NO_3^- \rightarrow CO_2 + 4\ NO_2^- + 2\ H_2O$ $\Delta G^{0'} = -523$ kJ/mol CH_4



109 Equation 2: $3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O$ $\Delta G^{0'} = -929$ kJ/mol CH_4



110 Equation 3: $CH_4 + SO_4^{2-} + 2\ H^+ \rightarrow CO_2 + H_2S + 2\ H_2O$ $\Delta G^{0'} = -21$ kJ/mol
111 CH_4

112 In contrast to sulfate-dependent AOM (Equation 3), it is evident that N-AOM (Equations 1
113 and 2) are highly exergonic metabolic processes that are not performed at the thermodynamic
114 limit of life (Krüger *et al.*, 2003; Hallam *et al.*, 2004).

115

116 The bacterium performing nitrite-dependent AOM, '*M. oxyfera*', belongs to the NC10 clade and
117 was found to contain a phylogenetically distinct particulate methane monooxygenase which
118 offered the possibility to use one of the encoding genes (*pmoA*) as a functional biomarker for
119 their specific detection in the environment next to its 16S rRNA gene (Luesken *et al.*, 2011c;
120 Han and Gu, 2013). The 16S rRNA genes of NC10 bacteria have been divided previously into
121 four groups (A, B, C, D) (Ettwig *et al.*, 2009, Figure 3), where all to date known N-AOM
122 performing '*M. oxyfera*'-like bacteria characterized from enrichment cultures fall into group A
123 (Ettwig *et al.*, 2010; Haroon *et al.*, 2013; He *et al.*, 2015). Very recently, a high-quality draft
124 genome of an NC10 bacterium belonging to group D could be re-constructed from an aquifer
125 sediment metagenome (Hug *et al.*, 2016). Notably, this genome does not contain the *pmo* operon
126 (encoding a methane monooxygenase) nor a quinol-dependent NO reductase so it is lacking the
127 essential genes required for methane activation to methanol. However, it does encode enzymes

128 associated to methylotrophy such as a methanol dehydrogenase and enzymes involved in
129 formaldehyde and formate oxidation (Hug *et al.*, 2016). The genome therefore suggests that this
130 organism is a methylotroph, but does not share the intra-aerobic pathway of methane oxidation
131 with the group A organisms. Group B and C are to date only represented by environmental
132 sequences and no details are known about the physiology of these organisms. In addition to
133 DNA-based biomarkers, it was shown that '*M. oxyfera*' contains high amounts of 10-
134 methylhexadecanoic acid and a unique monounsaturated 10-methylhexadecenoic acid with a
135 double bond at the Δ7 position, which comprised up to 10 % of the total membrane fatty acid
136 profile (Kool *et al.*, 2012). These lipids have been successfully recovered from samples of a
137 Dutch peatland harboring substantial amounts of '*M. oxyfera*' cells (Kool *et al.*, 2012; Zhu *et al.*,
138 2012) and therefore provide an alternative mode of detection. The environments where
139 '*M. oxyfera*' biomarkers have been detected are shown in Table 1. The table shows a wide
140 habitat spectrum including ecosystems from eutrophic to oligotrophic, freshwater to marine, and
141 pristine to hydrocarbon contaminated. Although both DNA and lipids can be used to show the
142 presence of '*M. oxyfera*' bacteria in various anoxic habitats, they cannot be used as a proxy for
143 its contribution to methane oxidation activity. Thus, a variety of complementary methods based
144 on RNA (usually used as cDNA) and proteins (direct shotgun proteomic sequencing) (Hanson
145 and Madsen, 2015; Padilla *et al.*, 2016) have been applied and could demonstrate a link between
146 observed methane disappearance and the presence of '*M. oxyfera*'. Furthermore, other features of
147 '*M. oxyfera*' physiology could be used to detect its activity. '*M. oxyfera*' was shown to be an
148 autotroph and assimilated CO_2 instead of methane actively into specific lipids and total biomass
149 (Rasigraf *et al.*, 2014). So far, most stable isotope methods used labeled methane for the
150 detection of active methanotrophs in field samples (Dumont and Murrell, 2005). A modified

151 method using ^{13}C-labeled CO_2 could potentially aid in the detection of labeled '*M. oxyfera*'
152 DNA or RNA in environmental samples. A further method for environmental detection is based
153 on ^{13}C fractionation of the environmental methane pool. It has been shown previously that
154 methanotrophic bacteria fractionate methane leading to an enrichment of heavier methane in the
155 remaining pool (Feisthauer *et al.*, 2011; Rasigraf *et al.*, 2012). Depending on the source of
156 methanogenesis and the presence of methanotrophic bacteria a specific signature could be
157 determined and linked to the extent of methanotrophy. So far, this tool of '*M. oxyfera*' activity
158 detection has not been applied.

159

160 After nearly a decade, the archaeal partner detected in the initial co-culture of Raghoebarsing *et*
161 *al.* (2006) was described in detail (Haroon *et al.*, 2013). In an enrichment fed with nitrate,
162 methane and ammonium, a stable co-culture of anammox bacteria and above mentioned archaea
163 was established. The archaeal counterpart was identified as the main organism responsible for
164 coupling nitrate reduction to methane oxidation and was named '*Candidatus* Methanoperedens
165 nitroreducens' (Haroon *et al.*, 2013). The successful enrichment of '*M. nitroreducens*' in this co-
166 culture was due to the differential use of nitrate by '*M. nitroreducens*' vs. nitrite by '*M. oxyfera'*.
167 Co-enrichment of both organisms in the culture described by Raghoebarsing *et al*. in 2006 was
168 most likely due to feeding of both nitrate and nitrite. The differentiating effect of the used
169 electron acceptor on enrichment of N-AOM archaea versus bacteria has been observed
170 previously (Hu *et al.*, 2011). Detailed metagenomic analyses revealed that the genome of '*M.*
171 *nitroreducens*' encoded pathways involved in the utilization of nitrate as electron acceptor (e.g.
172 by the nitrate reductase subunit NarG) as well as reverse methanogenesis, with methyl-CoM
173 reductase (McrA) as the key enzyme. Based on the available genomic data, CO_2 fixation in '*M.*

174 *nitroreducens*' may proceed via the acetyl-CoA pathway possibly leading to very depleted ^{13}C
175 biomarkers. Carbon isotope measurements in archaeal lipids from the original culture described
176 by Raghoebarsing *et al.* (2006) indeed revealed strong depletion compared to methane,
177 indicating methane as carbon source for biomass. The possibility to use '*M. nitroreducens*' lipids
178 for its environmental detection has so far not been explored. '*M. nitroreducens*' forms a new
179 cluster within the ANME 16S rRNA gene phylogeny and was classified as the ANME-2d clade
180 (Figure 2A). Few aspects about the physiology of '*M. nitroreducens*' are known, and its
181 environmental detection has been limited to molecular methods based on 16S rRNA and *mcrA*
182 genes (Ding *et al.*, 2015). The overview of environmental distribution based on those biomarkers
183 is summarized in Table 1. The table shows that *Methanoperedens*-like archaea have been found
184 in a variety of environments including mostly freshwater and some marine habitats. Based on
185 16S rRNA gene classification, the ANME-2d clade is referred to as GOM Arc I in the Silva 16S
186 rRNA gene database (Figure 2A, Quast *et al.*, 2013), as the first sequences were found in
187 environmental samples from the Gulf of Mexico (GOM). To date the GOM Arc I/ANME-2d
188 group consists of 96 high quality sequences, which split into three defined clusters A, B and C
189 (Figure 2B). The 16S rRNA sequences of the two known genomes from enrichment cultures
190 (Haroon *et al.*, 2013; Arshad *et al.*, 2015) cluster into group A, which is the largest and most
191 uniform group. With few exceptions of sequences found in marine and brackish environments,
192 this group consists of sequences detected in freshwater environments such as aquifers, lakes and
193 rivers (Li *et al.*, 2012; Flynn *et al.*, 2013). Group B and C have no cultured representatives so far
194 and consist exclusively of environmental sequences. The sequences of group B and C have been
195 found in extreme environments such as marine and terrestrial mud volcanoes, marine sediment
196 and hydrothermal vents (Inagaki *et al.*, 2006; Pachiadaki *et al.*, 2011; Yang *et al.*, 2012).

197

198 **Metabolic cooperation and competition of N-AOM microorganisms**

199 Physiological studies showed that nitrite was the main product of nitrate reduction by N-AOM
200 archaea (Haroon *et al.*, 2013; Zhu, 2014). In high concentrations, nitrite becomes toxic and must
201 be removed. The N-AOM archaea encode a membrane-bound nitrite reductase which could
202 convert some of the nitrite into ammonia (Arshad *et al.*, 2015). The presence of nitrate, nitrite
203 and ammonium creates a basis for metabolic co-operation with nitrite and ammonium
204 scavenging organisms (Figure 4). The first described co-culture of '*M. nitroreducens*' contained
205 anaerobic ammonium oxidizing (anammox) bacteria which use nitrite for respiration (Haroon *et*
206 *al.*, 2013). The original N-AOM culture described in 2006 also contained archaea closely related
207 to '*M. nitroreducens*'. The 16S rRNA gene sequence of the 2006 enrichment and the
208 '*Methanoperedens*' sp. BLZ1 are 99.2 % identical and cannot be resolved from each other in the
209 phylogenetic tree in Figure 2B. They were enriched together with '*M. oxyfera*' bacteria, the latter
210 being known by now to use nitrite as electron acceptor (Raghoebarsing *et al.*, 2006). Thus,
211 anammox and '*M. oxyfera*'-like bacteria are most likely common metabolic partners of N-AOM
212 archaea as both methane and ammonium derived from anaerobic food chains are often present at
213 oxic/anoxic interfaces. The co-occurrence of anammox and '*M. oxyfera*'-like bacteria with '*M.*
214 *nitroreducens*' would lead to a competition of the two for available nitrite. Previous studies have
215 shown that anammox bacteria can be co-enriched and form a stable co-culture with '*M. oxyfera*'
216 in a bioreactor system upon gradual increase of ammonium concentrations in the influent
217 medium (Luesken *et al.*, 2011b; Ding *et al.*, 2014; Zhu *et al.*, 2011). In contrast, Hu *et al.* (2015)
218 found that anammox bacteria successfully outcompeted '*M. oxyfera*' in bioreactor systems fed
219 with ammonium and methane and amended either with nitrate or nitrite. Environmental

220 molecular studies have shown that both co-occur in anoxic environments (Wang *et al.*, 2012;
221 Shen *et al.*, 2014c; Shen *et al.*, 2015). It is likely that different substrate affinities of different '*M.*
222 *oxyfera*' and anammox species/strains would determine the success of competition as well as
223 tolerance to harmful nitrogen oxide species (e.g. NO and NH_2OH). In natural settings, the
224 interactions will become more complex due to the activity of nitrifying bacteria and archaea.
225 Ammonium concentrations shape the community composition of nitrifying organisms with
226 ammonia oxidizing bacteria (AOB) typically dominating at higher concentrations and archaea
227 (AOA) mostly occurring at lower concentrations (Yan *et al.*, 2012). Moreover, higher
228 concentrations of nitrite would lead to the presence of nitrite oxidizing bacteria (NOB), with
229 *Nitrobacter*/*Nitrococcus* dominating at higher and *Nitrospira*/*Nitrospina* at lower nitrite
230 concentrations (Nowka *et al.*, 2015). Recently, a complete nitrification process has been
231 described in a *Nitrospira*-like organism (comammox), which seems to predominate at very low
232 substrate concentrations and thus become competitive with the "classical" two stage process
233 (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The comammox process produces nitrate and
234 bypasses the release of nitrite and could directly provide substrate to '*M. nitroreducens*'. This
235 scenario seems likely for oligotrophic environments with overall low concentration of
236 nitrogenous compounds and high methane (e.g. drinking water wells, Gülay *et al.*, 2016; Palomo
237 *et al.*, 2016; Pinto *et al.*, 2016). Thus, the co-occurrence of anammox and comammox bacteria
238 with N-AOM organisms might be a common scenario. The presence of other electron donors in
239 the environment (e.g. organic carbon, reduced iron and sulfur species) would potentially
240 intensify the competition for nitrate (and nitrite) in the form of denitrification and dissimilatory
241 nitrate/nitrite reduction to ammonium. Thus, various primary and secondary factors can
242 determine the outcome of each particular competition.

243

244 **Biochemistry and metabolism of N-AOM microorganisms**

245 Methane is quite inert due to the absence of functional groups and breaking the first C-H bond
246 poses an energetic barrier of ΔH_{298} = 439 kJ/mol (Blanksby and Ellison, 2003). Therefore,
247 oxidation of methane requires it to be activated first. Until now there are only two biological
248 processes known to activate methane, incorporation of oxygen by methane monooxygenases
249 utilized by aerobic methanotrophic bacteria and formation of methyl-CoM employing methyl-
250 CoM reductase in a reverse manner utilized by anaerobic methanotrophic archaea (Figure 5).

251

252 '*M. oxyfera*' is so far unique in its ability to couple anaerobic methane oxidation to nitrite
253 reduction. The biochemistry and general metabolism of '*M. oxyfera*' is not yet well explored.
254 The current metabolic model of nitrite-dependent methane conversion is therefore largely
255 inferred from the genome and based on homology. As there are no organisms sharing this
256 metabolism, a global comparative analysis is not available. Most of the metabolic modules,
257 however, are shared with canonical methanotrophs and denitrifiers, which allowed a metabolic
258 prediction for '*M. oxyfera*' (Ettwig *et al.*, 2010). Although '*M. oxyfera*' was cultivated under
259 strictly anaerobic conditions and displayed severe oxygen intolerance (Luesken *et al.*, 2012), its
260 genome encodes the complete aerobic methane oxidation pathway and is postulated to employ an
261 intra-aerobic pathway for the degradation of methane. Candidate enzymes for oxygen generation
262 are two nitric oxide reductase-like proteins that were hypothesized to disproportionate two
263 molecules of NO into N_2 and O_2 (Ettwig *et al.*, 2012). This dismutation reaction is highly
264 exergonic ($\Delta G^{0'}$ = -173 kJ/mol O_2) but due to complex bond reorganizations is expected to
265 present the rate limiting step in '*M. oxyfera*'s' energy metabolism. Activation of methane by

266 either NO or N_2O directly is thermodynamically feasible, but incompatible with the measured
267 substrate stoichiometries (Ettwig *et al.*, 2010; Reimann *et al.*, 2015). As in most aerobic
268 methanotrophs '*M. oxyfera*' employs a particulate methane monooxygenase for the activation of
269 methane into methanol (Figure 5). Methane has a high octanol-water partition coefficient and
270 accumulates in the hydrophobic membrane core in a ~12:1 ratio (Hansch *et al.*, 1995), making it
271 available in high effective concentrations to the particulate methane monooxygenase (pMMO).
272 Amino acid sequence comparison of the PmoA, PmoB and PmoC subunits from '*M. oxyfera*' to
273 canonical pMMOs suggested a similar overall architecture and conserved function for this
274 enzyme. Alternative reaction mechanisms involving NO, N_2O or NO_2 in methane activation are
275 difficult to justify in this context (Ettwig *et al.*, 2010; Reimann *et al.*, 2015).
276
277 '*M. oxyfera*' has three PQQ-dependent methanol dehydrogenases (MDH) at its disposal for the
278 conversion of methanol to formaldehyde. One gene cluster encodes for a calcium-dependent
279 MDH, which harbors all accessory genes, next to the canonical alpha (MxaF) and beta (MxaI)
280 subunits. The two additional MDHs belong to the recently described class of lanthanide-
281 dependent XoxF MDHs (Keltjens *et al.*, 2014). The XoxF methanol dehydrogenase from the
282 methanotroph *Methylacidiphilum fumariolicum* SolV was isolated as a homodimer and shown to
283 incorporate the rare earth element cerium believed to confer superior catalytic activity (Pol *et al.*,
284 2014). Purification of the dominant MDH from '*M. oxyfera*' resolved a unique combination of
285 the XoxF1 large subunit and the MxaI small subunit forming a heterodimeric complex ($\alpha_2\beta_2$)
286 (Wu *et al.*, 2015). It remains to be shown whether a rare earth element is indeed bound in the
287 enzyme. Although PQQ-biosynthesis genes were mostly absent in the '*M. oxyfera*' genome (Wu
288 *et al.*, 2011) spectroscopy on the purified MDH clearly confirmed the presence of the PQQ

289 cofactor (Wu *et al.*, 2015). It thus appears that formation of the holoprotein requires PQQ
290 acquisition from the environment as has been previously observed for glucose dehydrogenase in
291 PQQ-deficient enteric bacteria (Hommes *et al.*, 1991). Dependence on other microorganisms for
292 the production of this crucial cofactor could possibly explain why '*M. oxyfera*' has thus far not
293 been obtained as a pure culture. Formaldehyde is further oxidized to formate via two possible
294 pathways, a highly expressed methanopterin (H_4MPT) route likely used for energy conservation
295 and a lowly expressed folate (H_4F) route, where folate or methanopterin function as C1 carriers
296 for biosynthetic purposes (Reimann *et al.*, 2015). Three enzymes are available to '*M. oxyfera*' for
297 the oxidation of formate to CO_2, a highly expressed formyl-MFR dehydrogenase and two minor
298 expressed $NAD(P)^+$-dependent formate dehydrogenases (FDH) in which the extended N-
299 terminal parts show homology with bacterial complex I subunits NuoG for both FdhA subunits
300 and NuoE for the FdhB2 subunit. These alternative FDHs might provide extra reducing
301 equivalents in the form of NADH (Reimann *et al.*, 2015).
302
303 Although two nitrate reductases, NarGHI and NapAB, are present in the genome of '*M. oxyfera*'
304 low transcription and translation levels suggest that neither of the two systems is highly active.
305 Nitrite reduction to NO is catalyzed by cytochrome cd_1-type nitrite reductase (NirS), the only
306 nitrite reductase present in the genome. Produced NO is dismutated into N_2 and O_2 by two
307 putative NO dismutases (NOD). The genome does not code for a recognizable N_2O reductase
308 and N_2O was only measured in trace amounts in methane-driven nitrite reduction experiments of
309 '*M. oxyfera*' enrichments. The proposed NODs are homologous to the quinol-dependent NO
310 reductases, but display amino acid alterations in the catalytic site, the quinol-binding site and the
311 proposed proton channel . These changes hamper electron and proton entry into the active site

312 and could facilitate the disproportionation of NO to N_2 and O_2 rather than its reduction to N_2O
313 (Ettwig *et al.*, 2012). In addition to these two putative NO dismutases three nitric oxide
314 reductases (NOR) are encoded in the '*M. oxyfera*' genome, one canonical qNOR, one gNOR and
315 one sNOR (Hemp and Gennis, 2008). The product of these enzymes, N_2O, was only detected in
316 trace amounts under standard conditions. It therefore remains an open question what the
317 redundancy in NO reductases offers to '*M. oxyfera*'. NORs might be present to quickly respond
318 to external nitrosative stress and to ensure that concentrations of the metabolic intermediate NO
319 are kept below toxic levels. The NORs may also play a role in oxygen respiration. Since only
320 three of the four O_2 molecules produced from NO disproportionation are consumed during
321 methane activation the remaining O_2 molecule might be reduced to water, a side reactivity that
322 has been demonstrated for both c- and qNORs, with rates that could match the overall metabolic
323 rates of methane conversion (Reimann *et al.*, 2015).
324

325 The only known microorganisms capable of oxidizing methane with nitrate as electron acceptor
326 are '*M. nitroreducens*' and *Methanoperedens*-like archaea (Raghoebarsing *et al.*, 2006; Haroon
327 *et al.*, 2013). In contrast to '*M. oxyfera*' they do not use oxygen for methane activation but
328 instead utilizes the reverse reaction of methyl-CoM reductase (Figure 5, Krüger *et al.*, 2003;
329 Hallam *et al.*, 2004; Scheller *et al.*, 2010; Haroon *et al.*, 2013). Metabolic reconstructions from
330 environmental genomes (Haroon *et al.*, 2013; Arshad *et al.*, 2015) suggested that '*M.*
331 *nitroreducens*' oxidizes methane via reverse methanogenesis to CO_2. One of the key questions is
332 how electrons from methane oxidation are transferred to the final electron acceptor nitrate.
333 During reverse methanogenesis, electrons are transferred to yield cofactor $F_{420}H_2$, reduced
334 ferredoxin and the thiol cofactors coenzyme M (CoM-SH) and coenzyme B (CoB-SH). $F_{420}H_2$

335 and reduced ferredoxin can be re-oxidized by a canonical $F_{420}H_2$ dehydrogenase (Fqo) and an
336 Ech hydrogenase, respectively (Welte and Deppenmeier, 2014). CoM-SH and CoB-SH are either
337 oxidized via the reverse reaction of the membrane-bound heterodisulfide reductase (HdrDE) or
338 via the cytoplasmic heterodisulfide reductase (HdrABC). The latter enzyme has been
339 exemplified to perform electron bifurcation in methanogens (Costa *et al.*, 2010; Kaster *et al.*,
340 2011) and due to thermodynamic limitations provided by the reversal of methanogenesis in '*M.*
341 *nitroreducens*' would have to act as an electron confurcation enzyme here (Arshad *et al.*, 2015).
342 In methanogens, both HdrDE and Fqo interact with methanophenazine, a membrane-integral
343 electron carrier; in '*M. nitroreducens*', however, methanophenzine could not be detected but
344 instead a menaquinone biosynthesis pathway was encoded and expressed (Arshad *et al.*, 2015). It
345 is therefore likely that HdrDE and Fqo interact with menaquinones in this organism. The genome
346 also encodes a nitrate reductase subunit harboring the active site (NarG) for nitrate reduction to
347 nitrite. Electron transport to nitrate reductase seems to happen via a Rieske-cytochrome *b*
348 complex. The gene cluster encoding the Rieske-cytochrome *b* complex contains additional genes
349 for cytochrome *c* proteins whose function is unclear but may be connected to the electron
350 transport to nitrate reductase. The nitrate reductase gene cluster shows a highly unusual
351 composition that to our knowledge has not been observed in other prokaryotes (Arshad *et al.*,
352 2015). As in other archaea, NarG contains a signal peptide for the translocation of NarGH into
353 the pseudoperiplasm and nitrate may therefore be reduced non-cytoplasmically (Yoshimatsu *et*
354 *al.*, 2000; Martinez-Espinosa *et al.*, 2007; de Vries *et al.*, 2010). All other nitrate-reducing
355 archaea studied to date harbor NarM as a membrane anchor for the soluble NarGH complex (de
356 Vries *et al.*, 2010) that is absent in '*M. nitroreducens*'. Instead, a NapH like membrane anchor
357 together with membrane-integral heme-copper oxidase subunits was encoded in the same gene

358 cluster, along with a cytochrome *b* protein that is also found in the nitrate reductase complex of
359 halophilic archaea (Martinez-Espinosa *et al.*, 2007). A small part of the formed nitrite can be
360 further reduced to ammonium by a NrfAH type cytochrome *c* nitrite reductase, possibly to
361 prevent toxic accumulation of nitrite. The '*M. nitroreducens*' genomes do not encode other
362 denitrification enzymes illustrating that neither NO, N_2O nor N_2 are final products; instead,
363 nitrite is the main product of nitrate reduction with about 10 % of the nitrite reduced to
364 ammonium (Haroon *et al.*, 2013; Ettwig *et al.*, 2016). Another unusual feature of the ANME-2d
365 genomes are that they encode for a high number of cytochrome *c* proteins (Haroon *et al.*, 2013;
366 Arshad *et al.*, 2015; Kletzin *et al.*, 2015). The role of cytochrome *c* proteins in '*M.*
367 *nitroreducens*' remains enigmatic, as the metabolism of nitrate-dependent AOM does not require
368 electron transfer to a syntrophic partner microorganism as found for ANME-2a (McGlynn *et al.*,
369 2015; Wegener *et al.*, 2015). A recent publication detected ANME-2d archaea in a culture that
370 coupled Cr(VI) reduction to anaerobic methane oxidation (Lu *et al.*, 2016) which may require *c*-
371 type cytochromes. As many other microorganisms manage nitrate reduction without the
372 excessive use of cytochrome *c*, and furthermore closely related *Methanosarcina* strains harbor
373 only a few – if any – *c*-type cytochromes, the role of these proteins in N-AOM has to be further
374 investigated.

375

376 **Concluding remarks**

377 Methane oxidizing microorganisms play an essential role in counteracting biological methane
378 production and its release to the atmosphere. The widespread occurrence and substantial size of
379 potential habitats suggests an important role for nitrate- and nitrite-dependent methane oxidizers
380 that link the biogeochemical carbon and nitrogen cycles (Figure 5). Application of more specific

detection methods are needed and will hopefully broaden our insight into the environmental significance of N-AOM microorganisms. Physiological experiments with co-cultures of various nitrogen cycle organisms need to be further explored. Competition for nitrate and nitrite as well as composition of microbial communities in natural habitats is likely determined by the availability and relative concentrations of electron donors and acceptors. Further laboratory studies and environmental data sets are needed to understand substrate fluxes and microbial community development in relevant ecosystems to ultimately understand and possibly predict the fate of involved substrates.

Models describing the metabolic pathways for methane and nitrate/nitrite conversion and involved enzyme systems in *Methanoperedens* and *Methylomirabilis*-like microorganisms have been proposed (Figure 5). It is interesting to note that the degree of genetic innovation required to catalyze the two processes appears to be limited. Nitrite-dependent methane oxidation by '*M. oxyfera*' mostly employs enzymatic modules commonly found in denitrifiers and aerobic methanotrophs. The key novelty that seems to enable these organisms to respire methane with nitrite is the alteration of a canonical nitric oxide reductase into a nitric oxide disproportionating enzyme. Nitrate-dependent methane oxidation by '*M. nitroreducens*' is based on the reversal of the methyl-CoM reductase reaction and subsequent steps from the Wood-Ljungdahl/methanogenesis pathway. The key innovation is the acquisition of a nitrate reductase and accessory proteins. The exceptionally large number of Cyt*c* present in ANME organisms suggests the additional need to rewire the electron transfer routes to accommodate this metabolism or additional metabolic capacities that have not yet been discovered. Furthermore,

403 physiological and detailed biochemical studies are needed to test the current models for these

404 fascinating processes.

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734 **Table legends**

735 **Table 1:** Overview of the environmental distribution and relevant detection methods for N-AOM

736 microorganisms.

737 **Figure legends**

738 **Figure 1:** Microscopic image of a co-culture catalysing nitrite- and nitrate-dependent anaerobic
739 oxidation of methane that was subjected to fluorescence *in situ* hybridization. The
740 epifluorescence micrograph was obtained after hybridization with the ARCH-641 probe targeting
741 *Methanoperedens*-like archaea (green) and the *M. oxyfera* specific DBACT-193 probe (red). The
742 scale bar represents 20 µm.

743

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

753

754 **Figure 3:** Phylogenetic overview of NC10 bacteria based on 16S rRNA gene sequences.
755 Depicted is the clustering of the NC10 clade into groups A-D. *Candidatus* 'Methylomirabilis
756 oxyfera' of the group A is marked in bold. The calculation of the tree was carried out by
757 Neighbour-joining algorithm using the Jukes Cantor correction and filter over 1158bp and
758 Acidobacteria as an outgroup.

759

760 **Figure 4:** Simplified overview of how different bacterial and archaeal physiological groups
761 depend on or compete with each other including the anaerobic methanotrophs described in this
762 article. The nitrate-dependent methanotrophs ANME-2d compete with 'M. oxyfera' for methane
763 yet 'M. oxyfera' requires the provisioning of nitrite which is the final product of nitrate reduction
764 by ANME-2d. A key competitor for 'M. oxyfera' seems to be anammox bacteria that take up
765 nitrite very efficiently. Dotted arrow, diffusion. Solid line, metabolic conversion. For the
766 description of the individual groups, please see main text.
767
768 **Figure 5:** Schematic overview of central metabolism of the archaeon '*Methanoperedens*' (A)
769 and the bacterium '*Methylomirabilis oxyfera*' (B). Key enzymes in methane activation and
770 nitrogen conversion reactions are indicated with their encoding genes. Biochemical (solid
771 arrows) and electron transfer reactions (dashed arrows) are depicted schematically and do not
772 indicate stoichiometries. fdh, formate dehydrogenase; fmd, formylmethanofuran dehydrogenase;
773 ftr, formyl transferase; mch, methenyltetrahydromethanopterin cyclohydrolase; mcr, methyl-
774 CoM reductase; mdh, methanol dehydrogenase (XoxF and MxaFI type); mer, methylene
775 tetrahydromethanopterin reductase; mtd, methylene tetrahydromethanopterin dehydrogenase;
776 mtr, Na^+ translocating methyl transferase; nar, nitrate reductase; nir, cd_1 nitrite reductase; nod,
777 NO dismutase; nrf, ammonium-producing nitrite reductase; pmo, particulate methane
778 monooxygenase.

Table 1:

N-AOM organism (Environment type)	Environment	Location	Nutrient conditions	Detection method	Reference
Methylomirabilis* / *Methanoperedens					
Freshwater ditch	Freshwater ditch	Ooijpolder, Netherlands Twentekanaal, Netherlands	Eutrophic, high nitrite, high methane	Enrichment culture, 16S rRNA and *pmoA* gene clone libraries, FISH	Raghoebarsing *et al.*, 2006 Ettwig *et al.*, 2009
Methylomirabilis					
Wetlands	Various wetlands	China	Oligotrophic-eutrophic, temperatures -25°-80°, pH 5-9,	*pmoA* and 16S rRNA gene clone libraries, qPCR	Zhu *et al.*, 2015
	Minerotrophic peatland	Brunsummer-heide, Netherlands	Oligotrophic, influence by agricultural groundwater	*pmoA* and 16S rRNA gene clone libraries, qPCR, lipids, FISH	Kool *et al.*, 2012 Zhu *et al.*, 2012
Freshwater environments	Freshwater lakes	Yunnan Plateau, China	Various levels of reactive N and C	*pmoA* gene clone libraries, qPCR	Liu *et al.*, 2015
	Lake sediment	Lake Biwa, Japan	Mesotrophic	16S rRNA and *pmoA* gene clone libraries, DGGE, qPCR, CARD-FISH	Kojima *et al.*, 2012
	Lake sediment	Lake Constance, Germany	Oligotrophic	qPCR of 16S rRNA and *pmoA* genes, FISH	Deutzmann and Schink, 2011 Deutzmann *et al.*, 2014
	River sediment	Qiantang River, China	Eutrophic, polluted by domestic and industrial effluents, high reactive N and P contents	16S rRNA and *pmoA* gene clone libraries	Shen *et al.*, 2014b
	Agricultural soils	Nanjing City,	Slightly acidic pH,	16S rRNA gene amplicon	Shen *et al.*, 2016

		China	moderate N loading	sequencing, qPCR	
	Paddy field soils	Japan	Fertilized soils	enrichment cultures, *pmoA* gene clone library, FISH	Hatamoto *et al.*, 2014
	Paddy field soils	China	Fertilized soils, eutrophic, high N and C load	*pmoA* and 16S rRNA gene clone libraries, qPCR	Wang *et al.*, 2012 Shen *et al.*, 2014c
Wastewater & contaminated sites	Waste water treatment plants	Netherlands China	Long sludge retention times, low biological oxygen demand/N rations	Enrichment culture, *pmoA* and 16S rRNA gene clone libraries, FISH	Luesken *et al.*, 2011a Luesken *et al.*, 2011b Ding *et al.*, 2014 Kampman *et al.*, 2014
	Coal tar contaminated aquifer	South Glens Falls, USA	High CH_4 content, moderate reactive N load, contamination with naphthalene	Metaproteomic libraries	Hanson and Madsen, 2015
	Contaminated aquifers	Cape Cod, USA Banisveld, Netherlands	Elevated N load, contaminated with leachate and waste water	*pmoA* gene clone library	Luesken *et al.*, 2011b
Brackish environments	Coastal wetland	Mai Po, China	Brackish, eutrophic	16S rRNA and *pmoA* gene clone libraries	Chen *et al.*, 2014b
	Coastal sediments	Xiaogan Island, China	Marine	Enrichment culture, *pmoA* and 16S rRNA gene clone libraries, qPCR, FISH	He *et al.*, 2015
	Estuary	Jiaojiang, East Sea, China	Eutrophic, moderate salinity, high pollution with polycyclic aromatic hydrocarbons	*pmoA* and 16S rRNA gene clone libraries, qPCR	Shen *et al.*, 2014a
Marine environments	Sea sediments	South China Sea, China	n.a.	*pmoA* and 16S rRNA gene clone libraries	Chen *et al.*, 2014a
	Oceanic oxygen minimum zone	Mexico and Costa Rica	Anoxic core zone with detectable CH_4 and NO_2^-	*pmoA* gene clone library, 16S rRNA gene qPCR , metatranscriptome	Padilla *et al.*, 2016

Methanoperedens					
Peatlands	Peatland	Michigan Hollow, USA	Minerotrophic fen	16S rRNA gene clone library	Cadillo-Quiroz *et al.*, 2008
	Peatland	Ithaca, USA	Acidic ombrotrophic bog	16S rRNA gene clone library	Cadillo-Quiroz *et al.*, 2006
	Peatland	Zoige, Tibet	Neutral peatland	16S rRNA and *mcrA* gene clone libraries	Zhang *et al.,* 2008
Freshwater environments	Aquifer	Illinois, USA	High CH_4, low SO_4^{2-}	16S rRNA gene clone library	Flynn *et al.*, 2013
	Aquifer	Tokyo, Japan	NO_3^- ~7 µM	16S rRNA gene clone library	Kato *et al.*, 2013
	Subglacial sediment	Alberta, Canada	High C and N	16S rRNA and *mcrA* gene clone libraries	Boyd *et al.*, 2010
	Freshwater sediment	Green Bay, USA	Mn-/Fe-rich	16S rRNA gene clone library	Stein *et al.*, 2001
	River sediment from mining district	Idaho, USA	Heavy metal contaminated	16S rRNA gene clone library	Rastogi *et al.*, 2009
	Lake sediment	Lago di Cadagno, Switzerland	High SO_4^{2-} and S^{2-}, no NO_3^-	16S rRNA gene clone library	Schubert *et al.*, 2011
	Paddy field	Vercelli, Italy	Nitrogen loaded	*mcrA* gene clone libraries	Conrad *et al.,* 2008 Chin *et al.,* 2004 Xu *et al.*, 2012
Wastewater & contaminated sites	Lake sediment of oil seep	Baikal lake, Russia	Hydrocarbon rich, low NO_3^- and SO_4^{2-}	16S rRNA gene clone library	Lomakina *et al.*, 2014
	Contaminated soil	Shizuoka, Japan	Petroleum contaminated, Mn- and Fe-rich	16S rRNA gene clone library	Kasai *et al.*, 2005
	Freshwater lake sediment,	Australia	n.a.	Enrichment, metagenome, FISH	Haroon *et al.*, 2013

	digester sludge and activated sludge				
Mud volcanoes	Mud volcano	Usu City of Xinjiang, China	Alkaline, brackish	16S rRNA gene clone library	Yang *et al.*, 2012
	Mud volcano	Salse di Nirano, Italy	Hydrocarbon rich, brackish	16S rRNA gene clone library	Wrede *et al.*, 2012
	Mud volcano	Lei-Gong-Huo, Taiwan	Hydrocarbon rich, Mn- and Fe-rich, moderately saline	16S rRNA gene clone library	Chang *et al.*, 2012
Brackish environments	Estuary	Fujian, China	High CH_4 and SO_4^{2-}	16S rRNA and *mcrA* gene clone library	Li *et al.*, 2012
Marine environments	Seafloor sediment	Juan de Fuca Ridge, USA	High organic carbon	*mcrA* gene clone library	Lever *et al.,* 2013

Figure 1

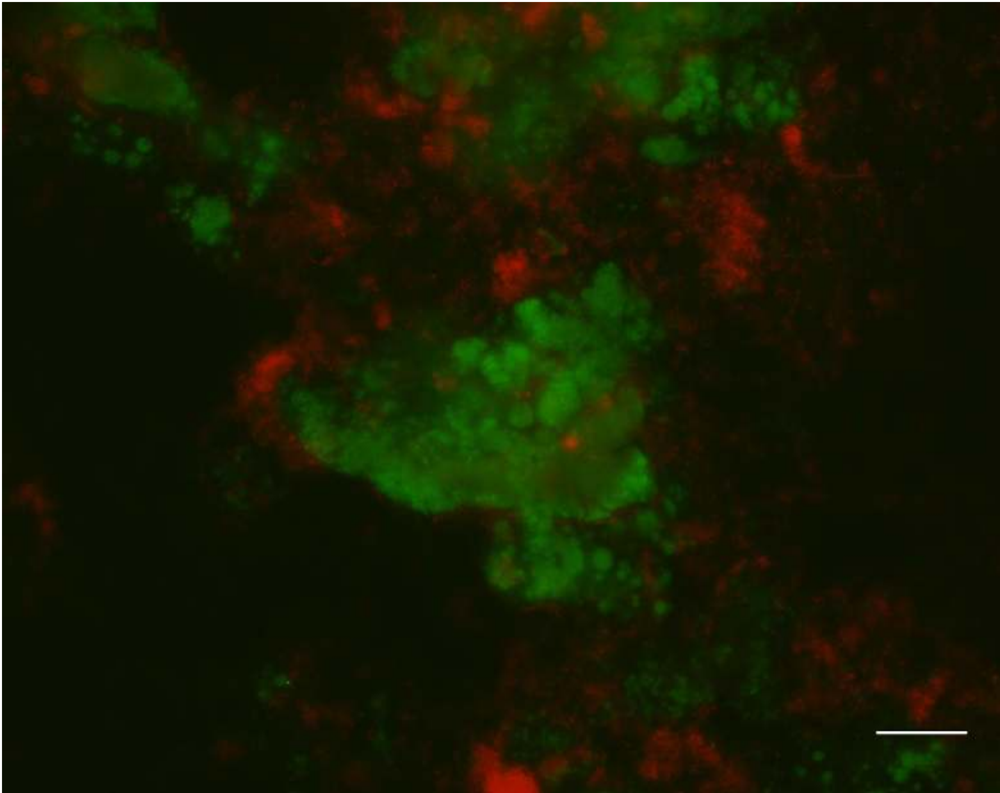

Figure 2

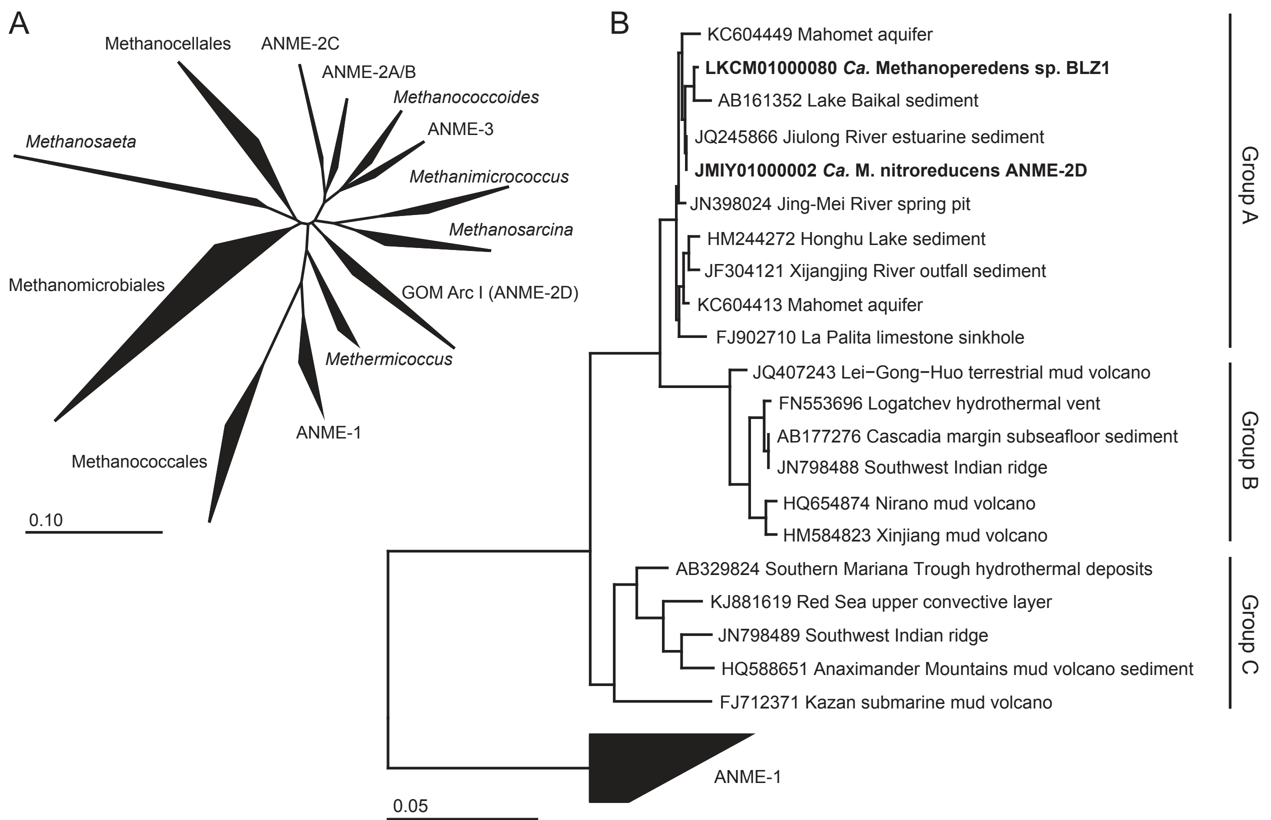

Figure 3

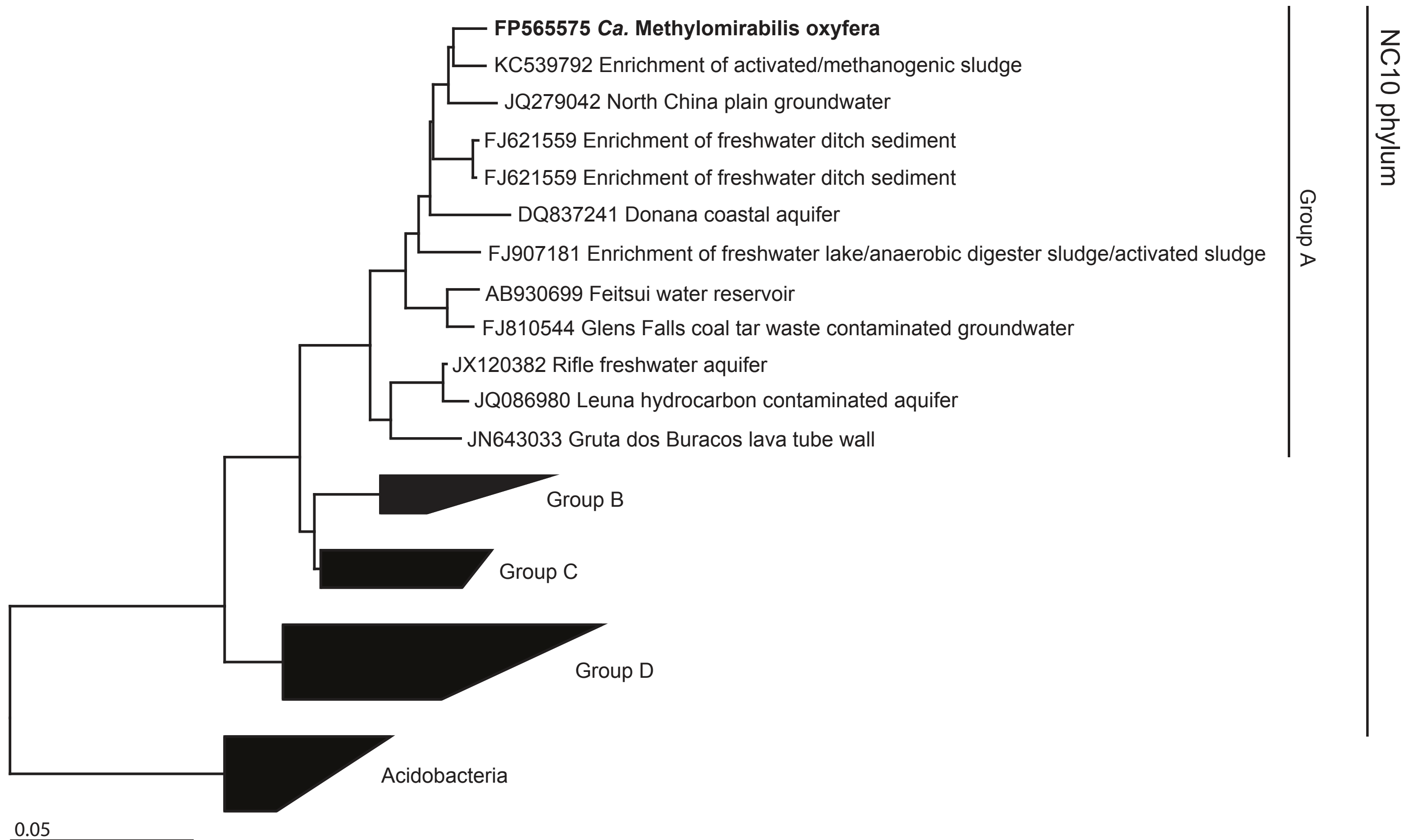

Figure 4

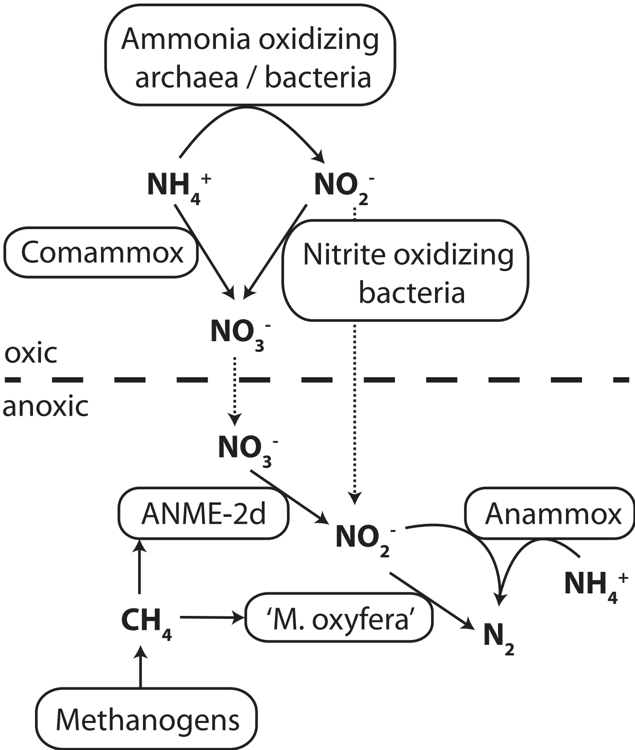

Figure 4