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ORIGINAL ARTICLE

Spatial change of reservoir nitrite-dependent methane-oxidizing microorganisms

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Abstract Nitrite-dependent anaerobic methane oxidation (n-damo), catalyzed by microorganisms affiliated with bacterial phylum NC10, can have an important contribution to the reduction of the methane emission from anoxic freshwater sediment to the atmosphere. However, information on the variation of sediment n-damo organisms in reservoirs is still lacking. The present study monitored the spatial change of sediment n-damo organisms in the oligotrophic freshwater Xinfengjiang Reservoir (South China). Sediment samples were obtained from six different sampling locations and two sediment depths (0–5 cm, 5–10 cm). Sediment n-damo bacterial abundance was found to vary with sampling location and layer depth, which was likely influenced by pH and nitrogen level. The presence of the n-damo *pmoA* gene was found in all these samples. A remarkable shift occurred in the diversity and composition of sediment n-damo *pmoA* gene sequences. A variety of distinctively different n-damo *pmoA* clusters existed in reservoir sediments. The *pmoA* sequences affiliated with *Candidatus Methylomirabilis oxyfera* formed the largest group, while a significant proportion of the obtained n-damo *pmoA* gene sequences showed no close relationship to those from any known NC10 species. In addition, the present n-damo process was found in reservoir sediment, which could be enhanced by nitrite nitrogen amendment.

Keywords Freshwater · Methane oxidation · Reservoir · Sediment

Introduction

Microbial communities in aquatic sediments can be involved in a variety of biogeochemical processes (Yang et al. 2015; Zhang et al. 2015). Methanogenesis in sediment ecosystems contributes a significant part of the total global emission of methane (CH_4), a greenhouse gas of critical significance to global climate change (Chaudhary et al. 2013). Aerobic methanotrophs are usually believed to play a crucial role in mitigating the methane emission from anoxic sediments to the atmosphere (Rahalkar et al. 2009; Lopes et al. 2011; Tsutsumi et al. 2012). However, the recent discovery of nitrite-dependent anaerobic methane oxidation (n-damo), biologically catalyzed by microorganisms within the bacterial phylum NC10 (Ettwig et al. 2009, 2010), suggests that the n-damo process might be another important sink of methane in natural aquatic environments (Deutzmann et al. 2014). So far, previous studies have investigated the distribution of n-damo bacteria in a number of sediment ecosystems, such as lake sediment (Deutzmann and Schink 2011; Kojima et al. 2012; Deutzmann et al. 2014; Liu et al. 2015), river sediment (Shen et al. 2014a), river estuary sediment (Yan et al. 2015), sea estuary sediment (Shen et al. 2014b), coastal wetland sediment (Chen et al. 2014), and marine sediment (Chen et al. 2015). However, the environmental factors regulating the distribution of sediment n-damo bacteria remain poorly known.

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Several previous studies suggested that the freshwater sediment n-damo community might be influenced by a number of environmental factors, such as ammonium nitrogen, nitrite nitrogen, total inorganic nitrogen, organic matter, and the ratio of organic matter to total nitrogen (Shen et al. 2014a; Liu et al. 2015; Long et al. 2016). Moreover, sediment is a stratified habitat, which can provide niches for metabolically diverse microorganisms (Zhao et al. 2008). Although the depth-related change of the sediment microbial community structure has been well documented (Shivaji et al. 2011; Liu et al. 2014; Pagès et al. 2015; Lu et al. 2016), the difference in n-damo bacteria among different sediment depths remains unclear.

The Xinfengjiang Reservoir, located in the subtropical climate zone, is the largest drinking water reservoir in Guangdong Province (South China), and the fourth largest drinking water reservoir in China. At full water level, this oligotrophic freshwater reservoir stores a volume of 13.9 billion m^3. The Xinfengjiang Reservoir covers 364 km^2, with a total catchment area of 5730 km^2, and its average water depth is 28.7 m (with a maximum water depth of 93 m) (Hu et al. 2008). The aim of this current study was to investigate the distribution of sediment n-damo bacteria in the Xinfengjiang Reservoir at different sampling locations and sediment depths. The possible environmental factors influencing sediment n-damo bacteria was also explored.

Materials and methods

Study locations and sampling

Sediment cores in triplicate at six sampling locations (A–F) in the Xinfengjiang Reservoir were collected using self-made stainless steel columnar sediment samplers (patent number ZL201420490790.1) in July 2015 (Figure S1). These sediment cores were immediately transported back to the laboratory after collection and were then sliced into layers. In the present study, the upper layer (0–5 cm) and the lower layer (5–10 cm) were used for further chemical and molecular analyses. Sediment samples UA and LA (23°50′21.00″ N, 114°36′23.00″ E), UB and LB (23°48′34.00″ N, E114°34′17.00″ E), UC and LC (23°45′36.00″ N, E114°30′7.00″ E), UD and LD (23°55′34.00″ N, 114°33′19.00″ E), UE and LE (23°54′3.00″ N, 114°28′21.00″ E), and UF and LF (23°51′5.00″ N, 114°31′23.00″ E) were referred to as the upper layer and lower layer sediments in sampling locations A–F, respectively. Sediment pH was determined using an IQ 150 pH meter. Sediment total organic carbon (TOC) and total phosphorus (TP) were analyzed using the potassium dichromate titration method and the molybdenum blue colorimetry method, respectively (Wang 2012). The levels of sediment ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), and total nitrogen (TN) were measured using Nessler's reagent method, the naphthalene ethylenediamine spectrophotometry method, phenoldisulfonic acid colorimetric method, and the Kjeldahl method, respectively (Wang 2012). The chemical parameters of these reservoir sediment samples are shown in Table 1. The levels of sediment pH, TOC, NH_4^+-N, NO_3^--N, NO_2^--N, TN, and the ratio of TOC to TN (C/N) were 5.12–5.85, 31.18–327.88 g/kg, 41.26–154.35 mg/kg, 0.33–3.01 mg/kg, 0.36–3.75 mg/kg, 69.63–643.26 mg/kg, and 343.1–899.1, respectively.

Quantitative PCR assay

Reservoir sediment DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The DNA concentration was quantified using

Table 1 Physicochemical features of reservoir sediment samples. Sediment samples UA and LA, UB and LB, UC and LC, UD and LD, UE and LE, and UF and LF refer to the upper layer and lower layer sediments in sampling locations A–F, respectively

Sample	pH	TOC (g/kg)	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	NO_2^--N (mg/kg)	TN (mg/kg)	C/N
UA	5.26 ± 0.02	5.11 ± 0.11	45.18 ± 1.76	0.91 ± 0.08	0.57 ± 0.03	69.63 ± 1.12	73.3 ± 1.2
UB	5.85 ± 0.02	19.38 ± 0.56	64.86 ± 2.31	0.58 ± 0.05	0.96 ± 0.08	305.27 ± 3.07	63.5 ± 1.5
UC	5.16 ± 0.01	16.5 ± 0.34	65.74 ± 1.45	2.75 ± 0.24	1.31 ± 0.05	270.87 ± 4.21	60.7 ± 2.1
UD	5.18 ± 0.01	21.4 ± 0.71	112.85 ± 2.22	1.6 ± 0.11	3.75 ± 0.16	511.18 ± 3.89	41.9 ± 1.6
UE	5.56 ± 0.01	32.79 ± 1.12	108.38 ± 1.98	2.69 ± 0.31	0.58 ± 0.04	366.59 ± 5.22	89.4 ± 1.3
UF	5.15 ± 0.01	31.78 ± 0.43	67.78 ± 1.34	0.87 ± 0.02	2.78 ± 0.13	643.26 ± 5.17	49.4 ± 2.3
LA	5.4 ± 0.02	3.12 ± 0.07	81.66 ± 0.98	2.91 ± 0.13	0.36 ± 0.01	90.89 ± 3.26	34.3 ± 1.2
LB	5.5 ± 0.01	28.09 ± 1.11	76.82 ± 2.15	0.33 ± 0.03	1.71 ± 0.07	427.41 ± 4.12	65.7 ± 2.2
LC	5.12 ± 0.02	14.53 ± 0.97	48.43 ± 2.11	2.48 ± 0.21	0.65 ± 0.02	273.53 ± 1.98	53.1 ± 3.1
LD	5.38 ± 0.01	28.83 ± 0.78	111.76 ± 2.08	2.43 ± 0.11	2.48 ± 0.12	497.1 ± 2.56	58 ± 1.4
LE	5.51 ± 0.01	28.64 ± 1.23	154.35 ± 1.76	1.03 ± 0.09	1.9 ± 0.12	463.99 ± 3.87	61.7 ± 2.1
LF	5.3 ± 0.01	25.39 ± 0.79	41.26 ± 1.87	3.01 ± 0.14	0.85 ± 0.03	282.42 ± 2.96	89.9 ± 1.8

NanoDrop® ND-1000 UV–vis spectrophotometry (USA). The abundance of the n-damo bacterial 16S rRNA gene was assessed using the primer set (qP2F/qP2R), according to the literature (Ettwig et al. 2009; Wang et al. 2012). Standard curves ranging from 10^3 to 10^9 gene copies/mL were obtained with serial dilutions of plasmid DNA containing the target gene. The amplification efficiency and coefficient (r^2) for n-damo bacterial 16S rRNA genes were 94% and 0.998, respectively. Negative control containing no template DNA was carried out following the same qPCR protocol to exclude any possible contamination.

Measurement of n-damo activity

In this study, the sediment sample with the highest n-damo bacterial abundance was used to perform the n-damo activity test using an incubation reactor (with a working volume of 1.0 L) (Figure S2). The sediment sample (300 g, dry weight) was added into the incubation reactor containing 450 mL nitrite solution (with 47.14 mg/L NO_2^--N) or deionized water (as control), and the incubation reactor was capped and sealed with glass cement. Before CH_4 (99.9% purity) injection, the headspace was flushed with argon gas (99.999% purity) for 15 min and subsequently evacuated. The initial volume content of CH_4 in the headspace was above 95%. The mixture in the reactor was magnetically stirred at 150 rpm at 25 °C. At each sampling date, 1 mL of gas was collected to measure the volume content of CH_4 and N_2, and a 3-mL liquid sample was sampled and centrifuged to measure nitrite level. The nitrite content in the liquid sample was measured according to the standard methods described by the Chinese Environmental Protection Agency (2002). The volume contents of CH_4 and N_2 were measured with a gas chromatographer (Fuli 9790, Fuli Analytical Instrument Co., Ltd., China) equipped with a PorparkQ packed column and a thermal conductivity detector. Argon gas was used as the carrier gas and its flow rate was 30 mL/min. The temperatures of the injector, oven, and detector were set at 50, 50. and 85 °C, respectively.

Clone library analysis

The n-damo *pmoA* gene was amplified using a nested approach (first-step primer pair A189_b/cmo682 and second-step primer pair cmo182/cmo568), as previously described (Wang et al. 2012). The PCR reactions were carried out as follows: 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1.5 min, followed by 72 °C for 10 min (Long et al. 2016). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen Inc.). The purified PCR products from triplicate sediment samples were pooled in equal amounts and then cloned into pMD19-T vector (Takara Corp, Japan). The clones containing the correct size were sequenced. The obtained valid n-damo *pmoA* gene sequences in this study were deposited in the GenBank database under accession numbers KT955749–KT955847, KU052366–KU052487, KU341779–KU341784, KU341792–KU341834, and KU605622–KU605628. The valid sequences were clustered into operational taxonomic units (OTUs) with a maximum distance of 7%, and OTU-based Chao1 richness estimator and Shannon and Simpson indices were then calculated using the mothur program (Schloss et al. 2009). Phylogenetic analysis of the retrieved reservoir sediment n-damo *pmoA* gene sequences was performed using the software MEGA 6.0 (Tamura et al. 2013), using the neighbor-joining method. Bootstrap analysis with 1000 replicates was applied to test the confidence levels. In addition, the similarity of the n-damo *pmoA* gene was determined based on the OTU-based Bray–Curtis similarity matrices. The relative abundance of each n-damo *pmoA* OTU equaled the ratio of the sequence number of each OTU to the total sequences of all OTUs in a given sample. Sample clustering was carried out with the unweighted pair group method with arithmetic mean (UPGMA) using the software PRIMER 5.0 (Clarke and Warwick 2001).

Statistical analysis

In this study, one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was applied to determine the significant difference ($P < 0.05$) in the number of n-damo bacterial 16S rRNA genes among reservoir sediment samples. Spearman's rank correlation analysis was used to discriminate the links between n-damo organisms and reservoir sediment physicochemical properties using the software SPSS 20.0. Moreover, redundancy analysis (RDA) with the software Canoco 4.5 was also applied to identify the relationship between n-damo *pmoA* gene composition and environmental factors. The proportion of n-damo *pmoA* gene sequences in each OTU was used as the species input, while the reservoir sediment physicochemical properties were assigned as the input for environmental variables. The significance test of Monte Carlo permutations was conducted to obtain a suitable model of the microbe–environment relationships.

Results

Abundance of n-damo bacteria

So far, the number of n-damo bacteria in the environment is usually estimated by quantifying their 16S rRNA genes (Wang et al. 2012; Shen et al. 2014a; Liu et al. 2015). In this study, the observed n-damo 16S rRNA gene copy number in reservoir sediments ranged between 8.43×10^4 and 1.03×10^6 copies per gram of dry sediment (Fig. 1). At each sampling

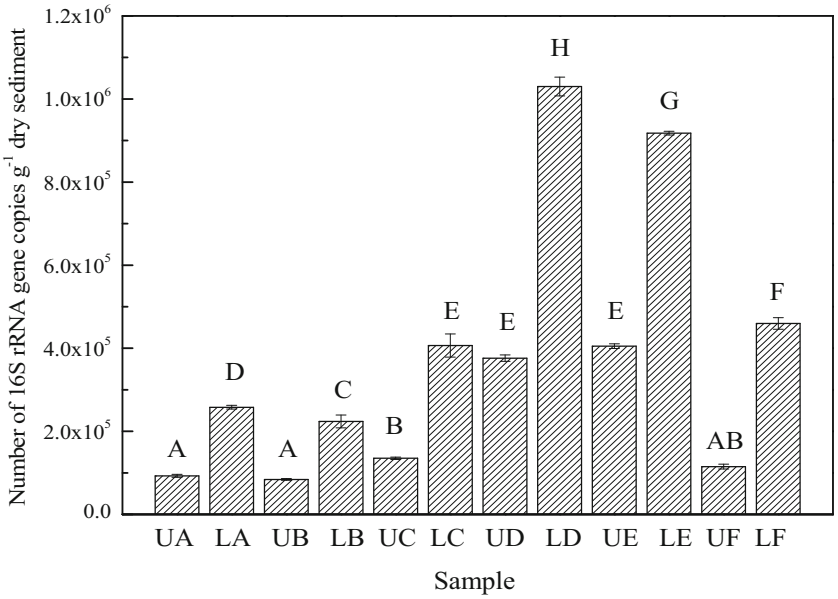


Fig. 1 Abundance of n-damo 16S rRNA genes in the different sediment samples. Values are the average of three independent experiments. The vertical bars indicate standard deviations. The different letters above the columns indicate the significant differences ($P < 0.05$)

location, the lower layer sediment showed higher n-damo bacterial abundance than the corresponding upper one ($P < 0.05$). Moreover, samples UD and UE had higher n-damo bacterial abundance than the other four upper sediments ($P < 0.05$). A significant difference in n-damo bacterial abundance was found between every two lower layer sediments ($P < 0.05$). These results illustrated the spatial variation of sediment n-damo bacterial abundance the Xinfengjiang Reservoir. In addition, Spearman's rank correlation analysis indicated that sediment n-damo bacterial abundance was positively correlated to the levels of sediment pH, TN, and NO_2^--N ($P < 0.01$) (Table 2).

Activity of the n-damo process

In this study, the sediment sample with the highest n-damo bacterial abundance (sample LD) was used to perform the n-damo activity test. The removal of CH_4 in the reactor added with nitrite was more rapid than that in the control reactor (Fig. 2). For the reactor added with nitrite, with the depletion of nitrite nitrogen from 47.1 to 8.9 mg/L, the content of CH_4 decreased from 95 to 26.6% after a 14-day incubation, but the N_2 content increased from below limit to 58.6%. In contrast, for the control reactor, after a 14-day incubation, the content of CH_4 decreased to 63.5%, while the N_2 content increased to 33.9%.

Richness and diversity of the n-damo *pmoA* gene

The n-damo *pmoA* gene has become a widely used functional biomarker to assess the diversity of n-damo organisms in natural environments (Deutzmann and Schink 2011; Kojima et al. 2012; Liu et al. 2015; Shen et al. 2015). In the present study, a total of 277 n-damo *pmoA* gene sequences were retrieved from sediments in the freshwater Xinfengjiang Reservoir. Each n-damo *pmoA* library contained 17–32 valid sequences. A remarkable variation in OTU number was found in either upper layer sediment samples (3–13) or lower layer sediment samples (1–9) (Table 3). The value of the Chao1 richness estimator in upper layer sediment samples ranged between 3 and 15.5, while lower layer sediment samples had a Chao1 richness estimator in the range 1–30. The value of the Shannon index also showed a remarkable variation in either upper layer sediment samples (1.02–2.34) or lower layer sediment samples (0–1.59). At four sampling locations, the upper layer sediment sample had higher *pmoA* diversity than the

Table 2 Spearman's rank correlation analysis of sediment n-damo organisms with environmental factors

	TOC	pH	NH_4^+-N	NO_3^--N	NO_2^--N	TN	C/N
Abundance	0.004	0.834*	0.557	−0.375	0.89*	0.977*	−0.116
OTUs	0.283	0.022	0.312	−0.28	0.483	0.559	−0.185
Chao1 estimator	0.448	0.140	0.322	0.046	0.287	0.480	0.088
Shannon index	−0.021	−0.203	−0.021	−0.392	0.336	0.336	−0.252
Simpson index	0.336	0.329	0.224	0.224	−0.161	−0.070	0.322

*Correlation is significant at the 0.01 level

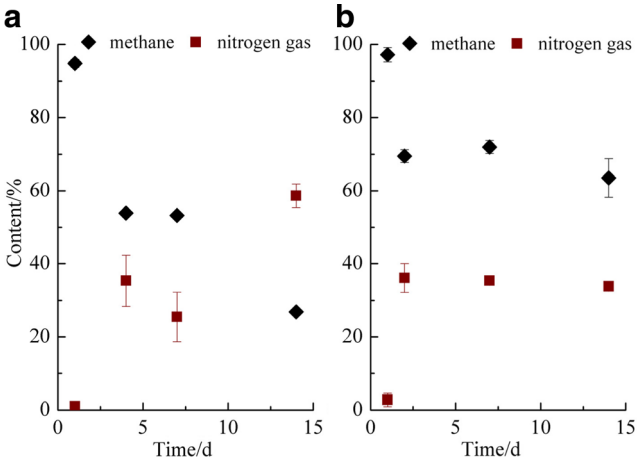


Fig. 2 Variations of CH_4 and N_2 levels in incubation reactors added with nitrite (**a**) and deionized water (**b**). Values are the average of three independent experiments. The vertical bars indicate standard deviations

corresponding lower layer one. In addition, a large difference in the value of the Simpson index also occurred in either upper layer sediment samples (0.09–0.4) or lower layer sediment samples (0.19–1). At four sampling locations, the upper layer sediment sample had lower evenness than the corresponding lower layer one. However, the result of Spearman's rank correlation analysis illustrated no significant correlation between the determined environmental factors and the n-damo *pmoA* gene ($P > 0.05$) (Table 2).

UPGMA clustering analysis of n-damo *pmoA* gene composition

Figure 3 illustrates the dendrogram constructed for the composition of sediment n-damo *pmoA* genes in the freshwater Xinfengjiang Reservoir. Four distinctive clades were found in the 12 studied sediment samples, indicating that distinctively different n-damo *pmoA* gene compositions could exist in these reservoir sediment samples. Sample LB was distantly separated from the other 11 reservoir samples. Sample UB alone formed a clade. Samples UD, UE, UF, and LD were clustered together. For either upper or lower layer sediments, they were distributed in three different clades. Moreover, at the sampling locations B, E, or F, the upper layer sediment was distantly separated from the corresponding lower layer sediment. These results suggested that both sampling location and sediment depth could affect n-damo *pmoA* gene composition in sediments of the Xinfengjiang Reservoir. In addition, the environmental factors in the first two RDA axes respectively explained 26.5 and 13.2% of the total variance in sediment n-damo *pmoA* OTU composition (Fig. 4). However, in the present study, none of the determined environmental parameters was found to significantly contribute to the n-damo *pmoA* composition–environment relationship.

Phylogeny of the n-damo *pmoA* gene

Figure 5 shows the phylogenetic relationships of the representative n-damo *pmoA* gene sequences from the major OTUs (with at least two members) and their close relatives reported in the GenBank database. The sequences from the major n-damo *pmoA* OTUs could be assigned to five distinctive clusters (clusters I, II, III, IV, and V). The sediment samples from the Xinfengjiang Reservoir showed a remarkable difference in the proportion of each n-damo *pmoA* cluster (Figure S3).

Cluster I was the largest n-damo *pmoA* group and was composed of 98 *pmoA* gene sequences. These sequences could be grouped together with the *pmoA* gene sequence from *Candidatus* Methylomirabilis oxyfera (Ettwig et al. 2010) and several uncultured n-damo *pmoA* sequences from a variety of ecosystems, such as wetland, freshwater lake and river sediment, and paddy soil. Cluster I-like n-damo organisms were present in all of the studied reservoir sediment samples (except for sample LB), and they predominated in samples LA, LD, LE, LF, UC, and UE (accounting for 50–81.3%). Cluster II

Table 3 Diversity indices of each reservoir sediment n-damo *pmoA* gene clone library

Sample	Sequences	OTUs	Chao1 estimator	Shannon index	Simpson index
UA	25	6	6	1.62	0.19
UB	20	7	7.3	1.75	0.16
UC	17	3	3	1.02	0.35
UD	26	13	15.5	2.34	0.09
UE	21	6	12	1.19	0.4
UF	32	7	7.5	1.65	0.2
LA	22	4	4	1.18	0.3
LB	20	1	1	0.00	1
LC	22	6	7	1.59	0.19
LD	23	6	6	1.51	0.23
LE	23	9	30	1.54	0.32
LF	26	6	9	1.36	0.3

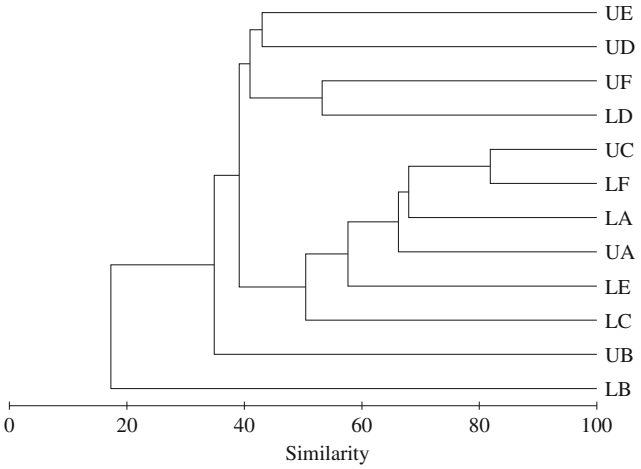


Fig. 3 UPGMA cluster diagram of n-damo *pmoA* gene composition similarity values for reservoir sediment samples. Similarity levels are indicated below the diagram

contained 43 sequence members that could be related to two uncultured n-damo bacterial *pmoA* gene sequences obtained from freshwater river and lake sediment ecosystems. The cluster II-like n-damo *pmoA* group showed a relatively high proportion in sample UD (45%), but they become much less abundant in other reservoir sediment samples (0–27.8%). Cluster III was the second largest n-damo *pmoA* group, containing 51 sequences. These sequences in cluster III were close to uncultured n-damo *pmoA* gene sequences retrieved from wetland, paddy soil, freshwater lake sediment, and reservoir water. The cluster III-like n-damo *pmoA* group showed relatively high proportions in samples LC, LD, UD, and UF

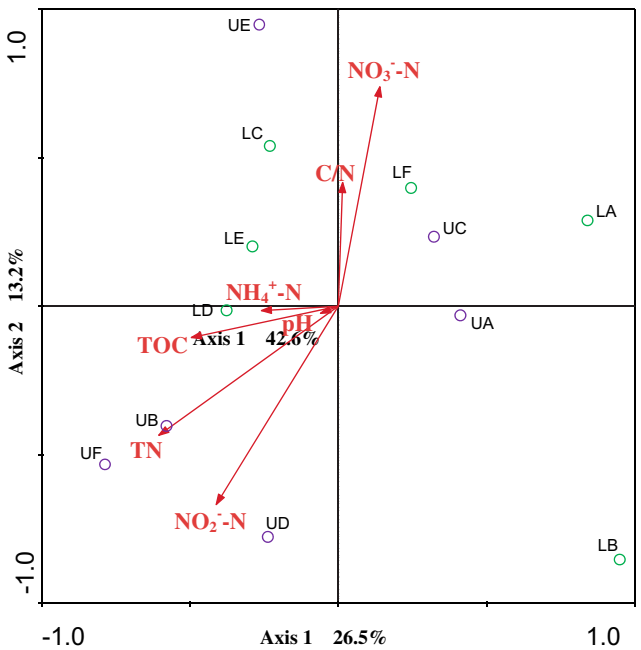


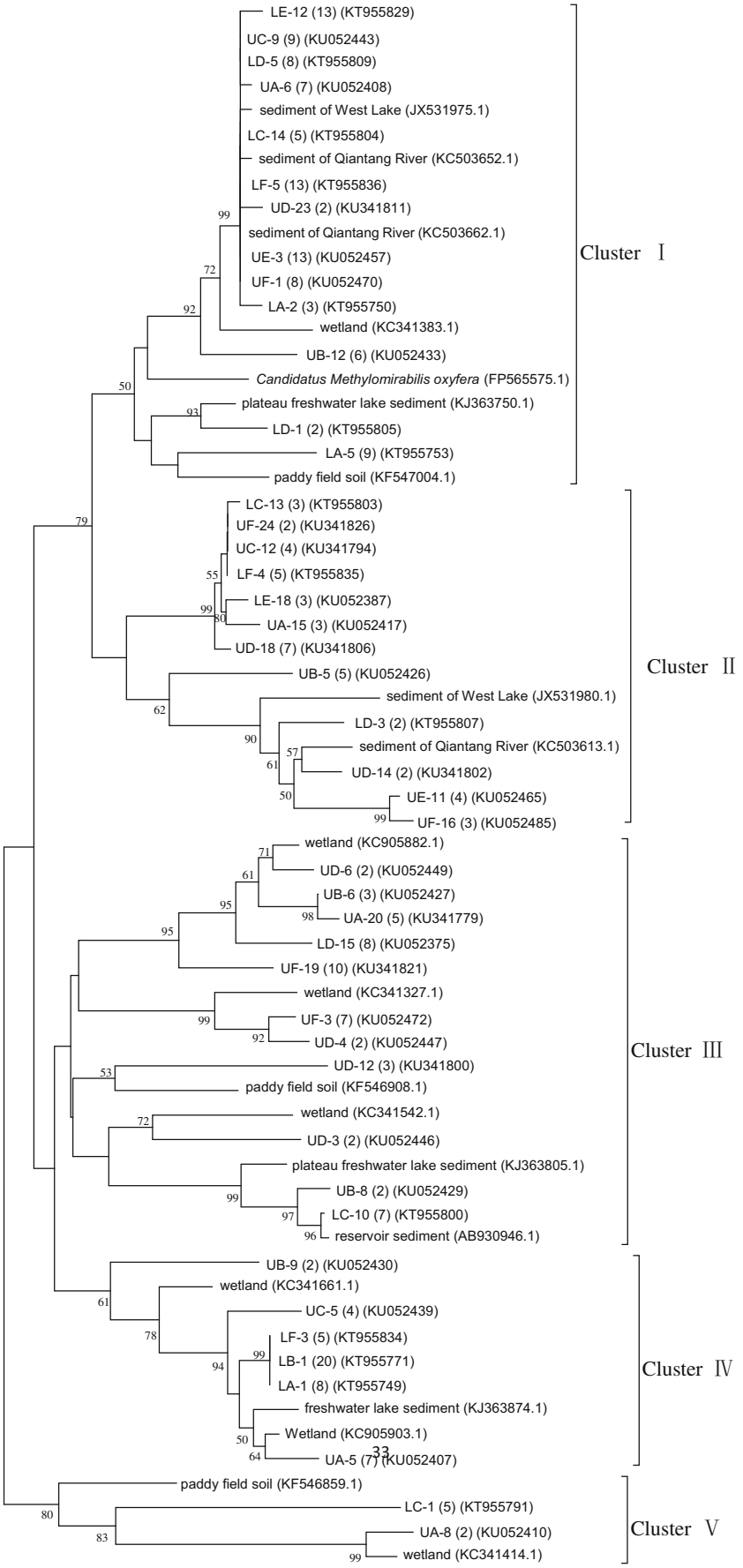
Fig. 4 RDA ordination plot for the first two principal dimensions of the relationship between n-damo *pmoA* OTU composition and the environmental factors

(35–56.7%), but became less abundant in samples UA and UB (20.8 or 27.8%). Moreover, cluster IV was a 46-member n-damo *pmoA* group. The sequences in cluster IV could be grouped with several uncultured wetland and lake sediment n-damo *pmoA* sequences. Sample LB was composed of only cluster IV-like n-damo *pmoA* sequences. Cluster IV-like n-damo *pmoA* sequences were also present in samples LA, LF, UA, UB, and UC (11.1–40%). In addition, cluster V was only composed of two sequences from sample UA and five from sample LC. They were close to uncultured wetland and paddy soil n-damo *pmoA* gene sequences.

Discussion

The presence of n-damo activity has been confirmed in freshwater lake sediments (Deutzmann and Schink 2011; Deutzmann et al. 2014), while information on n-damo activity in freshwater reservoirs is still lacking. Moreover, although nitrite nitrogen is one of the substrates of n-damo organisms, the effect of the nitrite nitrogen level on freshwater sediment n-damo activity has received no attention. In this study, the addition of nitrite nitrogen greatly improved the consumption of CH_4 by reservoir sediment, which also suggested the existence of an n-damo process in reservoir sediment. In addition, this result further suggested that the increase of nitrite nitrogen might promote sediment n-damo activity.

Shen et al. (2014a) and Long et al. (2016) showed the variation of freshwater sediment n-damo community abundance with sampling location in Qiantang River and Dongjiang River, respectively. Liu et al. (2015) reported that sediment n-damo bacterial abundance differed in freshwater lakes on the Yunnan Plateau. In this study, the number of sediment n-damo bacteria in the oligotrophic freshwater Xinfengjiang Reservoir ranged between 8.43×10^4 and 1.03×10^6 copies per gram of dry sediment, generally lower than that reported in Qiantang River and freshwater lakes on the Yunnan Plateau (Shen et al. 2014a; Liu et al. 2015). Moreover, for both upper and lower sediments, the variation of sediment n-damo bacterial abundance with sampling location was found in the Xinfengjiang Reservoir. At each sampling location, n-damo bacteria in the lower layer sediment was more abundant than the corresponding upper one. The present study provided, for the first time, that n-damo bacterial abundance varied with sediment layer depth. Biogenic methane is produced by the activity of strictly anaerobic methanogens. The methane produced in deep sediment habitat can be gradually consumed by n-damo bacteria. Compared with upper layer sediment (0–5 cm), lower layer sediment (5–10 cm) might favor the growth of n-damo bacteria, which might be attributed to the higher substrate methane in lower layer sediment.


LE-12 (13) (KT955829)
UC-9 (9) (KU052443)
LD-5 (8) (KT955809)
UA-6 (7) (KU052408)
sediment of West Lake (JX531975.1)
LC-14 (5) (KT955804)
sediment of Qiantang River (KC503652.1)
LF-5 (13) (KT955836)
UD-23 (2) (KU341811)
sediment of Qiantang River (KC503662.1)
UE-3 (13) (KU052457)
UF-1 (8) (KU052470)
LA-2 (3) (KT955750)
wetland (KC341383.1)
UB-12 (6) (KU052433)
Candidatus Methylomirabilis oxyfera (FP565575.1)
plateau freshwater lake sediment (KJ363750.1)
LD-1 (2) (KT955805)
LA-5 (9) (KT955753)
paddy field soil (KF547004.1)
Cluster Ⅰ
LC-13 (3) (KT955803)
UF-24 (2) (KU341826)
UC-12 (4) (KU341794)
LF-4 (5) (KT955835)
LE-18 (3) (KU052387)
UA-15 (3) (KU052417)
UD-18 (7) (KU341806)
UB-5 (5) (KU052426)
sediment of West Lake (JX531980.1)
LD-3 (2) (KT955807)
sediment of Qiantang River (KC503613.1)
UD-14 (2) (KU341802)
UE-11 (4) (KU052465)
UF-16 (3) (KU052485)
Cluster Ⅱ
wetland (KC905882.1)
UD-6 (2) (KU052449)
UB-6 (3) (KU052427)
UA-20 (5) (KU341779)
LD-15 (8) (KU052375)
UF-19 (10) (KU341821)
wetland (KC341327.1)
UF-3 (7) (KU052472)
UD-4 (2) (KU052447)
UD-12 (3) (KU341800)
paddy field soil (KF546908.1)
wetland (KC341542.1)
UD-3 (2) (KU052446)
plateau freshwater lake sediment (KJ363805.1)
UB-8 (2) (KU052429)
LC-10 (7) (KT955800)
reservoir sediment (AB930946.1)
Cluster Ⅲ
UB-9 (2) (KU052430)
wetland (KC341661.1)
UC-5 (4) (KU052439)
LF-3 (5) (KT955834)
LB-1 (20) (KT955771)
LA-1 (8) (KT955749)
freshwater lake sediment (KJ363874.1)
Wetland (KC905903.1)
UA-5 (7) (KU052407)
Cluster Ⅳ
paddy field soil (KF546859.1)
LC-1 (5) (KT955791)
UA-8 (2) (KU052410)
wetland (KC341414.1)
Cluster Ⅴ

◀ **Fig. 5** Phylogenetic tree of representative n-damo *pmoA* sequences and reference sequences from GenBank. The obtained *pmoA* sequences beginning with "UA" and "LA", "UB" and "LB", "UC" and "LC", "UD" and "LD", "UE" and "LE", and "UF" and "LF" referred to those retrieved from the upper layer and lower layer sediments in sampling locations A–F, respectively. The number in parentheses represents the numbers of the sequences in the same OTU in a given clone library. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets. Values less than 50 are not listed. The bar represents 1% sequence divergence

Shen et al. (2014a) suggested that sediment n-damo bacterial abundance might be influenced by the level of TOC, while Long et al. (2016) also suggested that the increase of sediment NO_2^--N level might increase the number of n-damo microorganisms. In this study, the result of Spearman's rank correlation analysis suggested that sediment n-damo bacterial abundance was positively influenced by sediment pH as well as TN and NO_2^--N. This also further confirmed the role of substrate NO_2^--N in determining sediment n-damo bacterial abundance. In addition, the environmental factors regulating sediment n-damo bacterial abundance could differ in various freshwaters.

Han and Gu (2013) reported a Shannon index of 1.89 for one sediment sample from the Tai Lam Chung freshwater reservoir of Hong Kong. Several recent studies further documented the spatial shift in the n-damo *pmoA* gene diversity in freshwater ecosystems, including Qiantang River (Shannon index = 0–1.98) (Shen et al. 2014a), Dongjiang River (0–2.58) (Long et al. 2016), and lakes on the Yunnan Plateau (0–2.4) (Liu et al. 2015). In the present study, the presence of the n-damo *pmoA* gene was detected in all of the 12 reservoir sediment samples that were obtained from six different sampling locations (A–F) and two sediment depths (0–5 cm, 5–10 cm) in the freshwater Xinfengjiang Reservoir. The value of the Shannon index ranged between 1.02 and 2.34 in upper layer sediment samples and 0 and 1.59 in lower layer sediment samples, illustrating the remarkable variation of n-damo *pmoA* diversity with sampling location. This was in agreement with the results found in other freshwater sediments (Shen et al. 2014a; Liu et al. 2015; Long et al. 2016).

So far, the environmental factors regulating freshwater sediment n-damo bacterial or *pmoA* gene diversity remain essentially unknown. Shen et al. (2014a) suggested that NH_4^+-N and total inorganic nitrogen might affect n-damo bacterial diversity in Qiantang River, while sediment C/N might play an important role in shaping n-damo *pmoA* diversity in freshwater lakes on the Yunnan Plateau (Liu et al. 2015). Long et al. (2016) indicated that both NO_2^--N and C/N were negatively correlated to n-damo *pmoA* diversity in Dongjiang River. These previous studies reported the different results. The environmental factors influencing freshwater sediment n-damo community diversity could differ in various freshwater ecosystems. In this current study, the environmental factors regulating sediment n-damo *pmoA* diversity in the Xinfengjiang Reservoir was not identified. Further efforts will be necessary in order to elucidate the links between n-damo *pmoA* diversity and environmental factors in freshwater sediment ecosystems.

So far, phylogenetic information on freshwater sediment n-damo community is still very limited. Several previous studies indicated that n-damo *pmoA* gene sequences from sediment of Lake Biwa, Lake Constance, and Qiantang River were mainly related to those from known NC10 bacteria (*Candidatus Methylomirabilis oxyfera* strains) (Deutzmann and Schink 2011; Kojima et al. 2012; Shen et al. 2014a). A small proportion of n-damo *pmoA* gene sequences obtained from one sediment sample from the Tai Lam Chung freshwater reservoir were also related to the *pmoA* gene sequence of *M. oxyfera* (Han and Gu 2013). In the present study, the *pmoA* sequences affiliated with *M. oxyfera* were the largest n-damo *pmoA* group in sediments of the Xinfengjiang Reservoir. However, most of the obtained n-damo *pmoA* gene sequences from sediments of the Xinfengjiang Reservoir showed no close relationship to those from any known NC10 species. This was also consistent with the results of previous investigations on sediments in Dongjiang River (Long et al. 2016) and lakes on the Yunnan Plateau (Liu et al. 2015). In addition, the *pmoA* sequences obtained in the study were related to those from diverse ecosystems, such as reservoir, lake, and river sediments, and wetland and paddy soils. This suggested that n-damo organisms might adapt to a variety of habitats and the n-damo organisms detected in one type of habitat might occur in other types of habitats.

The spatial variation of freshwater sediment n-damo community structure with sampling location has been found in Qiantang River (Shen et al. 2014a), Dongjiang River (Long et al. 2016), and lakes on the Yunnan Plateau (Liu et al. 2015). In this study, the results of both UPGMA clustering and phylogenetic analysis indicated that sediment n-damo *pmoA* composition in the Xinfengjiang Reservoir spatially varied. To date, little is known about the environmental factors driving freshwater sediment n-damo community structure. Shen et al. (2014a) suggested that sediment NH_4^+-N and total inorganic nitrogen might be the key determinants of n-damo community structure in Qiantang River, while sediment NO_2^--N as well as C/N might influence n-damo community structure in Dongjiang River (Long et al. 2016). These two previous studies suggested that the environmental factors governing sediment n-damo community structure could differ in various freshwater ecosystems. In this study, there were no obvious links between the determined environmental parameters and n-damo *pmoA* composition in the Xinfengjiang Reservoir.

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