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MINI-REVIEW

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).

$$5\,CH_4 + 8\,NO_3^- + 8\,H^+ \rightarrow 5\,CO_2 + 4\,N_2 + 14\,H_2O \quad \left(\Delta G^{\circ\prime} = -765\ \text{kJ mol}^{-1}\,CH_4\right) \tag{1}$$

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \rightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O \quad \left(\Delta G^{\circ\prime} = -928\ \text{kJ mol}^{-1}\,CH_4\right) \tag{2}$$

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

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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 nitrite-dependent anaerobic methane oxidation (n-damo) enrichments is presented in Table 1. Some important characteristics of n-damo inocula in these studies (Ettwig et al. 2008, 2009; Hu et al. 2009. 2011; Luesken et al. 2011a, b; Zhu et al. 2011) are the following: 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 *Methylomirabilis 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 1 to 2 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 M. 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. 2008, 2009, 2010). 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. *M. 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 *M. 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

Table 1 Comparison of n-damo bacteria enrichments available in the literature

Source	Origin of inoculum	Scale (L)	CH_4 supply	Anaerobic control	T (°C)	pH	Stir	Medium supply
Hu et al. (2009)	Mixture of sediments from freshwater lake, anaerobic digester sludge + return sludge from WWTP	2 (1.6 L liquid)	headspace was flushed periodically with mixed gas (N_2, CH_4 and CO_2)	……	22 35 45[d]	….	…	Nitrate added periodically
Hu et al. (2011)	Parent culture from Hu et al. (2009) [Culture A (320 ml) + Culture B (160 ml) inocula]	2; (1.6 L liquid)	5%N_2 + 90%CH_4 + 5%CO2 to flush headspace regularly with a P_{CH4}(0.5-1 atm)	N2 sparged into medium, then placed in an anaerobic chamber for 24 hours before it was used	35	7.0-7.5	200 rpm	20 min settling; 400 ml fresh medium exchanged/ 4 weeks[NO_3^-: 1-10 mM; NO_2^-: 1 ml/72 min-daily]
Ettwig et al. (2008)	Biomass (1.5 liters) from an enrichment culture in Raghoebarsing et al. (2006)[g] Biomass After 13 months enrichment from the above[b]	16; SBR (13 L liquid) 0.3 (0.22 liquid)	CH_4-CO_2 95:5 %; 10 ml/min …….	Ar-CO_2 95:5 % (Continuously sparged in reactor and medium) Ar-CO_2 95:5 %	30 30	7.3-7.6 ….	200 rpm Stirrer	Continuous supply; 0.2-0.8 L/ day with nitrite conc. 3-15 mM (keep NO_2^+: 0-1 mM) ……..
Ettwig et al. (2009)	Mixture sludge (2 liters) from four ditches draining agricultural land (upper 5 cm); Biomass After 230 days enrichment from the above	16; SBR (10-13 L liquid) Batch study according to Ettwig et al. (2008)	CH_4-CO_2 95:5 %; 10 ml/min[a]	6 cycles of vacuuming and subsequent gassing with Ar-CO_2 95:5, then 5 min of Ar-CO_2, leaving 0.5 10^5 overpressure (100 ml/min)	30	6.9-7.5	100 rpm Gently	0.3-1 L/day (pending on nitrite conc.: 0.2-1.5 mM)
Luesken et al. (2011b)	Enriched biomass from the bellow 15 L bioreactor WWTP in Lieshout	3; SBR 15; SBR	CH_4-CO_2 95:5 %; 10 ml/min	Ar-CO_2 95:5 % (Continuously sparged in reactor and medium)	————	6.8-7.3	100 rpm	23.2 h/day; 0.3-1 L/day pending on nitrite conc.; (To maintain nitrite: 0.1-1 mM)
Luesken et al. (2011a)	N-damo culture from Ettwig et al. (2009) (1 liter)	3; SBR	CH_4-CO_2 95:5 %; 12 ml/min (Continuously flushed)	Ar-CO_2 95:5 % 10 ml/min (Vessel flushed)	30	7.3-7.6	200 rpm	Synthetic medium from Ettwig et al. (2009)
Zhu et al. (2011)	500 mL Anammox granules from WWTP (van der Star et al. 2007)	2 liquid; SBR	>60 days, CH_4 10 ml/min[a] supplied with NO_2^+	SBR operated according to Luesken et al. (2011a)	Room temp.	7.2	…	Nitrite: ammonium = 4:3

Headspace-CH_4	Nitrite reduction/Test time (Operating days)	Methane oxidation/ Test time	Detection/Sample volume	FISH detection after X time	Observed stoichiometry
1.1–1.3 atm; He injected for confirming no gas leakage	0.065 mmol NO_3^-/day (day 220-260) 3.2 mmol NO_3^-/day (day 283-312)[h] **……..(320 days in total)**	0.038 mmol/day (day 220-260) 2.4 mmol/day (day 283-312) ……	GC/MS for 13C-SIP	270 day: no archaea 15 % NC10 297 d: 40 % archaea 50 % NC10 ….	Consuming 16.7 % more methane than predicted

Table 1 (continued)

Headspace-CH_4	Nitrite reduction/Test time (Operating days)	Methane oxidation/ Test time	Detection/Sample volume	FISH detection after X time	Observed stoichiometry
+80 ml He into for increasing P > 1.2 atm after each flushing	1.97 mmol/day for A (NO_2^- only, day 90-94) 0.062 mmol NO_3^-/day for B (NO_3^- only, day 50-105) **(150 days in total)**	A: close to calculated value B: 0.045 mmol/day	Test strip nitrite limit control daily FIA for NH_4^+ NO_2^- NO_3^-(1 ml) GC-40 °C for CH_4, N_2; 90 °C for He	Archaea disappeared after day 100 in A	B: 13.3 % > stoichiometrical predicted: 0.039 mmol CH_4/day
…… 6 % (2.5 % when measuring)	Around 3.5(mmol/day) for 13 months Only increased (≈9) till 450 ml new source added after 17, 19 months (**22 months in total**) 3.7 nmol/min/mg protein after 22 m	1.7 nmol/min/mg protein after 22 m; Test length: 36 h	Merckoquant test strip: NO_2^-, NO_3^- GC: 65 °C– N_b; 120 °C–CH_4; BCA assay: total protein 100 μl gas; 1 ml liquid collected HPLC-UV: NO_2^-:NO_c^-;10 μl used GC: CH4;	Monthly, >70 % NC10 all the time Archaea disappear from 10 to12 month	8:3.6 = Consumption of NO_2^-: CH_4 (? 8:3 Stated by Ettwig et al., 2009) 5:1 = NO_2^-:NO_3^- (21 h)
Final conc.: 2.5 to 10 %	Max = 33.5 (mmol/day); 217 (**243 days in total**) 4.7to5.1(nmol/min/mg protein^{-1}; red/grey stopper) Day 136-142 3.4to5.6(nmol/min/mg protein); 2 months period with 4.75 protein	1.6to2.2(nmol/min/mg protein; red/grey stopper); Day 136-142 with bioreactor stop; tested in batches; Test length: 32 h	The same to the above except: 50 μl gas GC/MS-80 °C for N_2 (5 min)	5 m-can be used 6 m-be dominant[f] 7 m-70 % n-damo	8:3.5 = Consumption of NO_2^-:CH_4
0.3 L; 4 % (5.3 %, 4.8 % when measured on day 308,315)	After 70 d, activity increased (**308 d in total**) 0.4 mmol/day (day 112); 1.1 after day 175; 0.9 (nmol/min/mg protein) on day 308,315; 1.2 after day 315 2.1 (nmol NO_2^-/**h**/mg protein); 9.2 (nmol NO_3^-/**h**/mg protein); Denitrifying activity decresed	0.3 (nmol/min/mg protein) on day 308,315; 0.5 after day 315 Test length: 10 h 1.7 (nmol/**h**/mg protein)	Routine nitrite analysis daily Batch exp. On day 308, 315 and Analysis according to Ettwig et al., 2008	64 days-detected 2-3 %; 308 days-60-70 % n-damo No n-damo detected	9.8NO_2^-:3CH_4 (day 308,315) 10.1NO_2^-:3CH_4 (day 308,315) …….
…….	8.1 (nmol/min/mg protein) on 49 d; 17.2 mmol/day after day151 (?No data shown) 15.4 (nmol/min/mg protein) on day 142 (with anammox); 5.0 after 3 h when ammonium depleted (its rate was 9.0) [**161 days in total**]	3.1 (nmol/min/mg protein) on 49 d (then NH_4^+ added); Coculture with anammox, (responsible for 77 % NO_2^+ ↓ and constituted 50 %	Analysis according to Ettwig et al. (2008)	45 days-80 % n-damo no anammox; 108 days- 5 % anammox 151 days, 50 % of each	8.1NO_2^-:3CH_4 (day 49)
			……..	1 year is necessary	8NO_2^-:3CH_4 (1 year later)

Table 1 (continued)

Headspace-CH_4	Nitrite reduction/Test time (Operating days)	Methane oxidation/ Test time	Detection/Sample volume	FISH detection after X time	Observed stoichiometry
Always above 20 % (255 μM)	21.8 mmol/day after 2 months **[1 year in total]** 13.9 mmol/l per day after 1 year; 8.4 for ammonium (Coculture with anammox,)	1.6 (mmol/min/mg protein)[0.7 mmol/ l per day] after 1 year Test length: 100 min			

[a] 3-7days supply + 1-2 h settling + 30-60 min pumping out liquid. As activity increased, after 6 months the cycle was shortened to 22.5 h of supply of medium, 1 h of settling, and 30 min of drawing off of liquid.

[b] After being made anoxic by thorough flushing with a mixture of Ar/CO_2 (95:5), culture liquid from the enrichment culture was being pumped through at a flow rate of 17.5 ml/min; After c. 24 h (accounting for at least 110 volume changes) it was disconnected from the main culture vessel by closing the influent and effluent tubes.

[c] 23.2 h supply + 20 min settling + 30 min pumping out liquid.

[d] Stopped on day 90 as no denitrification was detected after day 40.

[e] The use of black butyl rubber stoppers caused total inhibition of the activity of methane oxidation. Anoxic handling of the biomass, in contrast, was not crucial; exposure to oxygen during transfer to the bottles did not lead to lower activity.

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

[g] FISH showed that upon inoculation the population was dominated by a bacterium affiliated with the phylum NC10 (approximately 80 %) and an archaeon of the order *Methanosarcinales* (approximately 10 %)

[h] Decreased to 0.3 mmol NO_3—N/day in 40 days; Increased after 220d to 2.0 on day 312 (35 °C); 0.14 without methane supply; 0.11 for day 50-260 (22 °C)

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 as 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, 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

Table 2 A summary of 16S rDNA PCR primers currently used for detecting n-damo bacteria from environmental samples

Primer	Forward/reverse	Position	Sequence (5′-3′)	Specialty	References
202F	Forward	202–230	GAC CAA AGG GGG CGA GCG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
NC10-202Fdeg	Forward	202-230	RAC CAA AGG RGG CGA GCG	*M. oxyfera* 16S rDNA	Deutzmann and Schink (2011)
1492R	Reverse	1510–1529	GGT TAC CTT GTT ACG ACT T	Bacterial 16S rDNA	Lane (1991)
8F	Forward	8–27	AGA GTT TGA TYM TGG CTC AG	Bacterial 16S rDNA	Juretschko et al. (1998)
1043R	Reverse	1042–1060	TCT CCA CGT TCC CTT GCG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
1043Rb	Reverse	1042–1060	TCT CCA CGC TCC CTT GCG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
NC10-1043Rdeg	Reverse	1042–1060	TCT CCR CGY TCC CTT GCG	*M. oxyfera* 16S rDNA	Deutzmann and Schink (2011)
1545R	Reverse	1529–1545	CAK AAA GGA GGT GAT CC	Bacterial 16S rDNA	Juretschko et al. (1998)
qP1F	Forward	1001–1024	GGG CTT GAC ATC CCA CGA ACC TG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
qP1R	Reverse	1180–1201	CGC CTT CCT CCA GCT TGA CGC	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
qP2F	Forward	1169–1189	GGG GAA CTG CCA GCG TCA AG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)
qP2R	Reverse	1440–1460	CTC AGC GAC TTC GAG TAC AG	*M. oxyfera* 16S rDNA	Ettwig et al. (2009)

denitrifying AOM microbial consortium, Ettwig et al. (2009) developed a series of 16S ribosomal RNA (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, c). 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 steps 1 and 2 was based on the annealing gradient with temperatures of 50–60 or 53–63 °C for different samples (Luesken et al. 2011c), intending to minimize the effects of random polymerase errors and primer mismatches. The

Table 3 A summary of *pmoA* gene-based PCR primers specific for n-damo bacteria currently used

primer	Forward/reverse	Position	Sequence (5′–3′)	GC%	Tm (°C)	Test 1	Test 2	Test 3	Test 4	References
A189_b	Forward	121–138	GGN GAC TGG GAC TT**Y** TGG	55.6	64.5–69.4	+*	+	+	+	Luesken et al. (2011c)
cmo682	Reverse	637–654	AAA **Y**CC GGC **R**AA GAA CGA	44.4	61–65.8	–	–	+	+	Luesken et al. (2011c)
cmo182	Forward	167–184	TCA CGT TGA CGC CGA TCC	61.1	66.5	–	+	+	+	Luesken et al. (2011c)
cmo568	Reverse	537–556	GCA CAT AC***T*** CCA TCC CCA TC	55.0	63.9	–	–	+	+	Luesken et al. (2011c)
NA638Rdeg	Reverse	593–612	RAA TGT TCG RAG CGT VCC BC	45.0	59.5–70.0	+	+	+	+	Deutzmann and Schink (2011)
NA720R	Reverse	675–694	TCC CCA TCC ACA CCC ACC AG	65.0	69.6	–	–	+	+	Deutzmann and Schink (2011)
682_NC10	Reverse	637-654	AAA **T**CC GGC **G**AA GAA CGA	50.0	63.0	–	–	+	+	Kojima et al. (2012)
HP3F1	Forward	209–233	CCC AGT ACT TCA TGT GGG ARA ARA T	40.0	59.3–61.8	+	+	+	+	Han and Gu (2013)
HP3R1	Reverse	463–484	GGG GGC CAG CCA NRY CCA RTT	57.1	68.1–78.0	+	+	+	+	Han and Gu (2013)

*Test 1: Hairpins; Test 2: Self complementarity; Test 3: False priming in target group (FP565575 *M. oxyfera*); Test 4 False priming in excluded group (DQ119048 *Methylosinus sporium*); "+" means observed while "–" means not;

A189f (general *pmoA* primer; 5′-3′): GGN GAC TGG GAC TTC** TGG (Holmes et al. 1995); key differences within the same priming site are highlighted in bold

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, d; 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 italics (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

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

Unique fatty acid

New biomarkers were investigated by detecting the lipid composition of *M. oxyfera* in several enrichment cultures, where up to 46 % of the detected lipid profile was 10-methylhexadecanoic acid ($10MeC_{16:0}$) (Kool et al. 2012). Kool et al. (2012) also identified a unique fatty acid of monounsaturated 10-methylhexadecanoic acid with a double bond at the Δ7 position ($10MeC_{16:1\Delta7}$) comprised up to 10 % of the total fatty acid measured in multiple n-damo enrichments, which had not been reported previously. These branched fatty acids of $10MeC_{16:0}$ and $10MeC_{16:1\Delta7}$ were proposed to be important and characteristic chemical signatures of Ca. *M. oxyfera* and may serve as the biomarkers for detecting them from the environment (Kool et al. 2012). However, up to now, there is no other publication of n-damo bacteria with application of this method. The possible reasons are (1) although as a major chemical component, $10MeC_{16:0}$ is not only found in *M. oxyfera*, but also presents in other sulfate-reducing bacteria (i.e., *Desulfobacter*), actinobacteria, anammox bacteria, iron-reducing *Geobacter*, *Marinobacter*, and the marine denitrifier *Pseudomonas nautica* (Buhring et al. 2005; Doumenq et al. 1999; Londry et al. 2004; Rütters et al. 2002; Sinninghe Damste et al. 2005; Sittig and Schlesner 1993; Yoon et al. 2007; Zhang et al. 2003), which means positive signal of this biomarker in anaerobic methane oxidation enrichment cannot exclude either sulfate or nitrite-driven pathways, and the diagnosis could be more 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} day^{-1}) and measured by microsenor (31–437 μmol CH_4 m^{-2} day^{-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 types 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 5 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 plow 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 to 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.

Table 4 A summary and analysis of n-damo bacteria from different habitats based on *pmoA* gene sequences

Source/environment	N-damo related clone	OTU	Shannon-Wiener	Chao1	Type	Accession number in GenBank	Reference**
CF5S	21	2	0.6365	1	M/R	KJ023443, KJ023450	Chen et al. (2014)
CF5B	4	1	0	1	M/R	KJ023444	Chen et al. (2014)
E201S	8	2	0.3768	1	M/R	KJ023445-KJ023446	Chen et al. (2014)
E407S	6	2	0.4506	1	M/R	KJ023447-KJ023449	Chen et al. (2014)
E407B	24	2	0.3768	1	M/R	KJ023451, KJ023455	Chen et al. (2014)
E510S	16	2	0.2338	1	M/R	KJ023452, KJ023456	Chen et al. (2014)
E525S	24	1	0	1	M/R	KJ023453	Chen et al. (2014)
E525B	1	1	–	–	M/R	KJ023454	Chen et al. (2014)
SCS E704S	24	3	1.0055	1	M	KF528962-KF528968 KF528973-KF528984 KF742451-KF742455	Chen et al. (2015a)
SCS E401	13	3	0.9110	1	M	KF528961 KF528969-KF528972 KF742444-KF742450	Chen et al. (2015a)
Lake Constance	15	6	1.4878	10.5	F	HQ906565-HQ906579	Deutzmann and Schink (2011)
Lake Biwa	21	1	0	1	F	AB661605-AB661625	Kojima et al. (2012)
Reed bed	12	1	0	1	F	JX898463-JX898474	Han and Gu (2013)
Reservoir	9	7	1.8892	13.2500	F	JX898486-JX898494	Han and Gu (2013)
WWTP^	10	7	1.8344	19.5	E	JX898495-JX898504	Han and Gu (2013)
Paddy soil	11	2	0.3046	1	F	JX898475-JX898485	Han and Gu (2013)
Paddy soil	14	1	0	1	F	JN704402-JN704415	Wang et al. (2012)
Paddy field	20	2	0.1985	1	F	KC341611-KC341630	Zhu et al. (2015)
Jingshan paddy field	27	1	0	1	F	KC905857-KC905883	Hu et al. (2014)
Xixi wetland	21	3	0.3805	1	F	KC905884-KC905904	Hu et al. (2014)
XZ wetland	4	1	0	1	F	KC905905-KC905908	Hu et al. (2014)
FreshwaterWetlands (Jingshan Paddy; Xixi wetland; XZ wetland)	52	5	1.0575	1	F	KC905857-KC905908	Hu et al. (2014)
Baiyangdian Lake	24	1	0	1	F	KC341248-KC341271	Zhu et al. (2015)
Bosten Lake	16	2	0.4826	1	F	KC341301-KC341316	Zhu et al. (2015)
Chaohu Lake	19	6	1.1513	1	F	KC341317-KC341335	Zhu et al. (2015)
Dongting Lake	21	2	0.6920	1	F	KC341336-KC341356	Zhu et al. (2015)
Jiaxing C. wetland-winter	13	8	1.8393	26	F	KC341375-KC341387	Zhu et al. (2015)
Jiaxing constructed wetland-summer	11	8	2.0198	12.1667	F	KC341388-KC341398	Zhu et al. (2015)
Subsurface North canal	18	2	0.6870	1	U	KC341272-KC341289	Zhu et al. (2015)
North canal-12 m	3	1	0	1	U	KC341298-KC341300	Zhu et al. (2015)
North canal-15 m	8	1	0	1	U	KC341290-KC341297	Zhu et al. (2015)
Panjin swamp	21	1	0	1	F	KC341437-KC341457	Zhu et al. (2015)
Peat land China	23	6	1.3944	8	F	KC341414-KC341436	Zhu et al. (2015)
Poyang Lake	21	2	0.5983	1	F	KC341458-KC341478	Zhu et al. (2015)
Shahe River	18	5	1.2094	1	F	KC341479-KC341496	Zhu et al. (2015)
Songhuajiang River	18	7	1.6715	9.25	F	KC341497-KC341514	Zhu et al. (2015)
Tarim River	19	2	0.6918	1	F	KC341515-KC341533	Zhu et al. (2015)
Tiaoxi River	20	3	0.9973	1	F	KC341534-KC341553	Zhu et al. (2015)
Tibetan Lake	10	1	0	1	S/R	JQ429431-JQ429432	Yang et al. (2012)
Tulufan River	18	3	0.8487	3	F	KC341357-KC341374	Zhu et al. (2015)
Wuliangshuhai Lake	24	4	0.9366	4.5	F	KC341554-KC341577	Zhu et al. (2015)
Yellow River	15	1	0	1	F	KC341399-KC341413	Zhu et al. (2015)
Yuanmingyuan Lake	12	3	0.7215	3.5	F	KC341698-KC341709	Zhu et al. (2015)
Pearl River-winter	20	5	1.0098	6	F	KC341578-KC341597	Zhu et al. (2015)
Pearl River-summer	13	2	0.6172	1	F	KC341598-KC341610	Zhu et al. (2015)

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 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 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 groups 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

Table 4 (continued)

Source/environment	N-damo related clone	OTU	Shannon-Wiener	Chao1	Type	Accession number in GenBank	Reference**
Donghu Lake	15	3	0.8033	1	F	KC341631-KC341645	Zhu et al. (2015)
Honghaitan Tidal Land	17	4	1.1151	4.5	C	KC341646-KC341662	Zhu et al. (2015)
Shangqiu Reservoir	17	7	1.6100	8.5	F	KC341663-KC341679	Zhu et al. (2015)
Tianchi Lake	18	9	1.8121	18	F	KC341680-KC341697	Zhu et al. (2015)
WWTP^	128	3	0.8487	1	U	KC700842-KC700715	Gao et al. (2013)
Soils^	40	3	0.3813	1	U	KC700675-KC700714	Gao et al. (2013)
Qiantang River	50	16	2.1437	29.5	F	KC503613-KC503662	Shen et al. (2014)
West Lake	24	8	1.5359	12	F	JX531974-JX531997	Zhu et al. (2013)
Jiaojiang estuary	80	20	2.3787	30.1250	C	KC512302-KC512381	Shen et al. (2014c)
Freshwater habitats (Alpine peat bog WWTPs^ Drainage ditch Contaminated aquifer^)	12	9	NA	NA	F/R	HQ698926-HQ698937	Luesken et al. (2011c)
WWTPs^	19	10	NA	NA	F/R	JF706214-JF706196	Luesken et al. (2011b)
SBR co-enrichment with anammox^	7	3	NA	NA	E/R	JN006731-JN006737	Luesken et al. (2011a)
Sewage treatment plants^	12	8	NA	NA	E/R	KC112371-KC112382	Ho et al. (2013)
Minerotrophic peatland^	3	3	NA	NA	E/R	JX262153-JX262155	Zhu et al. (2012)
Lab-scale reactor^	13	3	1.0579	1	E/?	AB767281-AB767293	Masashi et al. (2013)

NA not applicable, *M* marine, *F* freshwater, *S* saline lake, *C* coastal, *U* unknown, *E* enrichment, *R* representative sequences available in the GenBank database only;

**Unpublished reference indicates that the related DNA sequences have already been released in the database without a published journal paper until 17 April 2014 when the information was accessed; OTU, Shannon-Wiener and Chao1 were recalculated using Fastgroup II (Yu et al. 2006) with the cutoff of 5 %

^Isolation sources/environments are not sediment

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. 2014, 2015a). The reconstructed phylogeny using amplified 16S rDNA sequences showed that none of the SCS sequences belonged to group a where the *M. oxyfera* 16S rDNA gene clustered within, but the majority of them grouped into clade e (Chen et al. 2014, 2015a). 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. 2014, 2015a). The gene copy numbers of n-damo bacterial 16S rRNA gene in SCS sediments ranged from 1.6×10^5 to 1.4×10^8 gene copies per gram dry sediment (Jing Chen and Ji-Dong Gu, unpublished data). NO_x^- significantly and positively correlated with 16S rDNA Group a and *pmoA* gene Cluster SCS-2, when NH_4^+ showed a directly adverse effect on the community structure based on either recovered 16S rRNA or *pmoA* genes-amplified sequences (Chen et al. 2014). This information of n-damo in SCS also showed similar information of anammox as previously observed in that unique species of anammox Ca. *Scalindua zhenhei* I, II, and III were discovered (Hong et al. 2011). Nitrate/nitrite-driven AOM was questioned for their roles in marine habitats (Orcutt et al. 2011). Molecular evidences on existence of n-damo

bacteria in coastal and marine environments now suggest the potential role of n-damo process in coastal and deep-sea sediments (Chen et al. 2014, 2015a, 2015b; Shen et al. 2014c; Zhu et al. 2015).

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

The amplified *pmoA* gene sequences of *M. oxyfera*-like bacteria were retrieved from GenBank database and summarized in Table 4. Fastgroup II (Yu et al. 2006) was applied to calculate the OTU, Shannon-Wiener, and Chao1 based on percentage sequence identity of 95 % *pmoA* gene sequences. Jiaojiang Estuary (Shen et al. 2014c), Qiantang River (Shen et al. 2014b), and Jiaxing constructed wetland (Zhu et al. 2015) had much higher Shannon-Wiener and Chao1 indexes compared with other samples. Lake Biwa, reed bed, Yellow River, Panjin swamp, two paddy fields, and XZ wetland showed very limited alpha diversity of n-damo bacterial community (Han and Gu 2013; Hu et al. 2014; Kojima et al. 2012; Wang et al. 2012; 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 pure culture of n-damo bacteria from either the shallow or deep-sea sediment sources with salinity of up to 34–36 ‰. For further understanding the ecophysiology, biochemistry, 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 essential basis for further research from characterization, phylogenetics, ecophysiology, and biochemistry to gene expression and evolutionary analysis to allow better understanding 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 limited information 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. 2014, 2015a), but, the low coverage of the PCR primer sets available is a serious limiting step to allow wider coverage and recovery of them from the environmental samples (Chen et al. 2015a). It is urgent to develop new specific PCR primers that can be applied efficiently to marine samples with high efficiency and specificity. On the other hand, the biochemical processes of n-damo bacteria involve two significant biochemical pathways of aerobic methane oxidation and denitrification under anaerobic condition. As far as is 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 to expand the scope of PCR primer design.

Successful enrichment of n-damo microorganisms from other ecosystems, including marine sediments, will pave the way for genome sequencing of the specific bacteria involved. The sequences can be used in the 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 linking to denitrification is largely overlooked in marine environments. The current investigations of anaerobic methane oxidation in coastal and ocean sediments focus more on sulfate than nitrite reduction as the electron acceptor. The contribution of n-damo process has not been reported in coastal or ocean ecosystems except the PCR amplification (Chen et al. 2014). 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 quantify their contribution to 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. 2014, 2015a, 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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Compliance with ethical standards

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

References

Buhring SI, Elvert M, Witte U (2005) The microbial community structure of different permeable sandy sediments characterized by the investigation of bacterial fatty acids and fluorescence in situ hybridization. Environ Microbiol 7:281–293

Chen J, Zhou ZC, Gu J-D (2014) Occurrence and diversity of nitrite-dependent anaerobic methane oxidation bacteria in the sediments of the South China Sea revealed by amplification of both 16S rRNA and pmoA genes. Appl Microbiol Biotechnol 98:5685–5696

Chen J, Jiang XW, Gu J-D (2015a) Existence of novel phylotypes of nitrite-dependent anaerobic methane-oxidizing bacteria in surface and subsurface sediments of the South China Sea. Geomicrobiol J 32:1–9

Chen J, Zhou ZC, Gu J-D (2015b) Complex community of nitrite-dependent anaerobic methane oxidation bacteria in coastal sediments of the Mai Po wetland by PCR amplification of both 16S rRNA and pmoA genes. Appl Microbiol Biotechnol 99:1463–1473

Deutzmann JS, Schink B (2011) Anaerobic oxidation of methane in sediments of Lake Constance, an oligotrophic freshwater Lake. Appl Environ Microbiol 77:4429–4436

Deutzmann JS, Stief P, Brandes J, Schink B (2014) Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake. Proc Natl Acad Sci U S A 111:18273–18278

Ding J, Fu L, Ding ZW, Lu YZ, Cheng SH, Zeng RJ (2016) Environmental evaluation of coexistence of denitrifying anaerobic methane-oxidizing archaea and bacteria in a paddy field. Appl Microbiol Biotechnol 100:439–446

Doumenq P, Acquaviva M, Asia L, Durbec JP, Le Dréau Y, Mille G, Bertrand JC (1999) Changes in fatty acids of *Pseudomonas nautica*, a marine denitrifying bacterium, in response to n-eicosane as carbon source and various culture conditions. FEMS Microbiol Ecol 28: 151–161

Ettwig KF, Shima S, van de Pas-Schoonen KT, Kahnt J, Medema MH, op den Camp HJM, Jetten MSM, Strous M (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of archaea. Environ Microbiol 10:3164–3173

Ettwig KF, van Alen T, van de Pas-Schoonen KT, Jetten MSM, Strous M (2009) Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl Environ Microbiol 75:3656–3662

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

Ettwig KF, Speth DR, Reimann J, Wu ML, Jetten MS, Keltjens JT (2012a) Bacterial oxygen production in the dark. Front Microbiol 3:273

Ettwig KF, Speth DR, Reimann J, Wu ML, Jetten MSM, Keltjens JT (2012b) Bacterial oxygen production in the dark. BBA-Bioenergetics 1817:S155–S155

Gao J, Luo X, Wu G, Peng Y (2013) Occurrence of nitrite-dependent anaerobic methane oxidizing bacteria from different soil and wastewater sludge. NCBI Genbank web http://www.ncbinlmnihgov/nuccore/ Accessed 17 April 2014

Han P, Gu J-D (2013) A newly designed degenerate PCR primer based on pmoA gene for detection of nitrite-dependent anaerobic methane-oxidizing bacteria from different ecological niches. Appl Microbiol Biotechnol 97:10155–10162

Han P, Gu J-D (2015) Further analysis of anammox bacterial community structures along an anthropogenic nitrogen-input gradient from the riparian sediments of the Pearl River Delta to the deep-ocean sediments of the South China Sea. Geomicrobiolgy. Journal 32:789–798

Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan Z, Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500:567–570

He Z, Geng S, Cai C, Liu S, Liu Y, Pan Y, Lou L, Zheng P, Xu X, Hu B (2015a) Anaerobic oxidation of methane coupled to nitrite reduction by halophilic marine NC10 bacteria. Appl Environ Microbiol 81: 5538–5545

He ZF, Geng S, Shen LD, Lou LP, Zheng P, Xu XH, Hu BL (2015b) The short- and long-term effects of environmental conditions on anaerobic methane oxidation coupled to nitrite reduction. Water Res 68: 554–562

Hill AR (1979) Denitrification in the nitrogen budget of a river ecosystem. Nature 281:291–292

Ho A, Vlaeminck SE, Ettwig KF, Schneider B, Frenzel P, Boon N (2013) Revisiting methanotrophic communities in sewage treatment plants. Appl Environ Microbiol 79:2841–2846

Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett 132:203–208

Hong YG, Li M, Cao HL, Gu J-D (2011) Residence of habitat-specific anammox bacteria in the deep-sea subsurface sediments of the South China Sea: analyses of marker Gene abundance with physical chemical parameters. Microb Ecol 62:36–47

Hu SH, Zeng RJ, Burow LC, Lant P, Keller J, Yuan ZG (2009) Enrichment of denitrifying anaerobic methane oxidizing microorganisms. Env Microbiol Rep 1:377–384

Hu SH, Zeng RJ, Keller J, Lant PA, Yuan ZG (2011) Effect of nitrate and nitrite on the selection of microorganisms in the denitrifying anaerobic methane oxidation process. Env Microbiol Rep 3:315–319

Hu BL, Shen LD, Lian X, Zhu Q, Liu S, Huang Q, He ZF, Geng S, Cheng DQ, Lou LP, Xu XY, Zheng P, He YF (2014) Evidence for nitrite-dependent anaerobic methane oxidation as a previously overlooked microbial methane sink in wetlands. Proc Natl Acad Sci U S A 111: 4495–4500

Jarman SN (2004) Amplicon: software for designing PCR primers on aligned DNA sequences. Bioinformatics 20:1644–1645

Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pommerening-Roser A, Koops HP, Wagner M (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microbiol 64:3042–3051

Keeney DR, Chen RL, Graetz DA (1971) Importance of denitrification and nitrate reduction in sediments to the nitrogen budgets of lakes. Nature 233:66–67

Kits KD, Klotz MG, Stein LY (2015) Methane oxidation coupled to nitrate reduction under hypoxia by the Gammaproteobacterium *Methylomonas denitrificans*, sp. nov. type strain FJG1. Environ Microbiol 17:3219–3232

Knowles R (2005) Denitrifiers associated with methanotrophs and their potential impact on the nitrogen cycle. Ecol Eng 24:441–446

Kojima H, Tsutsumi M, Ishikawa K, Iwata T, Mussmann M, Fukui M (2012) Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa. Syst Appl Microbiol 35:233–238

Kojima H, Tokizawa R, Kogure K, Kobayashi Y, Itoh M, Shiah FK, Okuda N, Fukui M (2014) Community structure of planktonic methane-oxidizing bacteria in a subtropical reservoir characterized by dominance of phylotype closely related to nitrite reducer. Scientific Reports 4:5728

Kool DM, Zhu BL, Rijpstra WIC, Jetten MSM, Ettwig KF, Damste JSS (2012) Rare branched fatty acids characterize the lipid composition of the intra-aerobic methane oxidizer "*Candidatus* Methylomirabilis oxyfera". Appl Environ Microbiol 78:8650–8656

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp. 115–175

Li M, Cao HL, Hong YG, Gu J-D (2011) Seasonal dynamics of anammox bacteria in estuarial sediment of the Mai Po nature reserve revealed by analyzing the 16S rRNA and hydrazine oxidoreductase (hzo) genes. Microbes Environ 26:15–22

Liu Y, Zhang J, Zhao L, Li Y, Yang Y, Xie S (2015) Aerobic and nitrite-dependent methane-oxidizing microorganisms in sediments of freshwater lakes on the Yunnan plateau. Appl Microbiol Biotechnol 99:2371–2381

Londry KL, Jahnke LL, Des Marais DJ (2004) Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. Appl Environ Microbiol 70:745–751

Lu YZ, Ding ZW, Ding J, Fu L, Zeng RJ (2015) Design and evaluation of universal 16S rRNA gene primers for high-throughput sequencing to simultaneously detect DAMO microbes and anammox bacteria. Water Res 87:385–394

Luesken FA, Sanchez J, van Alen TA, Sanabria J, Op den Camp HJM, Jetten MSM, Kartal B (2011a) Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation processes. Appl Environ Microbiol 77:6802–6807

Luesken FA, van Alen TA, van der Biezen E, Frijters C, Toonen G, Kampman C, Hendrickx TL, Zeeman G, Temmink H, Strous M, Op den Camp HJ, Jetten MS (2011b) Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. Appl Microbiol Biotechnol 92:845–854

Luesken FA, Zhu BL, van Alen TA, Butler MK, Diaz MR, Song B, den Camp HJMO, Jetten MSM, Ettwig KF (2011c) pmoA primers for detection of anaerobic methanotrophs. Appl Environ Microbiol 77: 3877–3880

Luesken FA, Wu ML, Op den Camp HJ, Keltjens JT, Stunnenberg H, Francoijs KJ, Strous M, Jetten MS (2012) Effect of oxygen on the anaerobic methanotroph '*Candidatus* Methylomirabilis oxyfera': kinetic and transcriptional analysis. Environ Microbiol 14:1024–1034

Masashi, H, Masafumi, K, Masato, K, Takashi, Y (2013) Enrichment of denitrifying anaerobic methane oxidizing microorganisms using nitrate or nitrite as electron acceptor. NCBI Genbank web http://wwwncbinlmnihgov/nuccore/ Accessed 17 April 2014

Mascarelli AL (2010) Methane-eating microbes make their own oxygen. Nature. doi:10.1038/news.2010.1146

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

McGarity JW (1961) Denitrification studies on some south Australian soils. Plant Soil 14:1–21

Meng H, Wang Y-F, Chan HW, Wu R-N, Gu J-D (2016) Co-occurrence of nitrite-dependent anaerobic ammonium and methane oxidation processes in subtropical acidic forest soils. Appl Microbiol Biotechnol. doi:10.1007/s00253-016-7585-6

Orcutt BN, Sylvan JB, Knab NJ, Edwards KJ (2011) Microbial ecology of the dark ocean above, at, and below the seafloor. Microbiol Mol Biol Rev 75:361–422

Oremland RS (2010) Biogeochemistry: NO connection with methane. Nature 464:500–501

Raghoebarsing AA, Smolders AJ, Schmid MC, Rijpstra WI, Wolters-Arts M, Derksen J, Jetten MS, Schouten S, Sinninghe Damste JS, Lamers LP, Roelofs JG, Op den Camp HJ, Strous M (2005) Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436:1153–1156

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

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

Shen LD, Liu S, Huang Q, Lian X, He ZF, Geng S, Jin RC, He YF, Lou LP, Xu XY, Zheng P, Hu BL (2014a) Evidence for the cooccurrence of nitrite-dependent anaerobic ammonium and methane oxidation

processes in a flooded paddy field. Appl Environ Microbiol 80: 7611–7619

Shen LD, Liu S, Zhu Q, Li XY, Cai C, Cheng DQ, Lou LP, Xu XY, Zheng P, Hu BL (2014b) Distribution and diversity of nitrite-dependent anaerobic methane-oxidising bacteria in the sediments of the Qiantang River. Microb Ecol 67:341–349

Shen LD, Zhu Q, Liu S, Du P, Zeng JN, Cheng DQ, Xu XY, Zheng P, Hu BL (2014c) Molecular evidence for nitrite-dependent anaerobic methane-oxidising bacteria in the Jiaojiang estuary of the East Sea (China). Appl Microbiol Biotechnol 98:5029–5038

Shen L, Liu S, He Z, Lian X, Huang Q, He Y, Lou L, Xu X, Zheng P, Hu B (2015a) Depth-specific distribution and importance of nitrite-dependent anaerobic ammonium and methane-oxidising bacteria in an urban wetland. Soil Bio Biochem 83:43–51

Shen LD, He ZF, Wu HS, Gao ZQ (2015b) Nitrite-dependent anaerobic methane-oxidising bacteria: unique microorganisms with special properties. Curr Microbiol 70:562–570

Shen LD, Huang Q, He ZF, Lian X, Liu S, He YF, Lou LP, Xu XY, Zheng P, Hu BL (2015c) Vertical distribution of nitrite-dependent anaerobic methane-oxidising bacteria in natural freshwater wetland soils. Appl Microbiol Biotechnol 99:349–357

Shen LD, Wu HS, Gao ZQ (2015d) Distribution and environmental significance of nitrite-dependent anaerobic methane-oxidising bacteria in natural ecosystems. Appl Microbiol Biotechnol 99:133–142

Shen LD, Wu HS, Gao ZQ, Li J, Liu X (2016) Presence of diverse *Candidatus* Methylomirabilis oxyfera-like bacteria of NC10 phylum in agricultural soils. J Appl Microbiol. doi:10.1111/jam.13119

Simon J, Klotz MG (2013) Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. Biochimica Biophysica Acta 1827:114–135

Sinninghe Damste JS, Rijpstra WI, Geenevasen JA, Strous M, Jetten MS (2005) Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox. FEBS J 272:4270–4283

Sittig M, Schlesner H (1993) Chemotaxonomic investigation of various prosthecate and/or budding bacteria. Syst Appl Microbiol 16:92–103

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

van der Star WRL, Abma WR, Blommers D, Mulder JW, Tokutomi T, Strous M, Picioreanu C, Van Loosdrecht MCM (2007) Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. Water Res 41:4149–4163

Wang Y, Zhu G, Harhangi HR, Zhu B, Jetten MS, Yin C, Op den Camp HJ (2012) Co-occurrence and distribution of nitrite-dependent anaerobic ammonium and methane oxidizing bacteria in a paddy soil. FEMS Microbiol Lett 336:79–88

Wang Y, Huang P, Ye F, Jiang Y, Song L, Op den Camp HJ, Zhu G, Wu S (2016) Nitrite-dependent anaerobic methane oxidizing bacteria along the water level fluctuation zone of the three gorges reservoir. Appl Microbiol Biotechnol 100:1977–1986

Wu ML, Ettwig KF, Jetten MS, Strous M, Keltjens JT, van Niftrik L (2011) A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium *Candidatus* 'Methylomirabilis oxyfera'. Biochem Soc Trans 39:243–248

Yan P, Li M, Wei G, Li H, Gao Z (2015) Molecular fingerprint and dominant environmental factors of nitrite-dependent anaerobic methane oxidation bacteria in sediments from the Yellow River estuary, China. PLoS One 10:e0137996

Yang J, Jiang HC, Wu G, Hou WG, Sun YJ, Lai ZP, Dong HL (2012) Co-occurrence of nitrite-dependent anaerobic methane oxidizing and anaerobic ammonia oxidizing bacteria in two Qinghai-Tibetan saline lakes. Front Earth Sci 6:383–391

Yoon JH, Lee MH, Kang SJ, Oh TK (2007) *Marinobacter salicampi* sp. nov., isolated from a marine solar saltern in Korea. Int J Syst Evol Microbiol 57:2102–2105

Yu YN, Breitbart M, McNairnie P, Rohwer F (2006) FastGroupII: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics 7:57

Zhang CL, Li Y, Ye Q, Fong J, Peacock AD, Blunt E, Fang J, Lovely DR, White DC (2003) Carbon isotope signatures of fatty acids in *Geobacter metallireducens* and *Shewanella algae*. Chem Geol 195:17–28

Zhou L, Wang Y, Long X, Guo J, Zhu G (2014) High abundance and diversity of nitrite-dependent anaerobic methane-oxidizing bacteria in a paddy field profile. FEMS Microbiol Lett 360:33–41

Zhu G, Jetten MS, Kuschk P, Ettwig KF, Yin C (2010) Potential roles of anaerobic ammonium and methane oxidation in the nitrogen cycle of wetland ecosystems. Appl Microbiol Biotechnol 86:1043–1055

Zhu B, Sanchez J, van Alen TA, Sanabria J, Jetten MS, Ettwig KF, Kartal B (2011) Combined anaerobic ammonium and methane oxidation for nitrogen and methane removal. Biochem Soc Trans 39:1822–1825

Zhu BL, van Dijk G, Fritz C, Smolders AJP, Pol A, Jetten MSM, Ettwig KF (2012) Anaerobic oxidization of methane in a Minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria. Appl Environ Microbiol 78:8657–8665

Zhu Q, Shen LD, Hu BL, Lou LP, Cheng DQ (2013) Molecular detection of denitrifying anaerobic oxidizing bacteria in the sediment of West Lake, Hangzhou. Acta Sci Circumst 33:1321–1325

Zhu G, Zhou L, Wang Y, Wang S, Guo J, Long XE, Sun X, Jiang B, Hou Q, Jetten MS, Yin C (2015) Biogeographical distribution of denitrifying anaerobic methane oxidizing bacteria in Chinese wetland ecosystems. Environ Microbiol Rep 7:128–138