Vertical and horizontal distribution of sediment nitrite-dependent methane-oxidizing organisms in a mesotrophic freshwater reservoir

Yan Long, Changbao Liu, Hengliang Lin, Ningning Li, Qingwei Guo, and Shuguang Xie

Abstract: In the present study, we investigated the spatial change of sediment nitrite-dependent anaerobic methane-oxidizing (n-damo) organisms in the mesotrophic freshwater Gaozhou Reservoir (6 different sampling locations and 2 sediment depths (0–5 cm, 5–10 cm)), one of the largest drinking water reservoirs in China. The abundance of sediment n-damo bacteria was quantified using quantitative polymerase chain reaction assay, while the richness, diversity, and composition of n-damo *pmoA* gene sequences were characterized using clone library analysis. Vertical and horizontal changes in sediment n-damo bacterial abundance occurred in Gaozhou Reservoir, with 1.37×10^5 to 8.24×10^5 n-damo 16S rRNA gene copies per gram of dry sediment. Considerable horizontal and vertical variations of n-damo *pmoA* gene diversity (Shannon index = 0.32–2.50) and composition also occurred in this reservoir. Various types of sediment n-damo *pmoA* genes existed in Gaozhou Reservoir. A small proportion of n-damo *pmoA* gene sequences (19.1%) were related to those recovered from "*Candidatus* Methylomirabilis oxyfera". Our results suggested that sediment n-damo *pmoA* gene diversity might be regulated by nitrite, while n-damo *pmoA* gene richness might be governed by multiple environmental factors, including total organic carbon, total phosphorus, nitrite, and total nitrogen.

Key words: denitrifying methanotrophs, bacterial community, methane oxidation, freshwater sediment.

Résumé : L'étude présente visait à examiner les changements dans l'espace d'organismes sédimentaires de dénitrification couplée à l'oxydation anaérobique du méthane (n-doam) dans le réservoir mésotrophique d'eau douce de Gaozhou (6 lieux d'échantillonnage différents et deux profondeurs de sédiment (0–5 cm, 5–10 cm)), un des plus grands réservoirs d'eau potable de Chine. L'abondance des bactéries n-doam sédomentaires a été quantifiée par un dosage par PCR quantitative, alors que la richesse, la diversité et la composition en séquences du gène de n-doam *pmoA* ont été caractérisées par une analyse d'une banque de clones. Des changements verticaux et horizontaux de l'abondance de bactéries n-doam sédimentaires survenaient dans le réservoir de Gaozhou, de $1,37 \times 10^5$ à $8,24 \times 10^5$ copies du gène de l'ARNr 16S de n-doam étant trouvées par gramme de sédiment sec. Des variations horizontales et verticales considérables de la diversité du gène de n-doam *pmoA* (indice de Shannon = 0,32–2,50) et de sa composition survenaient aussi dans ce réservoir. Différents types du gène de n-doam *pmoA* étaient présents dans les sédiments du réservoir de Gaozhou. Une petite proportion de séquences du gène de n-doam *pmoA* (19,1 %) était apparentée à celles récupérées de *Candidatus* « Methylomirabilis oxyfera ». Les résultats des auteurs suggèrent que la diversité du gène de n-doam *pmoA* des sédiments pourrait être régulée par le nitrite, alors que la richesse en gène de n-doam *pmoA* pourrait être gouvernée par de multiples facteurs environnementaux dont le carbone organique total, le phosphore total, le nitrite et l'azote total. [Traduit par la Rédaction]

Mots-clés : méthanotrophes dénitrifiants, communauté bactérienne, oxydation du méthane, sédiment d'eau douce.

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Introduction

Methane is an important greenhouse gas and can make an approximately 20% contribution to the global warming (IPCC 2007). A considerable amount of methane emission originates from methanogenesis in anoxic environments of aquatic ecosystems (Bastviken et al. 2004; Borrel et al. 2011). Freshwater ecosystems can contribute significantly to the global methane emissions (Bastviken et al. 2011). Microbial methane oxidation is known to play a crucial role mitigating methane emission into the atmosphere (Chowdhury and Dick 2013). Microbially mediated methane oxidation was believed to usually be performed by aerobic methanotrophs, mainly belonging to bacterial phylum *Proteobacteria*. Almost all of the known aerobic methanotrophs harbor the particulate methane monooxygenase (pMMO) that catalyzes the first step of methane oxidation (Deng et al. 2013; Yang et al. 2014). However, the discovery of anaerobic methane oxidation (AMO) using different electron acceptors (iron, manganese, sulfate, nitrite, or nitrate) suggested the presence of various pathways for methane oxidation (Beal et al. 2009; Schreiber et al. 2010; Haroon et al. 2013; Shen et al. 2015a). Recently, the potential ecological importance of the nitrite-dependent AMO (n-damo) process and n-damo organisms in natural ecosystems has aroused increasing attention (Shen et al. 2015a). N-damo organisms were first enriched from freshwater canal sediments (Raghoebarsing et al. 2006). By means of metagenomics, the n-damo process was found to be driven by "*Candidatus* Methylomirabilis oxyfera" (*Ca.* M. oxyfera) within the bacterial NC10 phylum through an "intra-aerobic" pathway involving the production of oxygen from nitric oxide (Ettwig et al. 2010). *Ca.* M. oxyfera can also catalyze methane oxidation using pMMO (Ettwig et al. 2010; Shen et al. 2015a). *Ca.* M. oxyfera can be present in various freshwater ecosystems, such as lake, reservoir, river, ditch, paddy soil, and wetland (Chen et al. 2016).

The *pmoA* gene, encoding the α subunit of pMMO, has become a favorite biomarker to study n-damo organisms in natural and man-made ecosystems on a functional level (Han and Gu 2013; Shen et al. 2014a, 2015a, 2015b; Chen et al. 2015a; Liu et al. 2015). Previous studies have investigated the presence and diversity of sediment n-damo organisms in a few freshwater lakes (Deutzmann and Schink 2011; Kojima et al. 2012; Deutzmann et al. 2014; Liu et al. 2015) and 1 freshwater river (Shen et al. 2014b), yet the environmental factors influencing the spatial distribution of n-damo organisms in freshwater sediments remain essentially unclear. In addition, compared with natural lakes, reservoirs usually show relatively short retention time and intense water level fluctuation. These 2 kinds of aquatic ecosystems can host different physical, chemical, and biological processes

(Lymperopoulou et al. 2012). To date, only Han and Gu (2013) reported the diversity of n-damo bacteria in a sole reservoir sediment, yet information on the spatial change of sediment n-damo bacteria in freshwater reservoir and the associated environmental factors is still lacking. Therefore, the main objective of the current study was to investigate the spatial variation of n-damo organisms in sediments of freshwater reservoir. The environmental factors influencing the distribution of freshwater sediment n-damo organisms were also explored.

Materials and methods

Study locations and sampling

The freshwater Gaozhou Reservoir, located in Maoming City, Guangdong Province, is one of the largest drinking water reservoirs in China. This reservoir covers 43.81 km^2 with the total catchment area of 1002 km^2, and its maximum water depth is 96 m. Gaozhou Reservoir can store a volume of 12.8 billion m^3 at full water level. The water quality of Gaozhou Reservoir is characterized as mesotrophic (Chen et al. 2012). The reservoir is separated into 2 parts, Shigu (south part) and Liangde (north part), but these 2 parts are connected with a canal.

In August 2015, sediment cores in triplicate were collected from Gaozhou Reservoir at 6 different sampling locations, including 3 in the south part (locations 1, 2, and 3) (22°2′29.00″–22°3′6.13″N, 111°1′10.00″–111°4′3.14″E) and another 3 in the north part (locations 4, 5, and 6) (22°8′38.00″–22°10′12.52″N, 110°59′18.40″–111°1′20.00″E) (Fig. S1[1]). These sediment cores were sampled using a stainless steel columnar sediment sampler (SCIES, China). To avoid possible contamination, each core was collected using a new sampler. These sediment cores were then placed into sterile containers, sealed, and immediately transported back to the laboratory on ice. In this study, these sediment cores were sliced into layers, and 2 layers (0–5 cm, 5–10 cm) of each core were used for subsequent analysis. Samples U1 and L1, U2 and L2, U3 and L3, U4 and L4, U5 and L5, and U6 and L6 denote the upper and lower layer sediments in the sampling locations 1–6, respectively.

Chemical analysis

Sediment pH was measured using an IQ150 pH meter (IQ Scientific Instruments, Inc.).

Sediment samples were dried using a freeze dryer (Alpha 1-2 LD, Martin Christ Instrument Co.). Sediment total organic carbon (TOC) was analyzed using the potassium dichromate oxidation–reduction titration method (Wang 2012). Sediment total phosphorus (TP) was determined using the molybdenum blue colorimetry method after 2 h combustion (500 °C) and 16 h extraction with 1 mol/L HCl (Wang 2012). The levels of sediment ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate ni-

trogen (NO$_3^-$-N), and total nitrogen (TN), respectively, were determined using Nessler's reagent method, naphthalene ethylenediamine spectrophotometry method, phenol disulfonic acid colorimetric method, and Kjeldahl method, after 2 h extraction using 2 mol/L KCl solution (Wang 2012).

Quantitative PCR assay

Sediment genomic DNA (0.5 g, dry mass) was extracted using a Powersoil DNA extraction kit (Mo Bio Laboratories, USA) in accordance with the manufacturer's instruction. The quality of DNA was evaluated by electrophoresis on 1.0% agarose gel, and the DNA yield was quantified using a biophotometer (Eppendorf, Germany). Since there has been no suitable quantitative PCR primers quantifying the abundance of n-damo *pmoA* gene, the number of n-damo bacteria is usually assessed by quantifying their 16S rRNA gene (Liu et al. 2015). In the present study, the abundance of n-damo bacterial 16S rRNA gene was determined using the primer pair (qP1F (5′-GGGCTTGACA-TCCCACGAACCTG-3′) and qP1R (5′-CGCCTTCCTCCAGC-TTGACGC-3′)) (Ettwig et al. 2010). The quantitative PCR was performed using an ABI 7500 FAST (Applied Biosystems, USA) in a 20 μL reaction mixture, including template DNA (2 μL), 2× SYBR Green PCR master mix (10 μL), and 10 μmol/L primers (each 0.8 μL). The amplification conditions was as follows: 95 °C for 3 min; 40 cycles of 1 min denaturation at 95 °C, 1 min annealing at 63 °C, and 1 min elongation at 72 °C; and a final 5 min elongation at 72 °C (Wang et al. 2016). Standard curves ranging from 10^2 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 95% and 0.997, respectively.

Clone library analysis

The n-damo *pmoA* gene was amplified using a nested approach (first-step primer pair A189_b (5′-GGNGACTGG-GACTTYTGG-3′) and cmo682 (5′-AAAYCCGGCRAAGAAC-GA-3′), and second-step primer pair cmo182 (5′-TCACG-TTGACGCCGATCC-3′) and cmo568 (5′-GCACATACTCCA-TCCCCATC-3′)) under the following PCR conditions: initial melting step at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1.5 min; and a final elongation at 72 °C for 10 min (Yang et al. 2016). PCR products were purified using a QIAquick PCR purification kit (Qiagen Inc.), and the equal amounts of PCR products from triplicate samples were mixed. The pooled amplicons were cloned into pMD19-T vector (Takara Corp., Japan) following the manufacturer's instruction. *Escherichia coli* clones were cultured on solid Luria–Bertani medium (containing 15 g/L agar and 50 μg/L ampicillin) at 37 °C for 15 h. The white colonies were further verified by PCR using the primer pair M13 F (5′-TGT-

AAAACGACGGCCAGT-3′) and M13 R (5′-AACAGCTA-TGACCATG-3′).

Clones containing correct size were sequenced at SinoGenoMax Co., Ltd. (Beijing), with a 3730xl DNA Analyzer (Applied Biosystems, USA). The obtained chimera-free *pmoA* gene sequences were deposited in the GenBank database under accession Nos. KT944916–944997 and KU301341–KU301677, and grouped into operational taxonomic units (OTUs) with 95% similarity (Chen et al. 2015*a*) by the MOTHUR program (Schloss et al. 2009). Chao1 richness estimator and Shannon index were also generated by the MOTHUR program (Schloss et al. 2009). Phylogenetic analysis of *pmoA* gene sequences was conducted with the software MEGA 6.0 (Tamura et al. 2013) by the neighbor-joining method. The similarity of n-damo *pmoA* gene composition was analyzed using the OTU-based Bray–Curtis similarity matrices. The relative abundance of each OTU equaled the ratio of the sequence number in each OTU to the total sequences in a given sample. Sample clustering was conducted with the unweighted pair group method with arithmetic mean (UPGMA), using the software PRIMER 5.0 (Clarke and Warwick 2001).

Statistical analysis

One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used to test the significant difference ($p < 0.05$) in the number of n-damo bacterial 16S rRNA genes among sediment samples. Detrended correspondence analysis (DCA) was applied to choose the suitable ordination analysis method. The detailed DCA data are shown in Table S1[1]. Since the longest DCA axis had a gradient length of more than 3 standard-deviation units (3.42614), canonical correspondence analysis (CCA) was selected to determine the links between n-damo *pmoA* gene composition and the determined environmental factors using CANOCO for Windows software (version 4.5) (Lepš and Šmilauer 2003). The relative abundance of the *pmoA* gene sequence in each OTU was used as species input, while the determined sediment physicochemical properties were used as environmental input. Moreover, Spearman's rank correlation analysis was also used to test the correlations between n-damo organisms and different environmental variables, using the software SPSS 20.0.
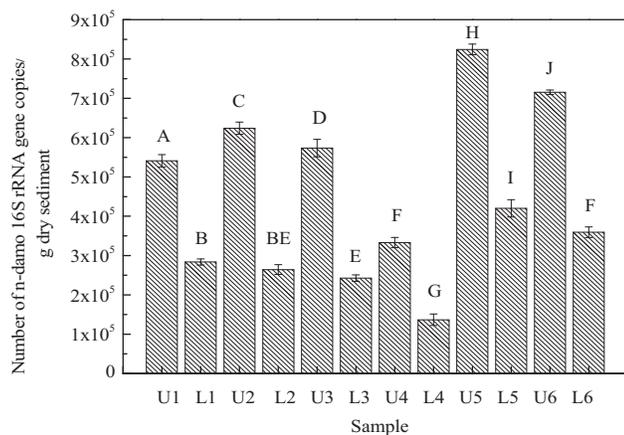
Results

Reservoir sediment properties

Table 1 displays the physicochemical features of sediments in mesotrophic freshwater Gaozhou Reservoir. The levels of pH, TOC, TP, NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N, TN, and ratio of TOC to TN (C/N) were 4.65–5.56, 22.52–35.62 g/kg, 309.83–559.63 mg/kg, 32.17–101.99 mg/kg, 0.05–1.46 mg/kg, 1.43–5.58 mg/kg, 591.86–1348.21 mg/kg, and 26.1–38.1, respectively. At most sampling locations, the upper layer sediment sample had higher TOC, TP, NH$_4^+$-N, NO$_2^-$-N, and TN but lower NO$_3^-$-N than the cor-

Table 1. Physicochemical features of reservoir sediments.

Sample	Longitude	Latitude	pH	TOC (g/kg)	TP (mg/kg)	NH$_4$$^+$-N (mg/kg)	NO$_2$$^-$-N (mg/kg)	NO$_3$$^-$-N (mg/kg)	TN (mg/kg)	C/N
U1	111°1′10.00″	22°2′29.00″	5.33	26.76	309.83	101.52	0.76	2.50	807.14	33.2
L1	111°1′10.00″	22°2′29.00″	5.37	23.14	356.92	53.13	0.20	2.61	753.91	30.7
U2	111°2′10.31″	22°3′6.13″	5.15	35.12	559.63	101.99	0.44	2.32	1348.21	26.1
L2	111°2′10.31″	22°3′6.13″	5.43	27.65	464.87	67.37	1.46	3.10	860.22	32.1
U3	111°4′3.14″	22°2′33.08″	5.56	31.18	407.41	35.86	0.55	3.49	933.56	33.4
L3	111°4′3.14″	22°2′33.08″	5.31	33.59	489.09	79.02	0.37	1.90	1015.77	33.1
U4	110°59′18.40″	22°8′43.28″	5.40	35.62	555.76	32.17	0.05	2.87	1214.34	29.3
L4	110°59′18.40″	22°8′43.28″	5.35	32.89	430.69	66.47	0.05	5.58	1194.97	27.5
U5	111°1′20.00″	22°8′38.00″	5.25	28.19	450.12	90.58	0.68	3.61	1074.87	26.2
L5	111°1′20.00″	22°8′38.00″	5.32	27.43	382.48	44.41	0.54	4.23	1025.43	26.8
U6	111°1′11.00″	22°10′12.52″	5.20	24.26	426.49	57.26	0.61	1.50	783.22	31.0
L6	111°1′11.00″	22°10′12.52″	4.65	22.52	373.27	40.12	0.23	1.43	591.86	38.1

Note: TOC, total organic carbon; TP, total phosphorus; TN, total nitrogen; C/N, ratio of TOC/TN.

Fig. 1. Abundance of n-damo 16S rRNA genes in the different sediment samples. Different letters above the columns indicate the significant differences ($p < 0.05$).

responding lower one. However, the sediment depth-related change patterns of pH and C/N were not clear.

Abundance of n-damo bacteria

In this study, the n-damo 16S rRNA gene copy number in sediments of the freshwater Gaozhou Reservoir ranged between 1.37×10^5 and 8.24×10^5 copies/g dry sediment (Fig. 1). At each sampling location, the lower layer sediment showed significantly lower n-damo bacterial abundance than the corresponding upper one ($p < 0.05$). Moreover, the significant difference in n-damo bacterial abundance was observed among upper layer sediments ($p < 0.05$). The n-damo bacterial abundance in lower layer sediments at sampling sites 5 and 6 was significantly higher than that at other sampling sites ($p < 0.05$). These results showed the spatial variation of n-damo bacterial abundance in sediments of Gaozhou Reservoir. However, n-damo bacterial abundance was not found to be significantly correlated with the determined sediment environmental factors ($p > 0.05$) (Table 2).

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

The n-damo *pmoA* gene has been the widely used functional biomarker to assess the richness and diversity of

n-damo organisms in the environment (Deutzmann and Schink 2011; Kojima et al. 2012; Liu et al. 2015; Shen et al. 2015b). In the current study, a total of 419 chimera-free n-damo *pmoA* gene sequences were obtained from the 12 studied sediment samples from the freshwater Gaozhou Reservoir. Each library was composed of 23–50 *pmoA* gene sequences. A substantial variation of OTU number was observed in both upper layer sediments (3–15) and lower layer sediments (3–15) (Table 3). Upper and lower layer sediments had Chao1 richness estimators of 3–21.5 and 3–22, respectively, indicating a remarkable horizontal change of n-damo *pmoA* gene richness in sediments of the Gaozhou Reservoir. The Shannon indices showed a remarkable horizontal variation in both upper layer (1.02–2.5) and lower layer sediments (0.32–2.46). A sediment-depth-related difference of n-damo *pmoA* gene diversity was also observed in the Gaozhou Reservoir. The upper layer sediment sample usually had higher n-damo *pmoA* gene diversity than the corresponding lower layer one. The result of Spearman rank correlation analysis indicated that the number of n-damo *pmoA* OTUs was positively correlated with sediment TOC and TN ($p < 0.01$ or $p < 0.05$) but negatively correlated with NO$_2$$^-$-N ($p < 0.05$) (Table 2). The n-damo *pmoA* Chao1 richness showed positive correlations with TOC, TP, and TN ($p < 0.01$ or $p < 0.05$) but a negative correlation with NO$_2$$^-$-N ($p < 0.05$). NO$_2$$^-$-N was also found to be negatively correlated with n-damo *pmoA* Shannon diversity ($p < 0.05$).

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

Figure 2 demonstrates the phylogenetic links of the representative n-damo *pmoA* gene sequences of the major OTUs (including at least 2 sequence members) and their close relatives reported in GenBank database. The *pmoA* gene sequences from the major OTUs could be grouped into 7 distinctive clusters (Clusters I–VII). The 12 studied reservoir sediment samples illustrated a remarkable difference in the proportion of each n-damo *pmoA* cluster, confirming the considerable horizontal

Table 2. Spearman rank correlation analysis of sediment n-damo organisms with the determined environmental factors.

	pH	TOC	TP	NH_4^+-N	NO_2^--N	NO_3^--N	TN	C/N
Abundance	−0.087	−0.142	0.005	0.324	0.259	−0.251	0.032	−0.237
OTUs	0.336	0.728**	0.445	−0.322	−0.676*	0.346	0.633*	−0.304
Chao1	0.133	0.767**	0.581*	−0.021	−0.596*	0.294	0.725**	−0.476
Shannon	0.385	0.566	0.238	−0.469	−0.655*	0.371	0.483	−0.203

Note: TOC, total organic carbon; TP, total phosphorus; TN, total nitrogen; C/N, ratio of TOC/TN; OTUs, operational taxonomic units. *, Correlation is significant at the 0.05 level; **, correlation is significant at the 0.01 level.

Table 3. Diversity indices of sediment *pmoA* gene clone libraries.

Sample	No. of sequences	OTUs	Chao1 estimator	Shannon index
U1	23	3	3.0	1.02
U2	35	11	21.5	1.78
U3	36	14	15.0	2.42
U4	42	15	16.0	2.50
U5	32	6	6.0	1.72
U6	37	7	7.0	1.85
L1	50	10	10.3	1.95
L2	27	3	4.0	0.32
L3	30	12	17.0	2.27
L4	33	15	22.0	2.46
L5	37	10	10.8	2.01
L6	37	3	3.0	0.52

Note: OTUs, operational taxonomic units.

and vertical change of sediment n-damo *pmoA* gene composition in Gaozhou Reservoir (Fig. 3).

Cluster I was the second largest n-damo *pmoA* group. It included 83 sequence members that could be related to several uncultured sequences retrieved from river and lake sediments and wetland ecosystems. Cluster-I-like n-damo *pmoA* gene sequences were the dominant group in Samples U1, U6, and L3 (accounting for 40.7%–55.8%), and they were also the largest group in Samples U3 and U4 (33.3% or 27.8%). However, they were absent in Samples U2, L1, L2, and L6. Cluster II was a 26-member n-damo *pmoA* group. The members in this cluster could be grouped together with the *pmoA* gene sequences from reservoir and wetland ecosystems. Cluster-II-like n-damo *pmoA* gene sequences were the largest group in Sample U5 (26.7%) and were also detected in Samples U4, U6, L1, and L3, with lower proportions (8.3%–17.6%). Cluster III contained only 5 *pmoA* gene sequences from Sample U5. These sequences showed no close relation to the *pmoA* gene sequences reported in the GenBank database. Cluster IV was the largest n-damo *pmoA* group, composed of 136 sequences. The sequences in Cluster IV showed close relation to reservoir or wetland *pmoA* gene sequences. The Cluster-IV-like n-damo *pmoA* gene dominated in Samples U2, L4, L5, and L6 (47.1%–90.2%) but showed a much lower proportion in other samples (0%–30.3%). Moreover, Cluster V consisted of 74 n-damo *pmoA* gene sequences that could be grouped together with the sequence from *Ca.* M. oxyfera (Ettwig et al. 2010) and with

several uncultured n-damo *pmoA* gene sequences from river and estuary sediments, paddy field soil, and wetland. The Cluster-V-like n-damo *pmoA* gene sequence showed a relatively high proportion in Samples U1, U2, and L1 (33.3%–56.3%) but became less abundant in other samples (0%–20%). Cluster VI consisted of only 4 *pmoA* gene sequences in Sample L4. They were close to an uncultured reservoir sequence. In addition, Cluster VII was composed of 60 *pmoA* gene sequences having close relation to several sequences from wetland and wastewater bioreactor. Cluster-VII-like n-damo *pmoA* gene was mainly distributed in Sample L2.

Figure 4 displays the dendrogram constructed for the compositions of n-damo *pmoA* gene in sediments of the Gaozhou Reservoir. Four distinctive groups existed in the 12 studied reservoir sediment samples, illustrating the existence of various sediment n-damo *pmoA* gene compositions in the Gaozhou Reservoir. Sample L6 was distantly separated from other sediment samples. Sample L2 also showed a large discrepancy with other samples. Samples U1 and U5 were grouped together, but they were distinguished from other sediment samples. Moreover, the 6 upper layer sediments were not grouped together, and an evident dissimilarity was also observed among the 6 lower layer sediments. This suggested an evident horizontal variation of sediment n-damo *pmoA* gene compositions in the freshwater reservoir. At a given sampling location, sediments of the upper and lower layers also displayed a large discrepancy, indicating the remarkable sediment depth-related change of n-damo *pmoA* gene compositions. In addition, the environmental factors in the first 2 CCA axes account for 40.3% and 35.6% of the total variance in sediment n-damo *pmoA* OTU composition (Fig. 5). However, in this study, none of the determined sediment variables significantly contributed to the n-damo organism – environment relationship ($p > 0.05$).

Discussion

The horizontal variation of sediment n-damo bacterial abundance with sampling location has been reported in a few aquatic ecosystems (Shen et al. 2014*a*, 2014*b*; Yan et al. 2015), while only Kojima et al. (2012) illustrated the layer depth-related change of n-damo bacterial abundance in freshwater sediment. In this study, n-damo bacterial

Fig. 2. Phylogenetic tree of representative n-damo *pmoA* sequences and reference sequences from GenBank. The obtained *pmoA* sequences beginning with "U1" and "L1", "U2" and "L2", "U3" and "L3", "U4" and "L4", "U5" and "L5", and "U6" and "L6" were referred to those retrieved from the upper layer and lower layer sediments in the sampling locations 1–6, respectively. The number in parentheses represents the numbers of the sequences in the same operational taxonomic unit in a given clone library. Numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled data sets. Values less than 50 are not listed. The bar represents 1% sequence divergence. Sequences from upper and lower layer sediments were highlighted in red and blue, respectively. [Colour online.]
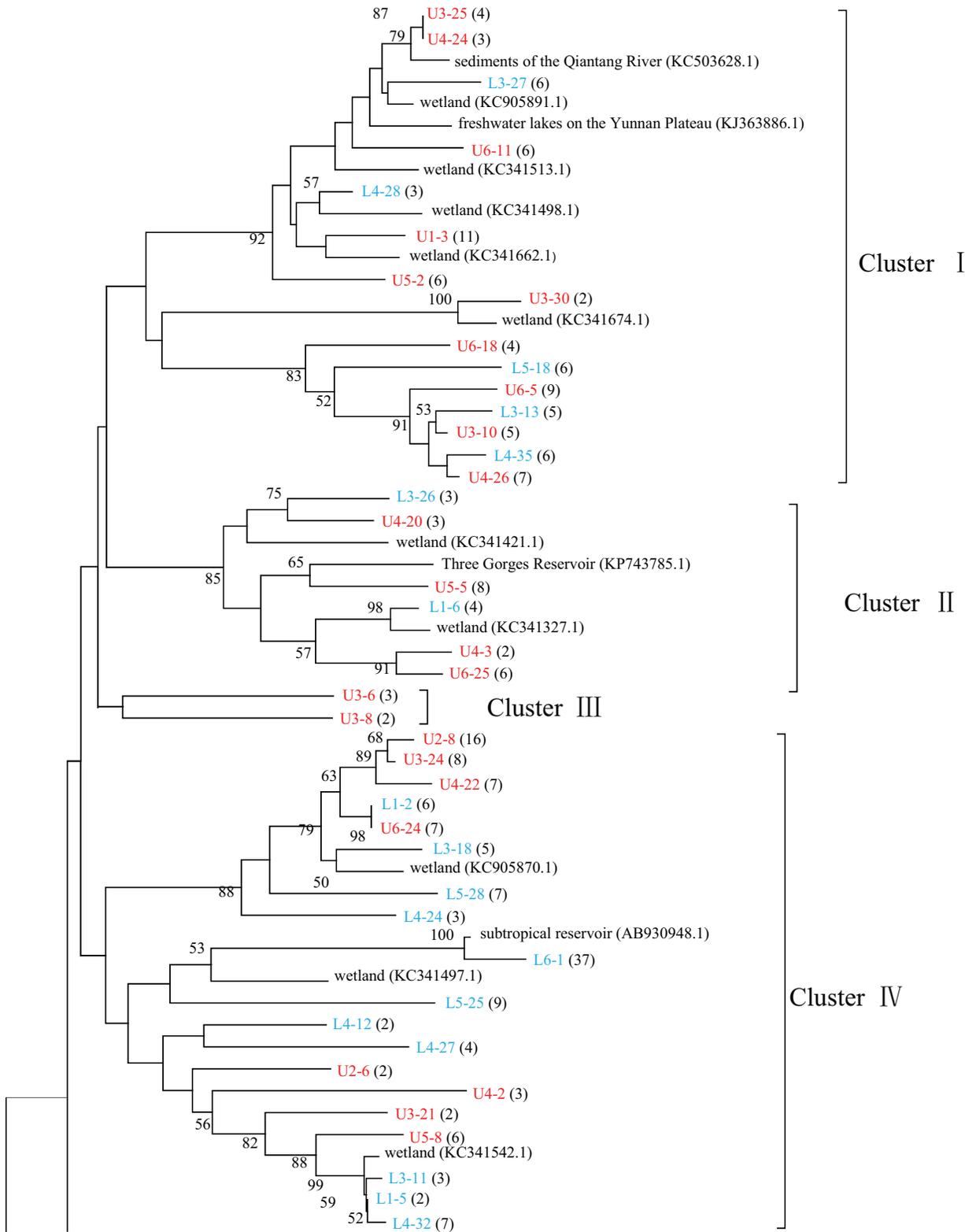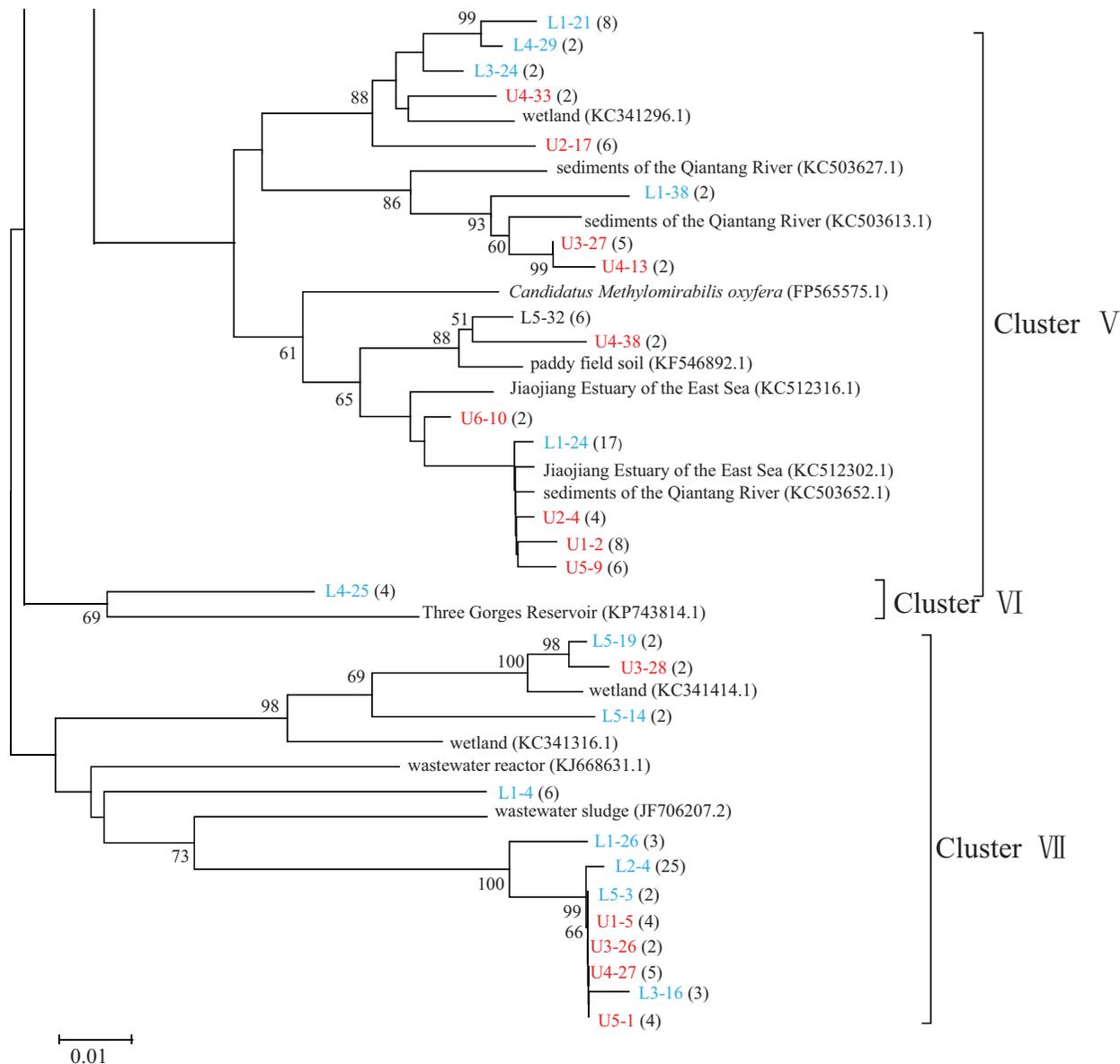
Fig. 2 (*concluded*).

abundance in sediments of Gaozhou Reservoir illustrated both vertical and horizontal changes.

There have been a number of previous investigations on sediment n-damo *pmoA* gene richness and diversity in saline environments. Chen et al. (2015*a*) found that sediment n-damo *pmoA* Chao1 richness and Shannon diversity in coastal intertidal wetland ranged between 1 and 15 and between 0.8605 and 1.4317, respectively, while Chen et al. (2014, 2015*b*) revealed lower marine sediment n-damo *pmoA* richness (1–2) and Shannon diversity (0–1.0055). Shen et al. (2014*a*) reported the remarkable spatial change of n-damo *pmoA* richness (1–11.5) and diversity (0–2.02) in marine estuary sediments. A considerable spatial change of n-damo *pmoA* richness (1–8.5) and diversity (0–1.887) was also found in sediments of the Yellow River Estuary (Yan et al. 2015). So far, information on the spatial change of n-damo *pmoA* gene richness and diversity

in freshwater sediments is still very limited. Shen et al. (2014*b*) showed the variation of sediment n-damo *pmoA* richness (1–9.2) and diversity (0–1.98) in freshwater Qiantang River. Liu et al. (2015) reported the n-damo *pmoA* diversity (0–2.4) in sediments of freshwater lakes on the Yunnan Plateau. Moreover, these previous studies focused on the horizontal variation of sediment n-damo *pmoA* gene richness and diversity, yet information on the sediment depth-related change is still lacking. In this study, the observed sediment n-damo *pmoA* gene Chao1 richness and Shannon diversity in the present study were 3–22 and 0.32–2.5, respectively. The highest values of sediment n-damo *pmoA* gene richness and diversity found in the current study outnumbered those reported in the previous studies (Chen et al. 2014, 2015*a*, 2015*b*; Shen et al. 2014*a*, 2014*b*; Liu et al. 2015; Yan et al. 2015). The horizontal changes of sediment n-damo *pmoA* gene

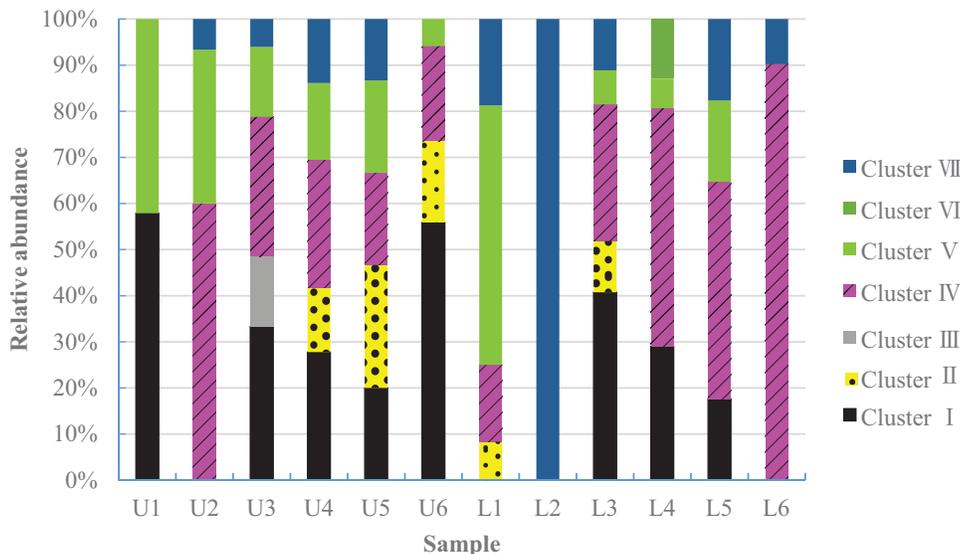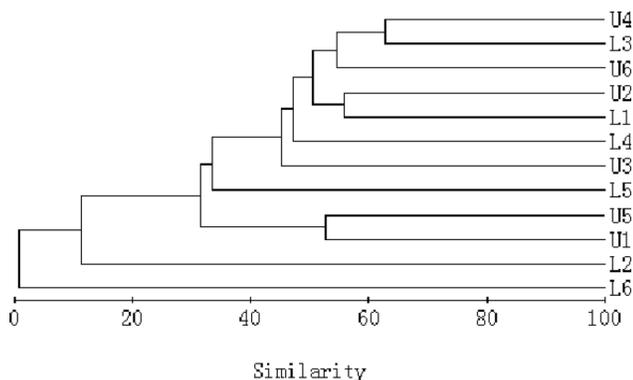**Fig. 3.** Compositions of n-damo *pmoA* clusters in reservoir sediments. [Colour online.]
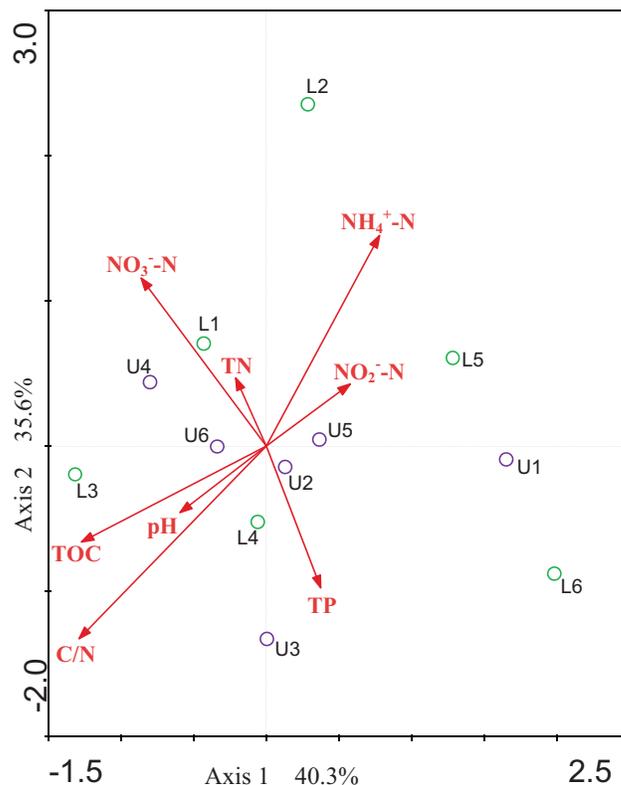
Fig. 4. UPGMA (unweighted pair group method with arithmetic mean) cluster diagram of n-damo *pmoA* gene composition similarity values for sediment samples from the Gaozhou Reservoir. Similarity levels are indicated below the diagram.

Fig. 5. Canonical correspondence analysis ordination plot for the first 2 principal dimensions of the relationship between n-damo *pmoA* operational taxonomic unit composition and the determined environmental factor. [Colour online.]

richness and diversity were also observed in this study, which was in agreement with the results reported in the previous studies (Chen et al. 2014, 2015*a*, 2015*b*; Shen et al. 2014*a*, 2014*b*; Liu et al. 2015; Yan et al. 2015). However, the present study provided evidence for the first time that sediment n-damo *pmoA* gene diversity was further influenced by sediment depth. At a given sampling location, the upper layer sediment sample usually had higher n-damo *pmoA* gene diversity than the corresponding lower layer one.

So far, the driving force for the variability of sediment n-damo *pmoA* gene richness and diversity remains essentially unclear. Several previous studies suggested that sediment n-damo *pmoA* gene Chao1 richness and Shannon diversity might be influenced by the levels of organic content (Shen et al. 2014*a*), pH (Yan et al. 2015), ammonia nitrogen (Shen et al. 2014*b*), total inorganic nitrogen (Shen et al. 2014*b*), and C/N (Liu et al. 2015). However, in this study, the results of Spearman rank

correlation analysis suggested that sediment n-damo *pmoA* Shannon diversity might be regulated by the level of nitrite. To the authors' knowledge, this was the first report on the potential role of nitrite level on n-damo *pmoA* Shannon diversity. Moreover, sediment n-damo *pmoA* gene Chao1 richness might be collectively influ-

enced by a number of environmental factors, including TOC, TP, NO$_2^-$-N, and TN. This was not consistent with the results found in previous studies (Shen et al. 2014*a*, 2014*b*; Liu et al. 2015; Yan et al. 2015).

Several previous studies showed the existence of diverse n-damo *pmoA* gene in sediment ecosystems (Chen et al. 2014, 2015*a*, 2015*b*; Shen et al. 2014*a*, 2014*b*; Liu et al. 2015; Yan et al. 2015). In this study, the result of phylogenetic analysis indicated that major n-damo *pmoA* OTUs from reservoir sediments could be grouped into 7 distinctive clusters, which suggested the presence of various types of sediment n-damo *pmoA* gene in Gaozhou Reservoir. The obtained n-damo *pmoA* gene sequences could be related to those from a variety of ecosystems, such as river, lake, reservoir and marine estuary sediments, wetland, paddy filed soil, and wastewater bioreactor. This suggested that n-damo organisms could be adapted to many kinds of habitats, and the n-damo bacterial species detected in one type of habitat might occur in other habitats. In addition, several previous studies revealed that sediment n-damo *pmoA* gene sequences from freshwater Lake Constance, Lake Biwa, and Qiantang River were mainly related to that from *Ca.* M. oxyfera (Deutzmann and Schink 2011; Kojima et al. 2012; Shen et al. 2014*b*), while all of the sediment n-damo *pmoA* gene sequences from freshwater lakes on the Yunnan Plateau had no close relationship with that from known NC10 bacteria (Liu et al. 2015). In this study, a small proportion of n-damo *pmoA* gene sequences (19.1%) were closely related to the *pmoA* gene sequence of *Ca.* M. oxyfera. This was not in agreement with the results found in other freshwater sediment ecosystems (Deutzmann and Schink 2011; Kojima et al. 2012; Shen et al. 2014*b*; Liu et al. 2015).

The horizontal change of n-damo *pmoA* gene composition has been documented in a variety of sediment ecosystems (Chen et al. 2014, 2015*a*; Shen et al. 2014*a*, 2014*b*; Liu et al. 2015; Yan et al. 2015). However, information on the sediment-depth-related change of n-damo *pmoA* gene composition is still lacking. In this study, the results of both phylogenetic analysis and UPGMA clustering illustrated the evident horizontal and vertical shift in n-damo *pmoA* gene composition in sediments of the freshwater Gaozhou Reservoir. To date, the links between sediment n-damo *pmoA* gene composition and environmental factors remain largely unclear. Several previous studies suggested that sediment n-damo *pmoA* gene composition might be shaped by ammonia (Chen et al. 2014, 2015*a*; Yan et al. 2015), nitrite (Chen et al. 2014, 2015*a*), nitrate (Chen et al. 2014), total inorganic nitrogen content (Shen et al. 2014*b*), and organic content (Shen et al. 2014*a*, 2014*b*; Yan et al. 2015). In contrast, in this study, the environmental factor driving the shift in sediment n-damo *pmoA* gene composition was not identified.

In conclusion, sediment n-damo bacterial abundance in the Gaozhou Reservoir displayed vertical and horizontal changes. The richness, diversity, and structure of sediment n-damo *pmoA* gene sequences showed a considerable spatial shift in the freshwater Gaozhou Reservoir. Nitrite might be a key determinant to sediment n-damo *pmoA* gene diversity, while TOC, TP, nitrite, and TN collectively shaped *pmoA* gene richness.

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