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1 **Current Advances in Molecular Methods for Detection of**
2 **Nitrite-dependent Anaerobic Methane Oxidizing Bacteria in Natural**
3 **Environments**

4
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Abstract

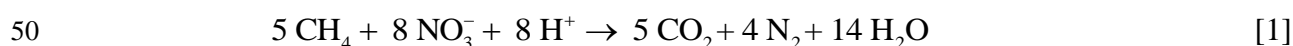
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Nitrite-dependent anaerobic methane oxidation (n-damo) process uniquely links microbial nitrogen and carbon cycles. Research on n-damo bacteria progresses quickly with experimental evidences through enrichment cultures. Polymerase chain reaction (PCR)-based methods for detecting them in various natural ecosystems and engineered systems play a very important role in the discovery of their distribution, abundance and biodiversity in the ecosystems. Important characteristics of n-damo enrichments were obtained and their key significance in microbial nitrogen and carbon cycles was investigated. The molecular methods currently used in detecting n-damo bacteria were comprehensively reviewed and discussed for their strengths and limitations in applications with a wide range of samples. The *pmoA* gene-based PCR primers for n-damo bacterial detection were evaluated and, in particular, several incorrectly stated PCR primer nucleotide sequences in the published papers were also pointed out to allow correct applications of the PCR primers in current and future investigations. Furthermore, this review also offers the future perspectives of n-damo bacteria based on current information and methods available for a better acquisition of new knowledge about this group of bacteria.

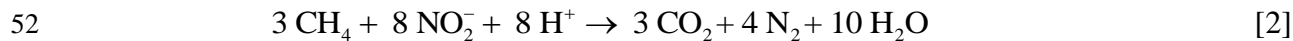
Keywords: n-damo; anaerobic methane oxidation; denitrification; *Methylomirabilis oxyfera*-like bacteria; molecular detection; PCR primer; *pmoA* gene

41 **Introduction**

42 Biological denitrification process has been investigated for more than half a century (Hill
43 1979; Keeney et al. 1971; McGarity 1961). However, the anaerobic methane oxidation
44 coupled to denitrification was once considered only thermodynamically feasible before the
45 experimental evidences obtained in 2006 (Raghoebarsing et al. 2006). There was no direct
46 evidence of any microorganisms capable of coupling methane oxidation and denitrification
47 under anoxic conditions (Knowles 2005; Mason 1977; Strous and Jetten 2004), although the
48 process can provide enough energy as shown in the following equations (Raghoebarsing et al.
49 2006).



$$51 \quad (\Delta G^{\circ'} = -765 \text{ kJ mol}^{-1} \text{ CH}_4)$$



$$53 \quad (\Delta G^{\circ'} = -928 \text{ kJ mol}^{-1} \text{ CH}_4)$$

54 Obtained from the anoxic sediments, a microbial consortium consisting of two
55 microorganisms (a bacterium belonging to NC10 phylum without any cultured species and an
56 archaeon distantly clustering with marine methanotrophic Archaea) showed a denitrification
57 rate of $21.5 \pm 2 \mu\text{mol N}_2 \text{ h}^{-1}$ with the simultaneous conversion of the added methane at a rate
58 of $22.0 \pm 2 \mu\text{mol CH}_4 \text{ h}^{-1}$ (Raghoebarsing et al. 2006). Using the enrichment culture of
59 Raghoebarsing et al. (2006) as inocula, Ettwig et al. (2008) demonstrated that the specific
60 inhibitor, Bromoethane at a concentration of 20 mM, for the key *mcr* gene of methanotrophic
61 and methanogenic archaea showed no effect on the subculture oxidizing methane and
62 reducing nitrite, which was further enhanced with the decline of the archaeal population.

63 Results showed a stoichiometry of 8: 3.5 for NO_2^- : CH_4 after 22 months of enrichment, very
64 close to the above equations (Ettwig et al. 2008).

65 A comparison of the parameters and results of several n-damo enrichments is presented in
66 Table 1. Some important characteristics of n-damo inocula in these studies (Ettwig et al. 2008;
67 Ettwig et al. 2009; Hu et al. 2009; Hu et al. 2011; Luesken et al. 2011a; Luesken et al. 2011b;
68 Zhu et al. 2011) are: first, no pure culture of n-damo bacteria is available and the enrichments
69 so far contained around 30-80% of NC10 phylum bacteria closely related to *M. oxyfera* (Shen
70 et al. 2015b); second, almost all of the n-damo enrichments were successfully established
71 from freshwater habitats as inocula, including wastewater treatment plant (WWTP). One
72 investigation reported that the highest n-damo activity was achieved without NaCl addition
73 into the culture medium in a study on the effect of a range of NaCl concentrations (0-20 g
74 NaCl L⁻¹) (He et al. 2015b). Only very recently, a halophilic denitrifying methanotrophic
75 culture (optimal salinity of 20‰) was obtained after 20 months of enrichment based on the
76 microbial community in the coastal mudflat sediment, of which the active species belonged to
77 NC10 bacteria (He et al. 2015a). Third, n-damo enrichment usually requires a very long
78 culturing and enriching period before the activity can be detected and stable. Ettwig et al.
79 (2009) reported that there was no measurable n-damo activity before 110 days in the
80 enrichment, and then it started to be detectable and increase. The estimated doubling time for
81 n-damo bacteria is one to two weeks under laboratory condition (Ettwig et al. 2008) with a
82 methane conversion rate of 1.7 nmol min⁻¹ mg protein⁻¹ (Ettwig et al. 2009). Finally, n-damo
83 bacteria are often simultaneously co-cultured with anaerobic ammonium oxidizing (anammox)
84 bacteria (Luesken et al. 2011a; Zhu et al. 2011), which also used nitrite as electron acceptor,

85 but utilized ammonium as electron donor instead of methane under anaerobic conditions. We
86 would like to make an updated evaluation of the current PCR primers available for detection
87 of n-damo and in particular point out the error in some of the published PCR primer set,
88 which has been widely used in molecular detection of n-damo. Such awareness is necessary
89 so that the science and new knowledge can be built systematically on sound foundation.

90

91 **Significance of n-damo bacteria in microbial nitrogen and carbon cycles**

92 *Microbial process couples anaerobic methane oxidation to denitrification*

93 Microbes capable of simultaneously oxidizing methane and denitrifying anaerobically had
94 not been found in nature nor isolated in pure culture (Knowles 2005; Strous and Jetten 2004)
95 before the first report of direct evidence of anaerobic methane oxidation with denitrification
96 (Raghoebarsing et al. 2006). Ettwig et al. (2008) further showed that the microbial
97 consortium in the study of Raghoebarsing et al. (2006) could perform the n-damo process
98 without the presence of archaea. The active bacterium was named as *Candidatus*
99 *Methylomirabilis oxyfera* that could reduce nitrite to dinitrogen (N₂) and utilize methane as
100 an electron donor under anaerobic conditions based on genomic analyses and experimental
101 results (Ettwig et al. 2010; Ettwig et al. 2008; Ettwig et al. 2009). This nitrite-driven
102 anaerobic oxidation of methane (AOM) provides a very unique link between the microbial
103 nitrogen and carbon cycles, previously unknown to science. Later on, Haroon et al. (2013)
104 reported a novel archaeal lineage, *Candidatus Methanoperedens nitroreducens*, which can
105 carry out AOM with reduction of nitrate to nitrite and needs the participation of anammox
106 bacteria to complete the denitrification process. Very recently, aerobic methanotroph

107 *Methylomonas denitrificans* sp. nov. strain FJG1T was suggested to couple nitrate reduction
108 to methane oxidation under oxygen limitation, but oxygen was still required because *M.*
109 *denitrificans* FJG1T could not grow under strictly anaerobic condition (Kits et al. 2015).
110 Nevertheless, *Ca. Methylomirabilis oxyfera* is so far the most important and unique
111 microorganism capable of carrying out the n-damo process.

112

113 *Novel denitrification pathway*

114 The significant biochemical pathways of *Methylomirabilis oxyfera* was summarized by
115 Ettwig et al. (2010). *M. oxyfera* encodes, transcribes and expresses the full biochemical
116 pathway for aerobic methane oxidation, which oxidizes methane through methanol,
117 formaldehyde and formate to CO₂ as the end product (Ettwig et al. 2010; Wu et al. 2011). On
118 the other side, the assembly and annotation of the genome indicated that *M. oxyfera* lacks the
119 gene cluster encoding the enzymes for reducing nitrous oxide to dinitrogen gas (N₂) in a
120 conventional denitrification pathway (Ettwig et al. 2010). Isotope and proteomic experiments
121 further suggested the production of N₂ by *M. oxyfera* was directly driven by a novel enzyme,
122 a putative NO dismutase (Ettwig et al. 2010; Wu et al. 2011).

123

124 *An intra-aerobic pathway and oxygen production without photosynthesis*

125 Interestingly, although the addition of oxygen into the enrichment culture (2% or 8%) directly
126 inhibited the methane and nitrite conversion rates by *M. oxyfera* (Luesken et al. 2012), the
127 organism utilizes the classical aerobic methane oxidation pathway in the absence of
128 externally supplied oxygen (Ettwig et al. 2010). The model of the unusual denitrification

129 pathway by *M. oxyfera* indicated electron transport in n-damo process (Simon and Klotz 2013)
130 and the production of oxygen when converting NO to N₂. Isotope experiments showed that
131 the majority of the oxygen produced via this oxygenic denitrification (75%) were used for
132 activation of the particulate methane monooxygenase (pMMO) to convert methane to
133 methanol, while the remaining might be consumed by other terminal oxidases (Ettwig et al.
134 2010). These incredibly integrated biochemical pathways using oxygen as an intermediate
135 were also incorporated into the naming of this n-damo bacterium (methyl (Latin): the methyl
136 group; mirabilis (Latin): astonishing, strange; oxygenium (Latin): oxygen; fera (Latin):
137 carrying, producing) (Ettwig et al. 2010). The production of its own supply of oxygen under
138 anoxic conditions makes *M. oxyfera* performing a peculiar and novel inter-aerobic
139 biochemical pathway in driving methane oxidation when compared with other
140 sulfate-reducing methanotrophs (Wu et al. 2011).

141 On the other hand, *M. oxyfera* is one of the only two known microorganisms so far that could
142 produce oxygen in the darkness (Ettwig et al. 2012a). Only three biochemical pathways are
143 known to produce oxygen before the discovery of n-damo bacteria: photosynthesis, bacterial
144 reduction of chlorates (chlorate-reducing bacteria), and the enzymatic conversion of reactive
145 oxygen species (Mascarelli 2010). Photosynthesis is considered the only biological source for
146 oxygen production and plays a critical role in the initial emission of oxygen, building up in
147 the atmosphere, and recycling of oxygen on Earth (Ettwig et al. 2010; Ettwig et al. 2012b).

148 The production of oxygen via bacterial n-damo process yields new aspect of the potential
149 aerobic biochemical pathways in a methane rich and oxygen limited environment before the
150 great oxidation event in the Archaean Earth (Oremland 2010).

151

152 **Molecular methods for the detection of n-damo bacteria**

153 *PCR approach*

154 Based on the fluorescence in situ hybridization (FISH) probes designed by Raghoebarsing et
155 al. (2006) for measuring the denitrifying AOM microbial consortium, Ettwig et al. (2009)
156 developed a series of 16S rRNA gene-based PCR primers specifically for denitrifying
157 methanotrophic bacteria of the NC10 phylum in the enrichment cultures (Table 2), which
158 were intensively used in the n-damo investigations. The combination of specific PCR primer
159 202F (1043R) and general primer 1492R (8F, 1545R) had been popularly applied for
160 detecting *M. oxyfera*-like bacteria in the early studies of n-damo bacterial enrichments and
161 their diversity in the environments, such as wastewater treatment plants, lake sediments,
162 natural and artificial forests, and paddy soils (Ettwig et al. 2009; Kojima et al. 2012; Luesken
163 et al. 2011b; Meng et al. 2016; Wang et al. 2012; Yang et al. 2012). But later on, a nested
164 PCR approach was developed with PCR primer set 202F-1545R in the first round and
165 qP1F-qP2R in the second round for retrieving n-damo 16S rDNA sequences from the
166 sediments of environmental samples, e.g., the sediments of Jiaojiang Estuary and Qiantang
167 River (Shen et al. 2014b; Shen et al. 2014c). It should be noted that the nucleotide sequence
168 of the PCR primer 8F in a recent review on n-damo research in the natural ecosystems (Shen
169 et al. 2015d) was incorrectly designated and the actual one referred to in the study was primer
170 202F. Primers qP1F/qP1R and qP2F and qP2R were used for qPCR analysis. With no
171 mismatches and 100% PCR efficiencies, it was found that primer pair qP1F and qP1R
172 generated a higher abundance than the gene copies numbers revealed by qP2F and qP2R

173 consistently along the enrichment period, and their deviation eventually reduced when the
174 biomass increased to a certain extent by the end of day 120 (Ettwig et al. 2009).

175 Unfortunately, there is no other specific PCR primer available currently for targeting n-damo
176 bacterial genes except for those based on 16S rRNA and *pmoA* genes although *M. oxyfera* has
177 some unique features, including the putative NO dismutase (Ettwig et al. 2010; Wu et al.
178 2011).

179 Because of the critical mismatches between *M. oxyfera*'s and other methanotrophs' sequences
180 in the gene fragments of the alpha subunit of particulate methane monooxygenase (PmoA),
181 specific PCR primers targeting the *pmoA* gene were designed for revealing n-damo bacteria
182 in several oxygen-limited freshwater environments (Luesken et al. 2011c). Primer pair of
183 A189_b and cmo682 was developed based on A189 and A682 (Holmes et al. 1995) with a
184 second set of primer cmo182 and cmo568 for a nested PCR approach specific for n-damo
185 bacteria (Luesken et al. 2011c). The combination of A189_b and cmo682 resulted in multiple
186 and faint PCR bands of the PCR products (Luesken et al. 2011c) and therefore an extremely
187 low coverage (Luesken et al. 2011b). Meanwhile, the thermal cycling for both PCR reactions
188 of step 1 and 2 was based on the annealing gradient with temperatures of 50-60 °C or
189 53-63 °C for different samples (Luesken et al. 2011c), intending to minimize the effects of
190 random polymerase errors and primer mismatches. The current available *pmoA* primers
191 specifically designed for detection of n-damo bacteria are summarized in Table 3. Amplicon
192 (Jarman 2004) was applied for the evaluation with the *pmoA* gene of *Methylosinus sporium*
193 (DQ119048) as the excluded group and that of *M. oxyfera* as the target group (DAMO_2450
194 downloaded from the complete genome FP565575, site 2106349-2107080). All these

195 biomarkers resulted in false priming either in the sequence of *M. oxyfera* or the excluded
196 group, and some of them might form hairpins and self-complementarity (Table 3), suggesting
197 the potential problems of their specificity and efficiency. However, nested PCR approach
198 with primer sets A189_b + cmo682, and cmo182 + cmo568 was soon popularly applied for
199 recovering *M. oxyfera*-like *pmoA* gene sequences from the freshwater environments (Hu et al.
200 2014; Kojima et al. 2012; Luesken et al. 2011b; Shen et al. 2014b; Wang et al. 2012; Zhu et al.
201 2015). Importantly, primers HP3F1 and HP3R1 were the only one developed for quantifying
202 the gene copy numbers of *M. oxyfera*-like sequences based on n-damo *pmoA* gene (Han and
203 Gu 2013). Furthermore, it is confirmed that the sequences of *pmoA* primers 682R, cmo682
204 and cmo568 in a previous publication by Luesken et al. (2011b) were incorrectly stated and
205 should be reversed for correct use (personal communication). It should also be mentioned that
206 there is a nucleotide ‘T’ missing in the sequence of PCR primer cmo568 in several
207 publications (Hu et al. 2014; Shen et al. 2015c; Shen et al. 2015d; Wang et al. 2012; Zhu et al.
208 2015) compared with the original paper where it was published (Luesken et al. 2011c) and
209 with the genome sequence of *M. oxyfera* (FP565575), which is shown in bold and italic
210 (Table 3). These differences and errors in the PCR primers of incorrect form used in their
211 investigations resulted in non-reliable data report and furthermore wrongly stated conclusions
212 deviated greatly from the genuine community of the different samples. This may also lead to
213 the propagation of the errors to a much great community because of unawareness of this
214 error.

215

216 *Fluorescence in situ hybridization (FISH)*

217 Raghoebarsing et al. (2006) designed the specific bacterial probes S^{*}-DBACT-0193-a-A-18
218 (5'-CGC TCG CCC CCT TTG GTC-3'), S^{*}-DBACT-0447-a-A-18 (5'-CGC CGC CAA
219 GTC ATT CGT -3') and S^{*}-DBACT-1027-a-A-18 (5'-TCT CCA CGC TCC CTT GCG-3')
220 based on the bacterial 16S rRNA gene sequences in a microbial consortium capable of
221 coupling anaerobic methane oxidation to denitrification that consisted of bacteria and archaea
222 following the FISH method of Raghoebarsing et al. (2005). These molecular probes were
223 later applied for identifying the *M. oxyfera*-like bacteria in the n-damo cultures after 7 months
224 of enrichment (Ettwig et al. 2008; Ettwig et al. 2009). Hu et al. (2009) also developed a FISH
225 probe S^{*}-NC10-1162-a-A-18 (5'-GCC TTC CTC CAG CTT GAC GCT G -3') to target the
226 NC10 phylum sequences. S^{*}-NC10-1162-a-A-18 hybridized around 15% (Enrichment
227 temperature of 22 °C) and 50% (Enrichment temperature of 35 °C) of the bacteria in n-damo
228 enrichments after culturing for 260 and 297 days, respectively. Considering as the first
229 application in the environmental sediments, catalyzed reporter deposition fluorescence *in situ*
230 hybridization (CARD-FISH) with the probe S^{*}-DBACT-1027-a-A-18 was used to examine
231 the n-damo bacteria in Lake Biwa sediments and resulted in a very low frequency of n-damo
232 cells on a singly occurrence with large amount of diatom debris, which failed to accurately
233 count the CARD-FISH-positive cells (Kojima et al. 2012). By applying the probes
234 DBACT1027 and DBACT193, Deutzmann et al. (2014) calculated the potential n-damo rate
235 in relation to the cell density in the profundal sediment core of Lake Constance. Luesken et al.
236 (2011b) reported that although specific FISH probes for *M. oxyfera* could detect
237 approximately 2-3% or 60-70% of the total microbial communities after 64 or 308 days of
238 inoculations, no n-damo cells could be revealed in the original inoculum. The application of

239 FISH in the environment is limited to the abundance of n-damo bacteria and is less sensitive
240 compared with PCR/qPCR approach.

241

242 *Unique fatty acid*

243 New biomarkers were investigated by detecting the lipid composition of *M. oxyfera* in several
244 enrichment cultures, where up to 46% of the detected lipid profile was
245 10-methylhexadecanonic acid (10MeC_{16:0}) (Kool et al. 2012). Kool et al. (2012) also
246 identified a unique fatty acid of monounsaturated 10-methylhexadecanonic acid with a double
247 bond at the $\Delta 7$ position (10MeC_{16:1 Δ 7}) comprised up to 10% of the total fatty acid measured in
248 multiple n-damo enrichments, which had not been reported previously. These branched fatty
249 acids of 10MeC_{16:0} and 10MeC_{16:1 Δ 7} were proposed to be important and characteristic
250 chemical signatures of *Ca. Methyloirabilis oxyfera* and may serve as the biomarkers for
251 detecting them from the environment (Kool et al. 2012). However, up to now there is no other
252 publication of n-damo bacteria with application of this method. The possible reasons are: (1)
253 although as a major chemical component, 10MeC_{16:0} is not only found in *M. oxyfera*, but also
254 presents in other sulfate-reducing bacteria (i.e., *Desulfobacter*), actinobacteria, anammox
255 bacteria, iron-reducing *Geobacter*, *Marinobacter* and the marine denitrifier *Pseudomonas*
256 *nautica* (Buhring et al. 2005; Doumenq et al. 1999; Londry et al. 2004; Rütters et al. 2002;
257 Sinninghe Damste et al. 2005; Sittig and Schlesner 1993; Yoon et al. 2007; Zhang et al. 2003),
258 which means positive signal of this biomarker in anaerobic methane oxidation enrichment
259 cannot exclude either sulfate or nitrite driven pathways, and the diagnosis could be more
260 complex in environmental samples; (2) 10MeC_{16:1 Δ 7} could represent up to 10% of the fatty

261 acid signatures in n-damo enrichment, but it accounted for a maximum of 0.5% of the total
262 fatty acid detected in a vertical soil profile in peatland (therefore up to 5% abundance of
263 n-damo bacteria in the samples), where qPCR analysis suggested that up to 8% of the total
264 bacterial community were *M. oxyfera*-like bacteria (Kool et al. 2012). The low proportion of
265 10MeC_{16:1Δ7} and the bias in extraction, measurement and calculation may affect the
266 application of these biomarkers in the analysis of environmental samples.

267

268 In addition to the above methods, universal 16S rRNA gene primers were developed for
269 high-throughput sequencing of n-damo bacteria in freshwater sediment and damo-anammox
270 co-culture (Lu et al. 2015).

271

272 **Distribution of n-damo bacteria in the environments**

273 *Lake*

274 The discovery of n-damo process in the wetland systems could have a drastic influence on the
275 conventional nitrogen cycling network, although it has only been reported in a very limited
276 number of freshwater habitats (Zhu et al. 2010). The study of n-damo bacteria in the different
277 environments began with the development of specific *pmoA* PCR primers for the detection of
278 denitrifying methanotrophs in the alpine peat bog, wastewater treatment plants and
279 contaminated aquifers (Luesken et al. 2011c).

280 Later, radiotracer experiments using the sediments of Lake Constance in Germany, were
281 conducted and indicated the formation of ¹⁴CO₂ from ¹⁴CH₄ in the presence of electron
282 acceptors of nitrate and nitrite, while the effect of sulfate addition on ¹⁴CO₂ production was

283 negligible (Deutzmann and Schink 2011). Molecular analyses suggested that the 16S rRNA
284 gene sequences belonging to Group a were retrieved in Lake Constance profundal sediments,
285 although Group b sequences were obtained from both littoral and profundal sediments, while
286 n-damo *pmoA* gene sequences were only recovered in the profundal sediments (Deutzmann
287 and Schink 2011). Despite the low diversity of n-damo Group a 16S rRNA and *pmoA* gene
288 sequences in the study of Lake Constance, this work provided the first indications that
289 anaerobic methane oxidation coupled to denitrification in the oligotrophic freshwater
290 ecosystems could be a widespread process that plays an important role in affecting the
291 methane production and consumption, flux (Deutzmann and Schink 2011). Further studies on
292 the sediment cores from Lake Constance by applying high-resolution micro-sensor and
293 culture-independent molecular approaches confirmed that n-damo could be the dominant
294 methane sink with the presence of nitrate in the stable and deep sediments of the freshwater
295 lake (Deutzmann et al. 2014). The potential n-damo rates calculated from cell densities
296 ($660\text{-}4890 \mu\text{mol CH}_4 \text{ m}^{-2} \text{ d}^{-1}$) and measured by microsensor ($31\text{-}437 \mu\text{mol CH}_4 \text{ m}^{-2} \text{ d}^{-1}$) were
297 both high enough to prevent the emission of methane from the profundal lake sediments
298 solely and showed a strong correlation with the abundance of *M. oxyfera*-like bacteria in the
299 sampling cores (Deutzmann et al. 2014).

300 The investigation on sediments in Lake Biwa, Japan also showed that n-damo 16S rRNA
301 gene Group a bacteria were detected in profundal sediments, when Group b sequences were
302 retrieved in shallow water sediments (Kojima et al. 2012). Similar to the previous study in
303 Lake Constance sediments (Deutzmann and Schink 2011), no PCR product targeting n-damo
304 *pmoA* gene was obtained in the Lake Biwa littoral sediments (Kojima et al. 2012).

305 Interestingly, the abundance of *M. oxyfera*-like bacteria was the highest in the surface layer of
306 the deep sediments where the oxygen penetration was higher (around 225 μM), and dropped
307 to the lowest with the decrease of dissolved oxygen along the sediment depth downward
308 (Kojima et al. 2012), suggesting the importance of anaerobic interface on the n-damo process.
309 Meanwhile, the examination of n-damo bacteria in two Qinghai-Tibetan saline lakes also
310 added new information on their distribution in the lake ecosystems (Yang et al. 2012). PCR
311 amplified sequences belonged to Group b clade (16S rRNA) and a unique *pmoA* gene lineage
312 (closely with other n-damo sequences), which suggested the occurrence and adaptation of
313 n-damo bacteria in the natural hypersaline ecosystems with salinity as high as 84 g/L (Yang et
314 al. 2012). Recently, the distribution of n-damo bacteria was reported in both oligotrophic and
315 eutrophic lake ecosystems and the oxic/anoxic interfaces in these habitats were hypothesized
316 to provide suitable conditions for the growth of *M. oxyfera*-like bacteria (Zhu et al. 2015). In
317 the sediments of various freshwater lakes on the Yunnan Plateau (China), novel *M.*
318 *oxyfera*-like sequences of *pmoA* gene were retrieved, where the ratio of organic matter and
319 total nitrogen showed a positive correlation with the n-damo *pmoA* gene diversity by
320 Pearson's correlation analysis (Liu et al. 2015).

321 On the other hand, the n-damo bacteria were reported in the deep water samples (90 m of the
322 water depth) of a subtropical reservoir and accounted for a larger portion than the Type I and
323 II methane oxidizing bacteria revealed by 16S rRNA gene analyses (Kojima et al. 2014).
324 However, they were not detected in the water sample at the depth of 10 m, possibly due to the
325 lack of methane (Kojima et al. 2014). Reconstructed phylogeny based on amplified *pmoA*
326 gene sequences indicated a close phylogenetic distance between those in the water column of

327 the subtropical reservoir (Kojima et al. 2014) and the sediments of Lake Biwa (Kojima et al.
328 2012). In the sediments of Shangqiu reservoir, *M. oxyfera*-like bacteria were very minor in
329 the total bacterial community by the analysis of amplified gene copy numbers (Zhu et al.
330 2015). In the water-level fluctuation zone of the Three Gorges Reservoir in China, qPCR
331 revealed the significant increase of their abundance to 10^3 - 10^4 copies g^{-1} ds after around 6
332 months' of flooding (Wang et al. 2016). In addition, the n-damo bacterial community based
333 on retrieved *pmoA* gene sequences in freshwater reservoir sediment of Hong Kong had a
334 closer relationship with that in wastewater treatment plant than in Lake Constance
335 (Deutzmann and Schink 2011) and Lake Biwa (Kojima et al. 2012) using unweighted
336 Jackknife Environmental Clusters (Han and Gu 2013).

337

338 *Wetland*

339 The freshwater wetland ecosystem was another environmental habitat that was intensively
340 studied for the distribution of n-damo bacteria in the last five years, especially in the paddy
341 fields. In agreement with the observations in lake sediments, n-damo bacteria were found to
342 be most abundant in the cultivated horizon, lower in the plough pan and steadily decrease
343 with the increase of soil depth (sediment core length: 100 cm) in the paddy fields (Wang et al.
344 2012). Phylogenetic analysis showed that the upper layers (0-30 cm) of the paddy sediments
345 hosted the sequences distantly related to the known n-damo bacteria, while the 16S rRNA
346 sequences from the lower layers (40-70 cm) clustered within Group a that contains *M.*
347 *oxyfera* sequence from the enrichment cultures (Wang et al. 2012). Stable isotope
348 experiments indicated that the potential n-damo rates ranged from 0.2-2.1 $nmol CO_2 g^{-1}$ dry

349 weight day⁻¹ in different layers of sediment cores from a flooded paddy field, and it is
350 estimated that the n-damo process contributed to a total of 0.14 g CH₄ m⁻² year⁻¹ consumption
351 in the paddy field based on the data in the layer of 20-30 cm (Shen et al. 2014a). The
352 diversity of *pmoA* gene based n-damo community in subsurface layer (10-20 cm) sediments
353 of paddy soil was lower than that in the WWTP and reservoir sediments in Hong Kong (Han
354 and Gu 2013). Furthermore, high abundances and diversity of n-damo bacteria were reported
355 in Jiangyin paddy soils [up to 1.0×10⁸ gene copies (g dry soil)⁻¹], whose portion to the total
356 bacteria reached the peak value of 2.80% (summer) and 4.41% (winter), respectively (Zhou et
357 al. 2014). Along the sampling core (0-200 cm), the groundwater level affected the abundance
358 of n-damo bacteria and highest Chao1 index was observed in layer 120-140 cm (summer) and
359 180-200 cm (winter), respectively (Zhou et al. 2014). Additionally, n-damo bacterial
360 sequences were also recovered in Jiaying paddy field with a similarity of 91.3-97.4% to *M.*
361 *oxyfera* 16S rRNA gene sequence (Zhu et al. 2015).

362 Molecular evidence proved that n-damo bacteria had a wide geographical distribution at the
363 oxic/anoxic interfaces of different wetlands (n=91) in China and contributed to up to nearly
364 0.62% of the total number of bacteria (Zhu et al. 2015). Isotope tracer experiments revealed
365 that the potential denitrifying AOM rates ranged from 0.31-5.43 nmol CO₂ g⁻¹ dry weight
366 day⁻¹ in various layers of soil cores in three freshwater wetlands (Hu et al. 2014), higher than
367 those examined in the flooded paddy field (Shen et al. 2014a). Around 0.51 g CH₄ m⁻² could
368 be linked to n-damo process annually in the tested wetlands, which predicted that n-damo
369 could reduce 4.1-6.1 Tg CH₄ m⁻² year⁻¹ in wetlands under anaerobic conditions, nearly 2-6%
370 of current global methane flux estimates for wetlands (Hu et al. 2014). Study on the vertical

371 distribution of *M. oxyfera*-like bacteria suggested that the deep wetland sediments (at the
372 depth of 50-60 cm and 90-100 cm) were the preferred habitat zones for n-damo bacteria, and
373 it was estimated that the CH₄ flux might increase 2.7-4.3% without n-damo in the largest
374 natural freshwater wetland (Xiazuhuhu) on the southern Yangtze River in China (Shen et al.
375 2015c). The n-damo process was also confirmed to be responsible for consuming 0.3-0.8 g
376 CH₄ m⁻² year⁻¹ in Xiazuhuhu wetland, therefore resulted in the loss of 0.7-1.9 g N m⁻² per year
377 based on the stoichiometry of 3 CH₄ -4 N₂ via this process (Raghoebarsing et al. 2006; Shen
378 et al. 2015c). In an urban wetland (Xixi), n-damo activity was mainly detected at the depth of
379 50-60 cm and 90-100 cm with the potential rates of 0.7-5.0 nmol CO₂ g⁻¹ dry weight day⁻¹,
380 and did not occur in the surface layer (0-10 cm) (Shen et al. 2015a). Molecular analysis
381 further implied that 16S rRNA Group a members were the dominant bacteria carrying out the
382 denitrifying AOM in the sediments of Xixi wetland (Shen et al. 2015a). Moreover, n-damo
383 *pmoA* sequences were also retrieved from the sediments of reed beds at Mai Po Nature
384 Reserve in Hong Kong and showed a lower diversity (Han and Gu 2013).

385 Recently, the co-existence of n-damo archaea and bacteria was investigated and confirmed in
386 the paddy fields by using next generation pyrosequencing (Ding et al. 2016). With the
387 available PCR primers for Illumina MiSeq sequencing (Lu et al. 2015), the molecular
388 detection of n-damo bacteria is now extended to high throughput sequencing to reveal their
389 diversity and community structure in the environmental samples. After Illumina-based 16S
390 rRNA gene sequencing for the samples from agriculture soils, NC10 related reads accounted
391 for 0.8-4.5% of 16S rDNA pools in the samples and showed a higher percentage in deep soils
392 (Shen et al. 2016).

393

394 *River*

395 The distribution of n-damo bacteria in the river ecosystems was firstly investigated in the
396 sediments of Qiantang River by molecular analysis (Shen et al. 2014b). Amplified 16S rRNA
397 and *pmoA* gene sequences showed 89.8%-98.9% and 85.1-95.4% identifies to those of *M.*
398 *oxyfera*, respectively (Shen et al. 2014b). Shen et al. (2014b) found that the total inorganic
399 nitrogen content and ammonium content in the river sediments were the most significant
400 factors affecting n-damo community based on *pmoA* gene-PCR amplified sequences, while
401 n-damo 16S rRNA gene abundance significantly related to the sediment organic carbon
402 content. By comparison, the gene copy number of n-damo bacteria and their ratio to total
403 bacteria was the highest in canal sediments, then lowered in the riparian sediments, and was
404 the lowest in the river sediments (Zhu et al. 2015).

405

406 *Coastal ecosystem*

407 The investigation of n-damo bacteria in the coastal ecosystems is very limited. Shen et al.
408 (2014c) reported the molecular evidence of n-damo bacteria in the surface sediments of the
409 Jiaojiang Estuary in China. In their work, the majority of the amplified 16S rRNA gene
410 sequences belonged to Group a, whereas others clustered within Group b. Meanwhile, the
411 highest abundances of n-damo bacteria were found in the sediments of the estuarine intertidal
412 zone other than the sub-tidal zone (Shen et al. 2014c). Sediment organic matter strongly
413 impacted the spatial variation and also significantly correlated with the diversity and
414 abundance of n-damo bacterial community by Redundancy analysis and Pearson Moment

415 Correlation (Shen et al. 2014c). *M. oxyfera*-like sequences were also recovered in the
416 sediment of Honghaitan tidal land, which was influenced by polluted seawater (Zhu et al.
417 2015). The abundance of n-damo bacteria in the tidal sediments was higher than that in the
418 sediments of rivers, paddy fields and reservoir (Zhu et al. 2015). More recently, 16S rDNA
419 sequences of n-damo Group a and b were retrieved from the Yellow River Estuary sediments
420 with 10^3 - 10^5 gene copies of 16S rRNA and *pmoA* genes per gram of wet sediment (Yan et al.
421 2015).

422 In the intertidal sediments of mudflat, mangrove and reed bed at Mai Po wetland of Hong
423 Kong, *M. oxyfera*-like sequences with high diversity were retrieved and analyzed (Chen et al.
424 2015b), which indicating that the *pmoA* gene-amplified sequences in MP wetland clustered
425 within both freshwater and marine subclusters, and were different from the so far reported
426 n-damo communities in other two coastal environments (Shen et al. 2014c; Zhu et al. 2015).
427 Community structures based on detected 16S rDNA sequences from dry season samples
428 distributed between the freshwater and marine groups toward the environmental changes in
429 PCoA plots, while those using amplified *pmoA* gene sequences grouped with the marine ones
430 only (Chen et al. 2015b). This observation on n-damo is mirrored the observation made at the
431 same site on anammox bacteria for its community composition shift between seasons due to
432 the ocean or terrestrial dominance in dry or wet seasons, respectively (Han and Gu 2015; Li
433 et al. 2011). Community of n-damo may respond to anthropogenic influence in a similar but
434 competitive fashion as anammox in coastal ecosystem (Han and Gu 2015).

435

436 *Marine sediments*

437 As a newly identified contributor to both N and C cycles, little information is known about
438 the diversity and distribution of n-damo bacteria in the marine environments. Marine *M.*
439 *oxyfera*-like sequences were poorly reported in published papers or GenBank database. Even
440 less studied is on the correlation of possible n-damo bacterial community and the associated
441 environmental factors to allow understanding of their relationship with environmental
442 variables. In the South China Sea (SCS) sediments sampled in the inner continental shelf,
443 outer continental shelf, the slope and the deep abyss, *M. oxyfera*-like sequences were
444 retrieved by applying the specific PCR primers of 16S rRNA and *pmoA* genes (Chen et al.
445 2015a; Chen et al. 2014). The reconstructed phylogeny using amplified 16S rDNA sequences
446 showed that none of the SCS sequences belonged to the Group a where the *M. oxyfera* 16S
447 rDNA gene clustered within, but the majority of them grouped into clade e (Chen et al. 2015a;
448 Chen et al. 2014). Amplified 16S rDNA sequences from surface sediments showed a higher
449 alpha diversity and formed more sub clusters compared with those from the subseafloor,
450 while retrieved *pmoA* gene sequences had lower diversity and richness compared with the
451 obtained 16S rDNA sequences (Chen et al. 2014). On the other hand, the SCS n-damo *pmoA*
452 gene sequences distinctively clustered within three newly identified clusters, which contained
453 none of the sequences amplified from the freshwater habitats and were tentatively named as
454 SCS-1, SCS-2 and SCS-3 (Chen et al. 2014). The analysis of the beta diversity based on
455 amplified 16S rRNA and *pmoA* gene sequences together with those available in the GenBank
456 database indicated that marine n-damo bacterial communities had a clear difference from
457 those recovered in freshwater environments (Chen et al. 2015a; Chen et al. 2014). The gene
458 copy numbers of n-damo bacterial 16S rRNA gene in SCS sediments ranged from 1.6×10^5 to

459 1.4×10^8 gene copies per gram dry sediment (Jing Chen and Ji-Dong Gu, unpublished data).
460 NO_x^- significantly and positively correlated with 16S rDNA Group a and *pmoA* gene Cluster
461 SCS-2, when NH_4^+ showed a directly adverse effect on the community structure based on
462 either recovered 16S rRNA or *pmoA* genes-amplified sequences (Chen et al. 2014). This
463 information of n-damo in SCS also showed similar information of anammox as previously
464 observed in that unique species of anammox *Ca. Scalindua zhenhei* I, II and III were
465 discovered (Hong et al. 2011). Nitrate/nitrite-driven AOM was questioned for their roles in
466 marine habitats (Orcutt et al. 2011). Molecular evidences on existence of n-damo bacteria in
467 coastal and marine environments now suggest the potential role of n-damo process in coastal
468 and deep-sea sediments (Chen et al. 2015a; Chen et al. 2014; Chen et al. 2015b; Shen et al.
469 2014c; Zhu et al. 2015).

470

471 **The diversity of n-damo bacteria based on *pmoA* gene in the environments**

472 The amplified *pmoA* gene sequences of *M. oxyfera*-like bacteria were retrieved from
473 GenBank database and summarized in Table 4. Fastgroup II (Yu et al. 2006) was applied to
474 calculate the OTU, Shannon-Wiener and Chao1 based on percentage sequence identity of
475 95% *pmoA* gene sequences. Jiaojiang Estuary (Shen et al. 2014c), Qiantang River (Shen et al.
476 2013) and Jiaxing constructed wetland (Zhu et al. 2015) had much higher Shannon-Wiener
477 and Chao 1 indexes compared with other samples. Lake Biwa, reed bed, Yellow River, Panjin
478 swamp, two paddy fields and XZ wetland showed very limited alpha diversity of n-damo
479 bacterial community (Han and Gu 2013; Hu et al. 2014; Kojima et al. 2012; Wang et al. 2012;
480 Zhu et al. 2015). Generally, the Shannon-Wiener indexes of *pmoA* gene sequences recovered

481 from paddy soils were significantly lower than in other environmental habitats. Meanwhile,
482 the diversity and richness based on amplified n-damo *pmoA* gene sequences in different lakes
483 and rivers varied drastically, which implied the niche adaptation of n-damo bacterial
484 community. Furthermore, Shannon-Wiener and Chao1 index of Pearl River samples collected
485 in winter were higher than those in summer, but the alpha diversity of Jiaxing constructed
486 wetland decreased in the winter samples, suggesting the complex effect of seasonal change
487 on the n-damo diversity in different environments. On the other hand, the alpha diversity of
488 n-damo bacterial community in the South China Sea sediments was lower than those in lake
489 and river sediments. In addition, n-damo enrichments had a relatively higher OTU numbers
490 than some pristine environments, where only one OTU was obtained.

491

492 **Future Perspectives and Trends**

493 Despite the fast development of n-damo study in recent years, the documentation and
494 knowledge of n-damo process and the microbial species responsible for it are still largely
495 limited. It is important and necessary to conduct further investigations to advance our
496 knowledge in the following directions.

497

498 *Marine enrichment of halophilic n-damo microbes*

499 There is still no culture of n-damo bacteria from either the shallow or deep-sea sediments
500 with salinity of up to 34-36‰. For further understanding the ecophysiology, biochemistry and
501 metabolisms of marine n-damo bacteria, it is important to obtain such cultures of n-damo
502 bacteria from the marine sediments, especially the aphotic and pelagic zones. Enrichment

503 cultures are basis for further research from characterization, phylogenetics, ecophysiology,
504 biochemistry to gene expression and evolutionary analysis to allow understand of this group
505 microorganisms more comprehensively from single cell to global climate change.

506

507 *The development of specific PCR primers*

508 It is apparent that there is a limitation of marine n-damo enrichment and the poor
509 understanding of them in the ocean. Recent works added a large amount of marine *M.*
510 *oxyfera*-like sequences into the GenBank database (Chen et al. 2015a; Chen et al. 2014).
511 However, the low coverage of the applied primer sets in this work was consistently
512 encountered (Chen et al. 2015a). It is urgent to develop additional specific PCR primers that
513 can be applied to marine sample with high efficiency and specificity.

514 On the other hand, n-damo bacteria involve in two significant pathways of aerobic methane
515 oxidation and denitrification under anaerobic condition. As far as known to us, the specific
516 PCR primers for n-damo bacterial detection are limited to 16S rRNA and *pmoA* genes. It is
517 interesting and important to investigate PCR primers targeting other genes unique to n-damo
518 bacteria.

519 Successful enrichment of n-damo microbes from other ecosystems, like marine sediments,
520 will pave the way for genome sequencing of the specific bacteria involved. The sequences
521 can be used in effective design of new PCR primers used for further amplification of n-damo
522 from a wide range of environmental samples. Through genome information, it is also possible
523 to decipher the genes involved in n-damo biochemical processes and to identify any novel
524 genes in this microorganism.

525

526 *The contribution of n-damo process in coastal and ocean environments*

527 Anaerobic methane oxidation tied to denitrification is largely overlooked in marine
528 environments. The popular investigation of anaerobic methane oxidation in coastal and ocean
529 sediments focused on sulfate reduction as the electron acceptor. The contribution of n-damo
530 process is not reported in neither coastal nor ocean ecosystems. Therefore, it is meaningful to
531 perform quantification of the n-damo activity in marine ecosystems to obtain the rate and flux
532 and a comparison with other methane oxidation or denitrification/nitrification processes to
533 identify their role in the nitrogen and carbon cycles.

534

535 *Investigation of n-damo bacteria in other marine ecosystems*

536 The understanding of n-damo bacterial diversity and distribution is currently confined in the
537 west Pacific region of the South China Sea (Chen et al. 2015a; Chen et al. 2014; Chen et al.
538 2015b) and the coastal areas of the East China Sea and Bohai Sea (He et al. 2015a; Shen et al.
539 2014c; Zhu et al. 2015). It is important to study n-damo bacteria in other marine sediments or
540 unique wetlands to further understand their diversity and contribution to the C and N cycling
541 in aquatic ecosystems.

542 Current available techniques, including pyrosequencing, transcriptomics, metabolomics and
543 single cell sequencing, can advance research on this topic significantly in the near future with
544 the selection of a research niche for the n-damo to be focused on. It is clear that enrichment
545 and possible pure culturing will be the major obstacle and bottleneck to further research and
546 development. Any pure culture of n-damo will allow a great leap in the research on this topic.

547 However, other approaches can also be used to assess the transformation processes and rates
548 in samples of interest to obtain important data on contribution of n-damo to the overall
549 transformation rate of CH₄ and NO₂⁻. At the same time, it is also necessary to recognize the
550 relationship between n-damo and other microorganisms, e.g., anammox bacteria, in the
551 natural ecosystems. Co-existence of them may have significant biological basis even though
552 the relationship may be a competitive one through the common substrate NO₂⁻.

553

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558

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562 **Conflict of Interest:** All authors declare that they have no conflict of interest.

563 **Ethical approval:** This article does not contain any studies with human participants or
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565

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