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Distribution and Diversity of Nitrite-Dependent Anaerobic Methane-Oxidising Bacteria in the Sediments of the Qiantang River

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Abstract Nitrite-dependent anaerobic methane oxidation (n-damo) process was reported to be mediated by “Candidatus Methylomirabilis oxyfera”, which belongs to the candidate phylum NC10. M. oxyfera-like bacteria have been detected in lake ecosystems, while their distribution, diversity and abundance in river ecosystems have not been well studied. In this study, both the 16S rRNA and the pmoA molecular biomarkers confirmed the presence of diverse NC10 phylum bacteria related to M. oxyfera in a river ecosystem—the Qiantang River, Zhejiang Province (China). Phylogenetic analysis of 16S rRNA genes demonstrated that the recovered M. oxyfera-like sequences could be grouped into several distinct clusters that exhibited 89.8 % to 98.9 % identity to the M. oxyfera 16S rRNA gene. Similarly, several different clusters of pmoA gene sequences were observed, and these clusters displayed 85.1–95.4 % sequence identity to the pmoA gene of M. oxyfera. Quantitative PCR showed that the abundance of M. oxyfera-like bacteria varied from $1.32 \pm 0.16 \times 10^6$ to $1.03 \pm 0.12 \times 10^7$ copies g (dry weight)$^{-1}$. Correlation analysis demonstrated that the total inorganic nitrogen content, the ammonium content and the organic content of the sediment were important factors affecting the distribution of M. oxyfera-like bacterial groups in the examined sediments. This study demonstrated the distribution of diverse M. oxyfera-like bacteria and their correlation with environmental factors in Qiantang River sediments.

Introduction

Methane is a key player in the carbon cycle [33], which is an important greenhouse gas and is approximately 20 times more effective in heat trapping in the atmosphere than carbon dioxide (CO$_2$) on a per-molecule basis [13]. It is estimated that methane is responsible for 20 % of global warming [16]. A major sink for methane in natural ecosystems is its microbial oxidation. Methane can be oxidised aerobically with oxygen as the electron acceptor, and anaerobic oxidation of methane can be coupled to sulphate reduction in anoxic environments [1, 25]. Sulphate-dependent anaerobic methane oxidation has been identified as a very important process in reducing the emission of the greenhouse gas methane from the ocean into the atmosphere [2, 17], and it is estimated that 80 % of the methane that arises from marine sediments is oxidised anaerobically by this process [8].

The process of nitrite-dependent anaerobic methane oxidation (n-damo), which refers to the oxidation of methane coupled to the reduction of nitrite under anaerobic conditions, was predicted to be a more thermodynamically favourable process than the process of sulphate-dependent anaerobic methane oxidation [30]. However, the occurrence of the n-damo process and the microorganisms responsible for carrying out this process were not confirmed until their discovery in an enrichment culture [26]. This process was reported to be...
performed by a bacterium named “Candidatus Methylomirabilis oxyfera” (M. oxyfera) [6], which is affiliated with the candidate division NC10, a phylum without a member of growing in pure culture [27].

The n-damo process constitutes a unique link between two major global elements essential to lives, the carbon and nitrogen, and can be an important and hitherto overlooked sink for methane in natural ecosystems [26, 29]. Although n-damo process is energetically favourable, proof of its occurrence in natural habitats is scarce. Two recent studies reported the presence of M. oxyfera-like bacteria in two freshwater lake ecosystems [3, 17]. Deutzmann and Schink [3] provided evidence for the presence of M. oxyfera-like bacteria and their activities in the natural freshwater sediments of Lake Constance, an oligotrophic freshwater lake. M. oxyfera-like 16S rRNA and pmoA gene sequences were also detected in the denitrifying zone of freshwater sediments of Lake Biwa, Japan [17]. In addition, Wang et al. [34] and Zhu et al. [36] reported the distribution of M. oxyfera-like bacteria in a paddy field that had been subjected to long-term fertilisation and a minerotrophic peatland, respectively.

The n-damo process is predicated to occur mainly proximal the oxic-anoxic interface where presents low concentrations of oxidisable substrates other than methane, and with low level of sulphate, but high level of nitrate [24, 33]. Environments with such ecological parameters in the anoxic freshwater river sediments that contaminated by agricultural run-off are prevalent. This suggests that, in addition to freshwater lake sediments, river sediments might also be suitable habitats for M. oxyfera.

Therefore, the present study aims to survey the distribution, diversity and abundance of M. oxyfera-like bacteria in the Qiantang River, a fresh water river located in Zhejiang Province (China), and to examine the main environmental factors influencing the distribution of these bacteria in this river system to better understand the microbial diversity and distribution of M. oxyfera-like bacteria in freshwater ecosystems.

**Materials and Methods**

**Sediment Collection and Chemical Analyses**

Qiantang River is an important river system located between 117.62° to 121.87° east longitude and 28.17° to 30.48° north latitude. Its total length is 688 km, with a watershed area of 55,600 km². The sediment samples (7 samples) examined in this study were collected along the river from Lanxi City (upstream) to Hangzhou City (downstream) in Zhejiang Province in September 2009 (Fig. S1) [10]. The sampling site JJY is located in Lanxi City and the sampling sites MC and XY are located in Jiande City. The sampling sites BQ and ZX are located in Tonglu City and the sampling site YS is located in Fuyang City. The sampling site JX is located in Hangzhou City. The average water depth and temperature of the examined sampling sites were approximately 6–7 m and 16 °C, respectively. Sediments were obtained using box-cores, and the top 3 cm of sediment was carefully collected. The samples were then put into sterile containers, sealed and transported to the laboratory on ice within 24 h. All of the collected samples were sectioned into two subsamples: one was stored at 4 °C for analysis of physical and chemical properties, and the other was frozen at −80 °C for molecular analysis. The physical and chemical properties of the collected sediments have previously been described in detail [10].

**DNA Extraction and PCR Amplification**

DNA was extracted using the Power Soil DNA kit (Mo Bio Laboratories, Carlsbad, California, USA) according to the manufacturer’s instructions. Quality of the extracted DNA was examined by electrophoresis on 1.0 % agarose gel.

Different combinations of primers (Table S1) were used for the amplification of the 16S rRNA and the pmoA genes of M. oxyfera-like bacteria. The 16S rDNA genes were finally amplified using a nested PCR approach based on the amplification yield. In the first round of PCR, the M. oxyfera-like bacterial specific forward primer 202F [5] and the general bacterial reverse primer 1545R [14] were used. In the second round, the PCR reaction was performed using primers qP1F and qP2R, which are specific for M. oxyfera-like bacteria [5]. The PCR reaction mixture and thermal cycling programs were performed as previously described [20].

A nested PCR approach was finally applied to amplify the M. oxyfera-like bacterial pmoA genes based on the amplification yield. In the first round of PCR, the forward primer A189_b [21] and the reverse primer cmo682 [21] were used. In the second round, the PCR reaction was conducted using the M. oxyfera-like bacterial specific primers cmo182 and cmo568 [21]. The PCR reaction mixture and thermal cycling programs were performed as previously described [21].

**Cloning and Sequencing**

The PCR products were cloned using the pMD19-T vector (TaKaRa, Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Randomly selected clones (at least 20 clones) from each sample were subjected to sequencing (Shenzhen Huada Genomics Institute, Shenzhen, China).

**Phylogenetic Analysis**

Phylogenetic analyses of the 16S rRNA and pmoA gene sequences were conducted with Mega 5 software [31] using the neighbour-joining method. Bootstrap analysis with 1,000 replicates was applied to examine the confidence levels of the clustering of the trees.
Quantitative PCR (qPCR)

The primer set qP1F–qP1R (Table S1) targeting the 16S rRNA gene was used to quantify the abundance of *M. oxyfera*-like bacteria. The qPCR was performed using an iCycler iQ5 thermocycler (Bio-Rad, California, USA) [9]. The PCR reaction mixture and thermal cycling program were performed as previously described [5]. The standard curve was constructed from a series of tenfold dilutions of a known copy number of plasmid DNA containing the target gene. The consistency of the qPCR assay makes it a good method for detection of the copy numbers of the 16S rRNA genes of *M. oxyfera*-like bacteria in the collected samples ($R^2$=0.995; Fig. S2). Triplicate qPCR reactions were performed for each sample and each dilution.

Statistical Analyses

According to a species delineation of 97% identity of the 16S rRNA gene for bacteria in general and 93% for the *pmoA* gene for methanotrophic bacteria [22], operational taxonomic unit (OTU) cut-off values of 3% and 7% were applied to determine the 16S rRNA gene and the *pmoA* gene diversity of *M. oxyfera*-like bacteria, respectively, as determined by DOTUR program [28]. DOTUR was also used to generate the Chao1 estimator and the Shannon index. The coverage of the clone libraries was calculated as previously described [7]. The distribution of *M. oxyfera*-like bacterial assemblages and their correlations with environmental factors were determined using principal components analysis (PCA) and canonical correspondence analysis (CCA), respectively, using the CANOCO software [32]. Pearson correlation analyses were used to test the correlations between *M. oxyfera*-like bacterial diversity, abundance and different environmental factors using the SPSS 18.0 software (SPSS, Chicago, Illinois, USA).

Nucleotide Sequence Accession Numbers

The sequences reported in this study have been deposited in the GenBank database under accession numbers KC503558-KC503613 (*M. oxyfera* 16S rRNA) and KC503614-KC503662 (*M. oxyfera pmoA*).

Results

Physicochemical Characteristics of the Studied River Sediments

The physicochemical characteristics of the collected sediment samples are summarised in Table S2. The collected samples contained relatively high content of total nitrogen (TN), ranging from 173.9 to 1,491.5 mg kg$^{-1}$. The total inorganic nitrogen (TIN) and organic nitrogen (OrgN) contents of the sediments varied from 37.4 to 183.6 mg kg$^{-1}$ and 136.5 to 1,385.6 mg kg$^{-1}$, respectively. The main component of TIN in the collected samples was in the form of ammonium, in the range of 37.0 to 182.0 mg kg$^{-1}$. The content of nitrate was much lower compared with ammonium, ranging from 0.4 to 16.9 mg kg$^{-1}$. The nitrite concentration was close to the detection limit (approximately 0.1 mg kg$^{-1}$). The organic carbon (OrgC) contents of the collected samples varied from 13.4 to 34.2 g kg$^{-1}$.

Phylogenetic Diversity of *M. oxyfera*-Like Bacterial 16S rRNA Genes in the Sediment

A total of 162 16S rRNA gene sequences were obtained from the collected sediment samples using a nested PCR approach. Cloning and sequence analyses confirmed that approximately 96.9% of the amplified sequences (157 sequences) were identified as NC10 phylum bacteria related to *M. oxyfera*. A total of 15 OTUs were observed in the collected samples based on DOTUR analysis using 3% differences in the nucleotide sequences. Phylogenetic analysis of 16S rRNA genes demonstrated that the 15 OTUs could be grouped into 5 distinctive clusters (Fig. 1). These clusters could be assigned to two groups of *M. oxyfera*-like bacteria, namely, group A and group B, according to Ettwig et al. [5] (Fig. 1). Clusters I and II fall into group A. Sequences of cluster I, which were recovered from samples BQ, JY, JX, YS and ZX, showed a high identity to the 16S rRNA gene of *M. oxyfera*, with 97.1–98.9% identity. The most abundant clones were grouped in this cluster, which accounted for approximately 58.0% of the retrieved 16S rRNA gene sequences. Sequences of cluster II, which could be detected in samples BQ, MC and XY, were somewhat less related to the 16S rRNA gene of *M. oxyfera*, with 95.6–96.1% identity. This cluster was most closely related to the clones recovered from paddy fields in Southern China [34], with 97% identity. Cluster II was the second-largest cluster and was responsible for approximately 15.3% of the recovered 16S rRNA gene sequences. The remaining clusters (clusters III, IV and V) fall into group B of *M. oxyfera*-like bacteria and accounted for 26.7% of the obtained 16S rRNA gene sequences. Sequences of cluster III, which were detected in samples BQ, MC, YS and ZX, were distantly related to *M. oxyfera*, with 93.3–93.9% identity of 16S rRNA gene. This cluster was also most closely related to the sequences retrieved from paddy fields [34], with 97% identity. Sequences of cluster IV, which were detected in samples MC, XY and ZX, showed relatively low identity (92.2–93.2%) to the 16S rRNA gene of *M. oxyfera*. Sequences of cluster V, which were recovered from samples JX, XY, YS and ZX, showed even lower identity (89.8–91.1%) to the 16S rRNA gene of *M. oxyfera*. Cluster IV was most closely related to the clones recovered from the inoculum of the n-damo enrichment
culture [5] with 97% identity, and the closest relatives of cluster V were the sequences retrieved from paddy soil [34] and Lake Biwa [17], with 96% identity.

The diversity of the 16S rRNA genes of \( M. \) oxyfera-like bacteria in each sample was compared based on the number of OTUs, the Shannon index and the \( S_{\text{cha}} \)1 estimators (Table 1). The library coverage values ranged from 0.90 to 1.00 (Table 1), indicating that the 16S rRNA gene sequences of \( M. \) oxyfera-like bacteria in Qiantang River sediments were sufficiently overrepresented in these clone libraries. Of all the collected samples, sample BQ showed the highest 16S rRNA gene diversity, and samples JX, MC and YS showed relatively lower 16S rRNA gene diversity. Samples JJY, XY and ZX exhibited a moderate level of 16S rRNA gene diversity (Table 1).

**Phylogenetic Diversity of \( M. \) oxyfera-Like Bacterial pmoA Genes in the Sediment**

Because the genome of \( M. \) oxyfera contains the complete pathway for the aerobic oxidation of methane [6], the pmoA gene, which encodes one of the subunits of the pMMO

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**Table 1** Diversity of \( M. \) oxyfera-like bacterial 16S rRNA and pmoA gene sequences recovered from Qiantang River sediments

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of sequences</th>
<th>Number of OTUs</th>
<th>Coverage</th>
<th>Shannon</th>
<th>Chao1</th>
</tr>
</thead>
<tbody>
<tr>
<td>JX</td>
<td>21</td>
<td>23</td>
<td>4 3</td>
<td>0.90</td>
<td>0.96</td>
</tr>
<tr>
<td>YS</td>
<td>21</td>
<td>24</td>
<td>4 1</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>ZX</td>
<td>21</td>
<td>21</td>
<td>5 1</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>BQ</td>
<td>26</td>
<td>26</td>
<td>7 9</td>
<td>0.96</td>
<td>0.92</td>
</tr>
<tr>
<td>MC</td>
<td>22</td>
<td>25</td>
<td>3 3</td>
<td>0.95</td>
<td>0.96</td>
</tr>
<tr>
<td>XY</td>
<td>25</td>
<td>26</td>
<td>5 4</td>
<td>0.96</td>
<td>0.92</td>
</tr>
<tr>
<td>JJY</td>
<td>21</td>
<td>28</td>
<td>4 4</td>
<td>1.00</td>
<td>0.93</td>
</tr>
</tbody>
</table>

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---

**Fig. 1** Neighbour-joining phylogenetic tree showing the phylogenetic affiliations of \( M. \) oxyfera-like bacterial 16S rRNA gene sequences (460 bp) recovered from the Qiantang River. Bootstrap values were 1,000 replicates, and the scale bar represents 2% sequence divergence.
complex, can be used as a functional marker to monitor and identify *M. oxyfera*-like bacteria on a functional level [21]. A total of 193 *pmoA* gene sequences were retrieved from the collected sediment samples using a nested PCR approach. Cloning and sequence analysis confirmed that approximately 90 % of amplified sequences (173 sequences) were related to the *pmoA* gene of *M. oxyfera*. A total of 13 OTUs were observed in the collected samples based on DOTUR analysis using a 7 % cut-off value. Phylogenetic analysis of the *pmoA* genes demonstrated that the 13 OTUs could be assigned to 5 distinctive clusters related to *M. oxyfera* (Fig. 2). Sequences of cluster I, which could be detected in all collected samples, showed 93.1–95.4 % identity to the *pmoA* gene of *M. oxyfera*. This cluster was the dominant cluster and was responsible for approximately 57.2 % of the retrieved *pmoA* gene sequences. Sequences of cluster II, which were found in samples BQ, JJY and XY, showed 90.9–92.0 % identity to the *pmoA* gene of *M. oxyfera*. Sequences of cluster III, which were detected in samples JJY and MC, showed 90.2–90.5 % identity to the *pmoA* gene of *M. oxyfera*. Cluster III was most closely related to the clones recovered from an enrichment culture of *M. oxyfera* [20], with 94 % identity. Sequences of cluster IV, which were detected in samples BQ, JX and XY, showed 88.7–89.9 % identity to the *pmoA* gene of *M. oxyfera*. The closest relatives of cluster IV were the sequences retrieved from Lake Biwa [17] and a peatbog [21], with 96 % identity. Sequences of cluster V, which were found in samples JX, MC and XY, showed 85.1–86.4 % identity to the *pmoA* gene of *M. oxyfera*. This cluster was most closely related to the clones recovered from paddy fields [34], with 88 % identity.

The diversity of the *pmoA* genes of *M. oxyfera*-like bacteria in each sample was also compared based on the number of OTUs, the Shannon index and the *S_{chao1}* estimators (Table 1). The values of *pmoA* gene library coverage ranged from 0.92 to 1.00 (Table 1), indicating that the *pmoA* gene sequences of *M. oxyfera*-like bacteria in the collected sediments were sufficiently overrepresented in these clone libraries. The diversity of *M. oxyfera*-like bacterial *pmoA* genes observed in each samples was similar to the observed diversity of the 16S rRNA genes. Among the collected samples, sample BQ showed the highest *pmoA* gene diversity, and samples YS and ZX showed the lowest *pmoA* gene diversity. Samples MC, JX, JJY and XY exhibited a moderate level of *pmoA* gene diversity (Table 1).

**Quantitative Analysis of *M. oxyfera*-Like Bacterial 16S rRNA Genes in the Sediment**

The abundance of *M. oxyfera*-like bacteria in each sample was determined by means of qPCR as previously described [5].
The copy number of \( M. \text{oxyfera} \)-like bacterial 16S rRNA genes ranged from \( 1.32\pm0.16\times10^6 \) to \( 1.03\pm0.12\times10^7 \) copies g (dry weight)\(^{-1} \) (Fig. 3). Different copy numbers of \( M. \text{oxyfera} \)-like bacterial 16S rRNA genes could be observed in different sediment samples, suggesting a heterogeneous distribution of these bacteria. The highest copy number was found in sample MC, and the lowest one was observed in sample JX. The remaining samples showed moderate copy numbers of \( M. \text{oxyfera} \)-like bacterial 16S rRNA genes (Fig. 3).

Spatial Distribution of \( M. \text{oxyfera} \)-Like Bacterial Assemblages and Their Relationships With Environmental Factors in the Sediment

The community structure of \( M. \text{oxyfera} \)-like bacterial assemblages in most collected samples appeared to differ substantially as revealed by the PCA test based on the recovered 16S rRNA and \( pmoA \) gene sequences (Fig. S3). According to the PCA analysis using the 16S rRNA gene sequences, \( M. \text{oxyfera} \)-like bacterial assemblages fell into four groups (Fig. S3a). Samples JJY, JX and ZX showed a similar community structure of \( M. \text{oxyfera} \)-like bacterial assemblages, while MC and XY shared a similar community structure. In contrast, the community structures of \( M. \text{oxyfera} \)-like bacterial assemblages in samples BQ and YS were sufficiently different from each other. PCA analysis using the \( pmoA \) gene sequence data showed a slightly different classification, with YS being more similar to JJY, and JX and ZX falling into the same group (Fig. S3b).

To find correlations between the distribution of \( M. \text{oxyfera} \)-like bacterial assemblages and the environmental variables of the Qiantang River sediments, CCA was conducted based on the 16S rRNA and \( pmoA \) gene sequences and the physicochemical parameters recovered from this river. CCA test with the \( pmoA \) gene sequences suggested that the sediment TIN content and the ammonium content appeared to be the most significant of the investigated environmental factors with respect to the variation of \( M. \text{oxyfera} \)-like bacterial assemblages in the examined samples (\( p<0.05 \), 1,000 Monte Carlo permutations; Fig. 4). Furthermore, Pearson moment correlation analyses showed that both the 16S rRNA gene diversity and \( pmoA \) gene diversity of \( M. \text{oxyfera} \)-like bacteria significantly correlated with the sediment TIN content and the ammonium content, and the 16S rRNA gene abundance strongly correlated with the sediment OrgC content (Table 2).

Discussion

Previous studies indicated that the slow-growing \( M. \text{oxyfera} \)-like bacteria were mainly present in only profundal sediments of lakes [3, 17] and deep layers of paddy soil [34] and peatland [36] characterised by stable environmental conditions. In the present study, both the 16S rRNA and \( pmoA \) genes confirmed the presence of \( M. \text{oxyfera} \)-like bacteria in the Qiantang River, where the environmental conditions change greatly (e.g. changes in pH, Eh, temperature and nutrient concentration) [10, 12, 18]. Quantitative PCR further confirmed the presence of \( M. \text{oxyfera} \)-like bacteria in this river ecosystem, with the abundance varying from \( 1.32\pm0.16\times10^6 \) to \( 1.03\pm0.12\times10^7 \) copies g (dry weight)\(^{-1} \). These values were greater than the previous reported abundance found in lake ecosystems (\( 10^5 \)–\( 10^6 \) copies g (dry weight)\(^{-1} \)) [17], and a paddy field, which contained \( 10^3 \)–\( 10^5 \) copies g (dry weight)\(^{-1} \) [34]. The results of this study suggested that \( M. \text{oxyfera} \)-like bacteria can adapt to the dynamic environmental conditions of the Qiantang River, thus significantly expanding the knowledge of the biogeography of \( M. \text{oxyfera} \).

Five different clusters of 16S rRNA genes related to \( M. \text{oxyfera} \) were recovered from the collected samples (Fig. 1), and a total of 15 OTUs (6 OTUs in group A and 9 OTUs in group B) could be observed based on 3 % differences in the detected 16S rRNA genes. Deutzmann and Schink [3] examined the diversity of \( M. \text{oxyfera} \)-like bacteria in an oligotrophic freshwater lake, Lake Constance, and found that only 3 OTUs (1 OTU in group A and 2 OTUs in group B) and 5 OTUs (all the 5 OTUs fall into group B) could be observed in profundal sediments (3 sampling sites) and littoral sediments (2 sampling sites), respectively, based on 3 % differences in the recovered 16S rRNA gene sequences. In addition, Kojima et al. [17] reported the presence of \( M. \text{oxyfera} \)-like in a freshwater lake, Lake Biwa, where 6 OTUs (1 OTU in group A and 5 OTUs in group B) were found in profundal sediments (2 sampling sites) based on 1 % differences in the recovered 16S rRNA gene sequences. In the current study, 5–7 OTUs could be observed in a single sample (samples BQ, XY and ZX), and at least 3 OTUs could be observed in each remaining sample based on 3 % differences in the recovered 16S rRNA
gene sequences (Table 1). Therefore, the overall diversity of *M. oxyfera*-like bacterial 16S rRNA genes in the Qiantang River was higher than that found in other reported habitats.

To detect *M. oxyfera*-like bacteria on a functional level, primers targeting the *pmoA* gene of *M. oxyfera* were used to construct clone libraries of *pmoA* genes. Thus far, the distribution and diversity of the *pmoA* genes of *M. oxyfera*-like bacteria in natural ecosystems was poorly known due to limited data available. The *pmoA* gene sequences could only be detected in the profundal sediments (3 sampling sites) of Lake Constance and showed a maximum sequence divergence of 1.1 % [3]. Similarly, *pmoA* gene sequences were only recovered from the profundal sediments (1 sampling site) of Lake Biwa and were very closely related to each other [17]. In addition, Wang et al. [34] and Zhu et al. [36] reported the presence of *pmoA* gene sequences in the deep layer of paddy soil (3 sampling sites) and peatland (1 sampling site), respectively, in which the recovered sequences showed high identity to each other. In the present study, *pmoA* gene sequences were successfully recovered from all samples collected from Qiantang River sediments. Five distinct clusters of *M. oxyfera* *pmoA* gene sequences have been recovered (Fig. 2), and a total of 13 OTUs were observed based on a 7 % cut-off in the detected *pmoA* genes. Furthermore, more than 3 OTUs could be recovered in a single sample except for samples YS and ZX. Thus the overall diversity of *M. oxyfera*-like bacteria in Qiantang River sediments was much higher than that in the reported lake sediments and wetlands based on the recovered 16S rRNA and *pmoA* gene sequences.

The different PCR amplification methods used could result in the different levels of *M. oxyfera*-like bacterial diversity that were observed. Direct amplification of the 16S rRNA and *pmoA* genes of *M. oxyfera*-like bacteria was applied in Lake Constance [3], Lake Biwa [17] and minerotrophic peatland [36]. In the present study, the nested PCR approaches developed by Luesken et al. [20, 21] were applied to examine the 16S rRNA and *pmoA* gene diversity of *M. oxyfera*-like bacteria in Qiantang River sediments. The nested PCR approach could help detect genes in low abundance compared with a conventional PCR approach [23]. A total of 11 OTUs and 5 OTUs of the 16S rRNA and *pmoA* genes of *M. oxyfera*-like bacteria were calculated in wastewater sludge based on the sequences deposited in GenBank by Luesken et al. [20] using 3 % and 7 % cut-off values, respectively, also exhibiting a relatively higher diversity of *M. oxyfera*-like bacteria.

Therefore, the nested PCR method applied in this study

### Table 2: Pearson correlation analyses of environmental factors and *M. oxyfera*-like bacterial diversity and abundance in Qiantang River sediments

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>Number of OTUs</th>
<th>Shannon</th>
<th>Chao1</th>
<th>Abundance</th>
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<tr>
<td></td>
<td>16S</td>
<td>pmoA</td>
<td>16S</td>
<td>pmoA</td>
</tr>
<tr>
<td>pH</td>
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<td>-0.322</td>
<td>-0.497</td>
<td>-0.270</td>
</tr>
<tr>
<td>OrgC</td>
<td>-0.284</td>
<td>0.250</td>
<td>0.316</td>
<td>0.206</td>
</tr>
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<td>-0.041</td>
<td>0.254</td>
<td>0.376</td>
<td>0.218</td>
</tr>
<tr>
<td>OrgN</td>
<td>-0.132</td>
<td>0.151</td>
<td>0.300</td>
<td>0.115</td>
</tr>
<tr>
<td>TIN</td>
<td>0.776*</td>
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<td>0.769*</td>
<td>0.961**</td>
</tr>
<tr>
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<td>0.977**</td>
<td>0.741</td>
<td>0.972**</td>
</tr>
<tr>
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<td>0.102</td>
<td>0.116</td>
<td>0.364</td>
<td>0.071</td>
</tr>
</tbody>
</table>

*p<0.05 and **p<0.01, respectively, as determined by SPSS version 18.0 program (SPSS, Chicago, Illinois, USA)
provided a higher sensitivity for detection of *M. oxyfera*-like bacteria in the environments. Previous studies indicated that only group A of *M. oxyfera*-like bacteria could be enriched from various habitats [4, 5, 15, 20, 26, 36]. In addition, Deutzmann and Schink [3] found that higher n-damo activities were found in sampling sites where group A members were the dominant *M. oxyfera*-like bacteria, while lower n-damo activities were found in sampling sites where group B members were the dominant *M. oxyfera*-like bacteria. These studies suggested that group A members are the dominant bacteria responsible for carrying out the n-damo process. In the present study, group A members were the dominant *M. oxyfera*-like bacteria (Fig. 1), and the pmoA gene sequences were detected in all of the collected samples. The ubiquitous distribution of group A of *M. oxyfera*-like bacteria and the pmoA gene sequences in the Qiantang River sediments may indicate that the detected *M. oxyfera*-like bacteria are active and thus contribute to the methane and nitrogen cycles in this freshwater habitat. Further studies on the direct measurement of n-damo activity and the abundance of pmoA genes are necessary to enable sound conclusions on the ecological importance of this process in the Qiantang River ecosystem.

An uneven distribution of *M. oxyfera*-like bacterial assemblages was observed in the collected sediments (Table 1 and Figs. 3 and S3), which may have been influenced by environmental factors. Correlation analysis suggested that the sediment TIN content and the ammonium content had a significant influence on the distribution of *M. oxyfera*-like bacterial assemblages in the examined Qiantang River sediments (Table 2). As shown in Table S2, the nitrite concentration was close to the detection limit in most sediment samples. Nitrite is very unstable in nature and quickly reacts with other compounds. This is the possible reason for the low nitrite concentration in the studied sites. It was already hypothesized that *M. oxyfera*-like bacteria may cooperate with other bacteria which reduce nitrate to nitrite or with ammonium-oxidising organisms [11, 35]. It is likely that ammonium oxidation at the sediment surface is the major source of nitrite for *M. oxyfera*-like bacteria because the sediment ammonium content was significantly greater than the nitrate content (Table S2; t test, p < 0.05). Previous study has confirmed the presence of ammonium-oxidising organisms in the sediments of Qiantang River [19]. The copy number of archaeal amoA genes ranged from $6.28 \times 10^7$ to $1.30 \times 10^8$ copies g (dry weight)$^{-1}$ in the examined sediments, and the number of bacterial amoA genes ranged from $2.61 \times 10^6$ to $6.99 \times 10^6$ copies g (dry weight)$^{-1}$. However, in nitrite-limited anoxic systems, *M. oxyfera*-like bacteria have to compete for nitrite with anaerobic ammonium-oxidising bacteria in this river system [37] because both groups depend on nitrite as substrate. Furthermore, the OrgC content was found to be positively correlated with *M. oxyfera*-like bacterial abundance in the examined Qiantang River sediments, suggesting that a higher OrgC content would be more favourable for the growth of *M. oxyfera*-like bacteria. Wang et al. [34] also reported that the abundance of *M. oxyfera*-like bacteria was significantly positively correlated with OrgC content in paddy fields. In addition, previous study indicated that the external oxygen could have adverse impact on the distribution of *M. oxyfera*-like bacteria in a paddy soil [34]. Besides, methane is the substrate of *M. oxyfera*-like bacteria, and the in situ methane concentration may also be the limiting factor for the growth of *M. oxyfera*-like bacteria in environments. Therefore, the oxygen concentration and methane concentration may also have important impact on the distribution of *M. oxyfera*-like bacteria in the examined Qiantang River.

Conclusively, we provided the first direct evidence for the presence of *M. oxyfera*-like bacteria in river systems, thus significantly expanding the knowledge of the biogeography of *M. oxyfera*. Phylogenetic analyses of the 16S rRNA and pmoA genes showed a higher diversity of *M. oxyfera*-like bacteria in the Qiantang River ecosystem than in other freshwater habitats reported to date. The sediment TIN content, the ammonium content and the OrgC content appeared to be the most important environmental factors among those investigated influencing the distribution of *M. oxyfera*-like bacterial assemblages in the examined sediments.

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