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

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Abstract

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

Introduction

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

$$5\,CH_4 + 8\,NO_3^- + 8\,H^+ \rightarrow 5\,CO_2 + 4\,N_2 + 14\,H_2O \quad [1]$$

$$(\Delta G^{\circ\prime} = -765\ \text{kJ mol}^{-1}\ CH_4)$$

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \rightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O \quad [2]$$

$$(\Delta G^{\circ\prime} = -928\ \text{kJ mol}^{-1}\ CH_4)$$

Obtained from the anoxic sediments, a microbial consortium consisting of two microorganisms (a bacterium belonging to NC10 phylum without any cultured species and an archaeon distantly clustering with marine methanotrophic Archaea) showed a denitrification rate of 21.5 ± 2 μmol N_2 h^{-1} with the simultaneous conversion of the added methane at a rate of 22.0 ± 2 μmol CH_4 h^{-1} (Raghoebarsing et al. 2006). Using the enrichment culture of Raghoebarsing et al. (2006) as inocula, Ettwig et al. (2008) demonstrated that the specific inhibitor, Bromoethane at a concentration of 20 mM, for the key *mcr* gene of methanotrophic and methanogenic archaea showed no effect on the subculture oxidizing methane and reducing nitrite, which was further enhanced with the decline of the archaeal population.

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

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

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

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

Microbial process couples anaerobic methane oxidation to denitrification

Microbes capable of simultaneously oxidizing methane and denitrifying anaerobically had not been found in nature nor isolated in pure culture (Knowles 2005; Strous and Jetten 2004) before the first report of direct evidence of anaerobic methane oxidation with denitrification (Raghoebarsing et al. 2006). Ettwig et al. (2008) further showed that the microbial consortium in the study of Raghoebarsing et al. (2006) could perform the n-damo process without the presence of archaea. The active bacterium was named as *Candidatus* Methylomirabilis oxyfera that could reduce nitrite to dinitrogen (N_2) and utilize methane as an electron donor under anaerobic conditions based on genomic analyses and experimental results (Ettwig et al. 2010; Ettwig et al. 2008; Ettwig et al. 2009). This nitrite-driven anaerobic oxidation of methane (AOM) provides a very unique link between the microbial nitrogen and carbon cycles, previously unknown to science. Later on, Haroon et al. (2013) reported a novel archaeal lineage, *Candidatus* Methanoperedens nitroreducens, which can carry out AOM with reduction of nitrate to nitrite and needs the participation of anammox bacteria to complete the denitrification process. Very recently, aerobic methanotroph

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

Novel denitrification pathway

The significant biochemical pathways of *Methylomirabilis oxyfera* was summarized by Ettwig et al. (2010). *M. oxyfera* encodes, transcribes and expresses the full biochemical pathway for aerobic methane oxidation, which oxidizes methane through methanol, formaldehyde and formate to CO_2 as the end product (Ettwig et al. 2010; Wu et al. 2011). On the other side, the assembly and annotation of the genome indicated that *M. oxyfera* lacks the gene cluster encoding the enzymes for reducing nitrous oxide to dinitrogen gas (N_2) in a conventional denitrification pathway (Ettwig et al. 2010). Isotope and proteomic experiments further suggested the production of N_2 by *M. oxyfera* was directly driven by a novel enzyme, a putative NO dismutase (Ettwig et al. 2010; Wu et al. 2011).

An intra-aerobic pathway and oxygen production without photosynthesis

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

pathway by *M. oxyfera* indicated electron transport in n-damo process (Simon and Klotz 2013) and the production of oxygen when converting NO to N_2. Isotope experiments showed that the majority of the oxygen produced via this oxygenic denitrification (75%) were used for activation of the particulate methane monooxygenase (pMMO) to convert methane to methanol, while the remaining might be consumed by other terminal oxidases (Ettwig et al. 2010). These incredibly integrated biochemical pathways using oxygen as an intermediate were also incorporated into the naming of this n-damo bacterium (methyl (Latin): the methyl group; mirabilis (Latin): astonishing, strange; oxygenium (Latin): oxygen; fera (Latin): carrying, producing) (Ettwig et al. 2010). The production of its own supply of oxygen under anoxic conditions makes *M. oxyfera* performing a peculiar and novel inter-aerobic biochemical pathway in driving methane oxidation when compared with other sulfate-reducing methanotrophs (Wu et al. 2011).

On the other hand, *M. oxyfera* is one of the only two known microorganisms so far that could produce oxygen in the darkness (Ettwig et al. 2012a). Only three biochemical pathways are known to produce oxygen before the discovery of n-damo bacteria: photosynthesis, bacterial reduction of chlorates (chlorate-reducing bacteria), and the enzymatic conversion of reactive oxygen species (Mascarelli 2010). Photosynthesis is considered the only biological source for oxygen production and plays a critical role in the initial emission of oxygen, building up in the atmosphere, and recycling of oxygen on Earth (Ettwig et al. 2010; Ettwig et al. 2012b). The production of oxygen via bacterial n-damo process yields new aspect of the potential aerobic biochemical pathways in a methane rich and oxygen limited environment before the great oxidation event in the Archaean Earth (Oremland 2010).

Molecular methods for the detection of n-damo bacteria

PCR approach

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

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

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

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

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

Fluorescence in situ hybridization (FISH)

Raghoebarsing et al. (2006) designed the specific bacterial probes S-*-DBACT-0193-a-A-18 (5'-CGC TCG CCC CCT TTG GTC-3'), S-*-DBACT-0447-a-A-18 (5'-CGC CGC CAA GTC ATT CGT -3') and S-*-DBACT-1027-a-A-18 (5'-TCT CCA CGC TCC CTT GCG-3') based on the bacterial 16S rRNA gene sequences in a microbial consortium capable of coupling anaerobic methane oxidation to denitrification that consisted of bacteria and archaea following the FISH method of Raghoebarsing et al. (2005). These molecular probes were later applied for identifying the *M.oxyfera*-like bacteria in the n-damo cultures after 7 months of enrichment (Ettwig et al. 2008; Ettwig et al. 2009). Hu et al. (2009) also developed a FISH probe S-*-NC10-1162-a-A-18 (5'-GCC TTC CTC CAG CTT GAC GCT G -3') to target the NC10 phylum sequences. S-*-NC10-1162-a-A-18 hybridized around 15% (Enrichment temperature of 22 °C) and 50% (Enrichment temperature of 35 °C) of the bacteria in n-damo enrichments after culturing for 260 and 297 days, respectively. Considering as the first application in the environmental sediments, catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with the probe S-*-DBACT-1027-a-A-18 was used to examine the n-damo bacteria in Lake Biwa sediments and resulted in a very low frequency of n-damo cells on a singly occurrence with large amount of diatom debris, which failed to accurately count the CARD-FISH-positive cells (Kojima et al. 2012). By applying the probes DBACT1027 and DBACT193, Deutzmann et al. (2014) calculated the potential n-damo rate in relation to the cell density in the profundal sediment core of Lake Constance. Luesken et al. (2011b) reported that although specific FISH probes for *M. oxyfera* could detect approximately 2-3% or 60-70% of the total microbial communities after 64 or 308 days of 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 Δ7 position ($10MeC_{16:1\Delta7}$) 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 $10MeC_{16:1\Delta7}$ were proposed to be important and characteristic
250 chemical signatures of *Ca*. Methylomirabilis 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) $10MeC_{16:1\Delta7}$ could represent up to 10% of the fatty

acid signatures in n-damo enrichment, but it accounted for a maximum of 0.5% of the total fatty acid detected in a vertical soil profile in peatland (therefore up to 5% abundance of n-damo bacteria in the samples), where qPCR analysis suggested that up to 8% of the total bacterial community were *M. oxyfera*-like bacteria (Kool et al. 2012). The low proportion of $10MeC_{16:1\Delta7}$ and the bias in extraction, measurement and calculation may affect the application of these biomarkers in the analysis of environmental samples.

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

Distribution of n-damo bacteria in the environments

Lake

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

Later, radiotracer experiments using the sediments of Lake Constance in Germany, were conducted and indicated the formation of $^{14}CO_2$ from $^{14}CH_4$ in the presentence of electron acceptors of nitrate and nitrite, while the effect of sulfate addition on $^{14}CO_2$ production was

negligible (Deutzmann and Schink 2011). Molecular analyses suggested that the 16S rRNA gene sequences belonging to Group a were retrieved in Lake Constance profundal sediments, although Group b sequences were obtained from both littoral and profundal sediments, while n-damo *pmoA* gene sequences were only recovered in the profundal sediments (Deutzmann and Schink 2011). Despite the low diversity of n-damo Group a 16S rRNA and *pmoA* gene sequences in the study of Lake Constance, this work provided the first indications that anaerobic methane oxidation coupled to denitrification in the oligotrophic freshwater ecosystems could be a widespread process that plays an important role in affecting the methane production and consumption, flux (Deutzmann and Schink 2011). Further studies on the sediment cores from Lake Constance by applying high-resolution micro-sensor and culture-independent molecular approaches confirmed that n-damo could be the dominant methane sink with the presence of nitrate in the stable and deep sediments of the freshwater lake (Deutzmann et al. 2014). The potential n-damo rates calculated from cell densities (660-4890 μmol CH_4 m^{-2} d^{-1}) and measured by microsenor (31-437 μmol CH_4 m^{-2} d^{-1}) were both high enough to prevent the emission of methane from the profundal lake sediments solely and showed a strong correlation with the abundance of *M. oxyfera*-like bacteria in the sampling cores (Deutzmann et al. 2014).

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

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

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

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

Wetland

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

weight day^{-1} in different layers of sediment cores from a flooded paddy field, and it is estimated that the n-damo process contributed to a total of 0.14 g CH_4 m^{-2} $year^{-1}$ consumption in the paddy field based on the data in the layer of 20-30 cm (Shen et al. 2014a). The diversity of *pmoA* gene based n-damo community in subsurface layer (10-20 cm) sediments of paddy soil was lower than that in the WWTP and reservoir sediments in Hong Kong (Han and Gu 2013). Furthermore, high abundances and diversity of n-damo bacteria were reported in Jiangyin paddy soils [up to 1.0×10^8 gene copies (g dry soil)$^{-1}$], whose portion to the total bacteria reached the peak value of 2.80% (summer) and 4.41% (winter), respectively (Zhou et al. 2014). Along the sampling core (0-200 cm), the groundwater level affected the abundance of n-damo bacteria and highest Chao1 index was observed in layer 120-140 cm (summer) and 180-200 cm (winter), respectively (Zhou et al. 2014). Additionally, n-damo bacterial sequences were also recovered in Jiaxing paddy field with a similarity of 91.3-97.4% to *M. oxyfera* 16S rRNA gene sequence (Zhu et al. 2015).

Molecular evidence proved that n-damo bacteria had a wide geographical distribution at the oxic/anoxic interfaces of different wetlands (n=91) in China and contributed to up to nearly 0.62% of the total number of bacteria (Zhu et al. 2015). Isotope tracer experiments revealed that the potential denitrifying AOM rates ranged from 0.31-5.43 nmol CO_2 g^{-1} dry weight day^{-1} in various layers of soil cores in three freshwater wetlands (Hu et al. 2014), higher than those examined in the flooded paddy field (Shen et al. 2014a). Around 0.51 g CH_4 m^{-2} could be linked to n-damo process annually in the tested wetlands, which predicted that n-damo could reduce 4.1-6.1 Tg CH_4 m^{-2} $year^{-1}$ in wetlands under anaerobic conditions, nearly 2-6% of current global methane flux estimates for wetlands (Hu et al. 2014). Study on the vertical

distribution of *M. oxyfera*-like bacteria suggested that the deep wetland sediments (at the depth of 50-60 cm and 90-100 cm) were the preferred habitat zones for n-damo bacteria, and it was estimated that the CH_4 flux might increase 2.7-4.3% without n-damo in the largest natural freshwater wetland (Xiazhuhu) on the southern Yangtze River in China (Shen et al. 2015c). The n-damo process was also confirmed to be responsible for consuming 0.3-0.8 g CH_4 m^{-2} $year^{-1}$ in Xiazhuhu wetland, therefore resulted in the loss of 0.7-1.9 g N m^{-2} per year based on the stoichiometry of 3 CH_4 -4 N_2 via this process (Raghoebarsing et al. 2006; Shen et al. 2015c). In an urban wetland (Xixi), n-damo activity was mainly detected at the depth of 50-60 cm and 90-100 cm with the potential rates of 0.7-5.0 nmol CO_2 g^{-1} dry weight day^{-1}, and did not occur in the surface layer (0-10 cm) (Shen et al. 2015a). Molecular analysis further implied that 16S rRNA Group a members were the dominant bacteria carrying out the denitrifying AOM in the sediments of Xixi wetland (Shen et al. 2015a). Moreover, n-damo *pmoA* sequences were also retrieved from the sediments of reed beds at Mai Po Nature Reserve in Hong Kong and showed a lower diversity (Han and Gu 2013).

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

River

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

Coastal ecosystem

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

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

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

Marine sediments

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

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

Future Perspectives and Trends

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

Marine enrichment of halophilic n-damo microbes

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

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

The development of specific PCR primers

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

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

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

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

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

Investigation of n-damo bacteria in other marine ecosystems

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

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

However, other approaches can also be used to assess the transformation processes and rates in samples of interest to obtain important data on contribution of n-damo to the overall transformation rate of CH_4 and NO_2^-. At the same time, it is also necessary to recognize the relationship between n-damo and other microorganisms, e.g., anammox bacteria, in the natural ecosystems. Co-existence of them may have significant biological basis even though the relationship may be a competitive one through the common substrate NO_2^-.

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Ethical Statement

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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