Existence of Novel Phylotypes of Nitrite-Dependent Anaerobic Methane-Oxidizing Bacteria in Surface and Subsurface Sediments of the South China Sea

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Nitrite-dependent anaerobic methane oxidation (n-damo) is a recently discovered new microbial process performed by the Candidatus Methylomirabilis oxyfera with an unusual intra-aerobic pathway, but there is no report about n-damo bacteria in marine environments. M. oxyfera-like sequences were successfully retrieved for the first time from both surface and subsurface ocean sediments of the South China Sea (SCS) using both 16S rRNA and pmoA genes as biomarkers and PCR amplification in this study. The majority of M. oxyfera-like 16S rRNA gene-based PCR amplified sequences from the SCS sediments formed a new group distinctively different from those detected in freshwater habitats and the information is consistent phylogenetically with those obtained from the pmoA gene. This study showed the existence of n-damo in ocean sediments and suggests that marine sediments harbor n-damo phylotypes different from those in the freshwater. This finding here expands our understanding on the distribution of n-damo bacteria to marine ecosystem and implies their potential contribution to the marine C and N cycling.

Keywords: marine sediment, n-damo, nitrite-dependent anaerobic methane oxidation, pmoA gene, South China Sea

Introduction

Anaerobic oxidation of methane (AOM) is a very important microbial process to the current global methane cycle as it consumes a large portion of annual CH4 flux into the atmosphere on earth (Smemo and Yavitt 2011). Because the mass-based warming potential of methane in atmosphere is 72 times greater than that of carbon dioxide (IPCC 2007), AOM is also considered as an effective mitigation process against global warming. On the other hand, human activities have transformed global nitrogen cycle significantly and continuously with the large increase of inorganic N load into the environment in the last decades (Galloway et al. 2008). Both denitrification and anaerobic ammonium oxidation (Anammox) play a key role for N loss from the eutrophicated ecosystems (Gruber 2005). Thus, AOM coupled with nitrate/nitrite reduction linking the C and N cycles, has become an attractive research topic in recent years.

Unlike the microbial AOM with sulphate reduction (Boetius et al. 2000), AOM linking to denitrification once was considered to be only thermodynamically possible prior to the successful enrichment of this kind of microbes (Knowles 2005; Raghoebarsing et al. 2006). These biological processes refer to the following reactions (Raghoebarsing et al. 2006):

\[
\begin{align*}
5\text{CH}_4 + 8\text{NO}_3^- + 8\text{H}^+ & \rightarrow 5\text{CO}_2 + 4\text{N}_2 + 14\text{H}_2\text{O} \quad [1] \\
(\Delta G') &= -765 \text{ kJ mol}^{-1}\text{CH}_4 \\
3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ & \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O} \quad [2] \\
(\Delta G') &= -928 \text{ kJ mol}^{-1}\text{CH}_4
\end{align*}
\]

The main dominant microbes in the enrichment culture were bacteria not archaea (Ettwig et al. 2008, 2009). Further studies revealed that the bacteria responsible for the AOM coupled to denitrification were affiliated with NC10 phylum (Ettwig et al. 2009) and no pure culture representatives have been obtained so far (Deutzmann and Schink 2011; Ettwig et al. 2010). This member of the uncultured phylum NC10 (Raghoebarsing et al. 2006) was later named as Candidatus ‘Methylomirabilis oxyfera’ by Ettwig et al. (2010) and formed a novel taxonomic group among bacterial methanotrophs based on 16S rRNA gene sequences (Wu et al. 2011b). Later, the co-localization of particulate methane monooxygenase (pMMO) and the cd1 nitrite reductase (NirS) was established in M. oxyfera (Wu et al. 2012a,b). Recently, Candidatus ‘Methanoperedens nitroreducens’, a novel...
archaeal lineage, was found to be able to reduce nitrate to nitrite and have a syntrophic relationship with an anaerobic ammonium oxidizing bacterium to complete AOM with nitrite reduction (Haroon et al. 2013).

Since the first report on the enrichment culture from freshwater canal sediment (Ettwig et al. 2008; Raghoebarsing et al. 2006), specific PCR primers for detection of \textit{M. oxyfera} based on 16S rRNA (Ettwig et al. 2009) and \textit{pmoA} genes (Deutzmann and Schink 2011; Luesken et al. 2011c) made it possible to study the n-damo microbial community in natural habitats. Deutzmann and Schink (2011) studied denitrifying methanotrophs in sediments of oligotrophic Lake Constance, Germany and provided the first indication that anaerobic methane oxidation coupling to denitrification might be a widespread process in freshwater ecosystem. The distribution of putative n-damo bacteria in sediment of Lake Biwa, Japan was reported and the denitrifying methanotrophs were found to prefer the upper layer of the profundal sediments (Kojima et al. 2012), but abundant in deep layers (30–60 cm) of the paddy soil (Wang et al. 2012). Later, n-damo bacteria were enriched from a peatland sample (Zhu et al. 2012). The discovery of n-damo bacteria from freshwater sediments implies the potential ecological importance of anaerobic oxidation of methane coupling to denitrification (Shen et al. 2012).

However, whether the n-damo process plays an ecological important role in the marine methane oxidation process is still unknown. Recently, co-occurrence of n-damo bacteria and anammox bacteria was reported in the surface sediments of two Qinghai-Tibetan saline lakes (Yang et al. 2012), suggesting n-damo bacteria might reside in marine sediments. Since there is no report about n-damo from marine habitats or publication focusing on the distribution of n-damo bacteria in marine environments as far as we know, it is undoubtedly meaningful to study the diversity and distribution pattern of n-damo bacteria in ocean sediments.

The objectives of the present work were to investigate whether n-damo bacteria exist in the South China Sea (SCS) sediments, where the habitat specific anammox bacteria involved in the N removal were observed in our previous studies (Hong et al. 2011; Li et al. 2013), and to unravel the diversity and distribution pattern of the detected n-damo bacteria compared with those detected in freshwater lakes.

**Materials and Methods**

**Sampling Site Selection and Description**

Four sampling sites, namely E704S, CF5, E702S, and E401 were selected from the continental shelf, the slope and the deep ocean in the northern part of the South China Sea (Figure 1). Sediments were collected during South China Sea Open Cruise by R/V Shiyan 3 in the summer of 2008, transferred aseptically into plastic bags and kept immediately at −20°C in refrigerator. Subsurface sediment CF5 (7.5 m below the seafloor) and E401 (3.7 mbsf) were chosen because surface samples of sediment cores were discarded due to exposure to the air. The site location, in situ seawater depth, salinity, and the temperature of each sample site were measured with a CTD (Sea-bird, NE). Chemical analyses of ammonium-N, nitrate-N and nitrite-N were conducted on pore water obtained after centrifugation according to Hong et al. (2011). All these sediments were confirmed to harbor anammox communities capable of the inorganic N removal process (Hong et al. 2011; Li et al. 2013). The physicochemical characteristics of sediment samples used in the present study are listed in Table 1.

![Fig. 1. Map of sampling sites at the South China Sea. The four sampling sites (CF5, E401, E702S, and E704S) are indicated in the map. The environmental characteristics of the sampling sites are described in Table 1. The map was drawn with Google Maps available online (http://maps.google.com.hk). Sample locations were displayed by using LatLng Tool Tip and Marker through Google Maps Labs.](Image 311x507 to 560x718)

**Molecular Detection of n-Damo Bacteria**

Genomic DNA in the sediment (~0.25 g) was extracted according to the instruction manual of PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA, USA). The yield of DNA was determined using a biophotometer (Eppendorf AG 22331, Hamburg, Germany). PCR reaction mixtures contain (in 50 μl): dNTPs 1 μl (10 mM; Promega, USA), MgCl2 5 μl (25 mM; Promega, Hong Kong), Primer_forward 1 μl (20 μM), Primer_reverse 1 μl (20 μM), DNA 2 μl (30–50 ng/μl), GoTaq Flexi Buffer 10 μl (Promega, Hong Kong), Bovine serum albumin 2 μl (100 mg/ml, Roche), GoTaq Flexi polymerase 0.5 μl (5 U/ml; Promega, Hong Kong), and H2O 27.5 μl. Forward primer 202F specific for NC10 phylum (Ettwig et al. 2009) was used in combination with universal reverse primer 1492R for the amplification of 16S rRNA gene. PCR condition was based on the previous study (Kojima et al. 2012) with the following thermal cycling: initialized with 2 min denaturation at 94°C, followed by 34 cycles of 30 s at 94°C, 30 s annealing at 57°C and
Table 1. Physicochemical characteristics of sediment samples used in the present study*

| Sample name | Latitude  | Longitude | Seawater depth (m) | Temperature (°C) | Salinity (%) | NH₄⁺ (μM) | NO₂⁻ + NO₃⁻ (μM) |
|-------------|-----------|-----------|--------------------|------------------|--------------|-----------|------------------|
| CF5         | 19°55N    | 115°13E   | 1153               | 2–4              | 34.56        | 1.76      | 2.15             |
| E401        | 21°31N    | 119°59E   | 3300               | 2.5              | 34.39        | 5.21      | 6.39             |
| E702S       | 19°38N    | 115°13E   | 2370               | 2.9              | 34.58        | 660.6     | 5.90             |
| E704S       | 20°15N    | 114°44E   | 175                | 13.5             | 34.57        | 120.3     | 12.88            |

*Seawater depth, temperature and salinity refer to the characteristics of bottom water, while NH₄⁺, NO₂⁻, and NO₃⁻ correspond to the values of the pore water in each sediment sample (Hong et al. 2011; Li et al. 2013);
** Meters below seafloor.

60 s elongation at 72°C, and finalized with an additional extension at 72°C for 10 min.

For the PCR amplification of pmoA gene, a nested PCR approach was applied according to Luesken et al. (2011c): 95°C for 5 min; 35 cycles of 95°C for 1 min, an annealing temperature gradient (53–63°C) for 1 min, followed by 72°C for 1.5 min; and finally 72°C for 10 min. After the amplification with forward primer A189_b (Luesken et al. 2011c) and reverse primer cmo682R (Holmes et al. 1995) in nested PCR step 1, primer cmo182 and cmo568 (Luesken et al. 2011c) were used to detect the M. oxyfera-like bacteria in the following nested PCR step 2. Once purified PCR products were examined and resulted in almost no visible band on electrophoresed gel, a “short PCR” was applied using these purified PCR products with the following thermal procedure: 95°C for 5 min; 15 cycles of 95°C for 1 min, an annealing temperature of 55°C for 1 min, followed by 72°C for 1.5 min; and finally 72°C for 10 min.

All PCR products were electrophoresed on 1.2% agarose gels (Promega, Madison, WI, USA), purified using Gel Advanced Gel Extraction System (Viogene-Bio Tek Corporation, Taipei, Taiwan), and stored at −20°C.

Purified PCR products were used to construct the clone libraries with DH5α E. coli cells and pMD-18 T-vector (Takara, Otsu, Japan). A maximum of about 40 clones per library was selected randomly for sequencing. Primer set M13F and M13R were used to verify the inserted DNA fragment through PCR amplification. Sequencing was performed using the Big Dye Terminate kit (Applied Sciences, Foster City, USA) and an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) by Center for Genomic Sciences, The University of Hong Kong.

**Phylogenetic and Community Analyses**

The sequences were analyzed and trimmed together with their phylogenetic relatives retrieved from the GenBank database using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned by ClustalW (Thompson et al. 1994) using MEGA 5.0 software (Tamura et al. 2011) for the reconstruction of phylogenetic trees.

The DNA Models were tested and suggested by Find Best DNA/Protein Models function using MEGA 5.0. The confidence values of the tree nodes were tested by 1,000 cycles of bootstrap sampling and gaps/missing data treatment was completely excluded. For 16S rRNA gene, the phylogeny was reconstructed using the statistical method of maximum likelihood and the substitution model of Kimura-2-parameter. Gamma Disturbed was used for rates among sites while the number of Discrete Gamma Categories was set to be 4. For pmoA gene, the phylogeny was reconstructed using the statistical method of neighbor-joining and the substitution model of p-distance. Uniform rates were used for rates among sites while the pattern of lineages was homogenous.

For the community diversity analyses, n-damo sequences were retrieved from GenBank according to the references (Deutzmann and Schink 2011; Kojima et al. 2012) (Biwa, sequences recovered in Lake Biwa sediments: AB661479-AB661520 [except AB661503, AB661504, AB661514 and AB661519], AB661586 for 16S rRNA gene and AB661605-AB661625 for pmoA gene; Constance, sequences detected in Lake Constance sediments: HQ906501-HQ906564 for 16S rRNA gene and HQ906565-HQ906579 for pmoA gene).

Fast group II (Yu et al. 2006) was applied to analyze the operational taxonomic units (OTUs) by defining 3% differences for 16S rRNA and 5% for pmoA nucleotide sequences, and to generate the diversity index Shannon-Wiener (Magurran 1988) and richness index Chao1 (Chao 1984).

Unifrac (Lozupone and Knight 2005) and FastUnifrac (Hamady et al. 2010) was used to analyze the β diversity of the n-damo community in the present study together with those from two freshwater lakes (Deutzmann and Schink 2011; Kojima et al. 2012) using 16S rRNA and pmoA gene sequences, respectively. Phylogenetic contexts were reconstructed with the same method stated above except neighbor-joining method was used for 16S rRNA tree. Environmental information was prepared according to the Unifrac tutorial (Lozupone et al. 2006).

The GenBank accession numbers reported in the present study are KF528941 to KF528960 and KF742456 to KF742496 for 16S rRNA gene; and KF528961 to KF52898 and KF742444 to KF742455 for pmoA gene.

**Results**

**The Existence of n-Damo Bacteria in South China Sea Sediments**

Based on 16S rRNA gene, PCR amplified sequences with close relatedness to M. oxyfera were found in both E401 and
Fig. 2. Consensus phylogenetic trees constructed after subjecting 16S rRNA gene (a) and pmoA gene (b) sequences to maximum likelihood and neighbor joining analysis, respectively. OTUs were defined with a Percent Sequence Identity of 97% (16S rRNA gene) or 95% (pmoA gene) similarity by Fastgroup II. Sequences in the present study were colored in blue and red for those from sediment E401 and E704S, respectively. Numbers in parenthesis refer to how many clones were retrieved using respective primer sets (16S rRNA and pmoA gene). Numbers at the nodes represent the levels of bootstrap support based on 1000 re-sampled data sets (only >50% values are shown).
E704S of the SCS using primer 202F and 1492R (Figure 2a). Sequences retrieved from E702S and CF5 were not closely related to M. oxyfera (data not shown). The majority of the 16S gene PCR-amplified sequences from the clone library E401 and E704S formed a distinctive new cluster (Cluster e) from the established Clusters a, b, c and d, which were proposed by Ettwig et al. (2009) (Figure 2a). The reference sequences within the Cluster e were mainly uncultured environmental clones from anaerobic methane oxidation or denitrification fields retrieved from GenBank database through BLAST.

Based on pmoA gene, the diversity of PCR amplified sequences from sediment E401 and E704S was much lower than that of 16S rRNA gene (Figure 2b). The PCR amplified products were shown as multibands and barely visible on electrophoresed gel after nested PCR step 1 using primer A189_bF and cmo 682R. For nested PCR step 2, there was no observed product of E702S. The amplification products of E401 and E704S were much brighter but still with multiple bands on electrophoresed gel using primer cmo 182F and cmo 568R, while those of CF5 were shown as faint multiple bands. The bands of expected sizes were excised and purified. After ligated to vector and transformed into competent E. coli, only several clones for all three libraries could be obtained (CF5, E401 and E704S). The purified PCR products were examined and resulted in almost no observed band on electrophoresed gel. Thus, a “short PCR” was applied and resulted in a very bright and single band except sample CF5 (still faint). Clone libraries were constructed using the above re-PCR products. Probably due to the mismatches, recovered sequences of CF5 did not have enough query coverage (around 10%) using BLASTN (Figure S1). The sequences with low query coverage were also found in E401 and E704S (data not shown).

**Community Structure of n-damo Bacteria**

The community diversity of n-damo sequences detected in this study together with those from lake sediments (Deutzmann and Schink 2011; Kojima et al. 2012) was analyzed with software Fastgroup II (Yu et al. 2006) and Unifrac (Lozupone et al. 2006). “Biwa” and “Constance” represent n-damo bacteria detected in Lake Biwa, Japan and Lake Constance, Germany, respectively. Because the 16S rRNA gene sequences of n-damo bacteria from paddy soil (Wang et al. 2012) could not be aligned well with those from Lake Constance (Deutzmann and Schink 2011) due to the lack of overlap region, they could not be taken into account for any comparison in this study.

Results of α diversity analyses revealed that n-damo 16S rRNA gene diversities were much greater than pmoA gene diversities observed in both marine and freshwater sediments, which was consistent with the values of the Shannon-Wiener index (Table 2). The OTU numbers in E704S could be higher if more n-damo related sequences were detected based on rarefaction curves (Figure S2). The β community diversity for each pair of the samples including E401, E704S, Constance and Biwa was performed for the p-test and Unifrac significance (Table 3). Both the p-test and Unifrac significance test between marine sediments (E401 and E704S) and freshwater sediments (Biwa and Constance) were highly significant (p < 0.001). Significance for both tests indicates that less genetic diversity within each community type than between marine and freshwater phylogenetically. The significant p-test for 16S rRNA gene and a non-significant Unifrac test imply that the communities harbor high levels of diversity considering the data obtained. No significant difference was observed only within marine sediments (E401 and E704S) or freshwater sediments (Biwa and Constance) considering Unifrac significance analysis.

Unifrac significance on each environment individually with 1000 tree permutations and no abundance weights showed that pmoA gene-PCR amplified sequences from sample Biwa (p ≤ 0.001) and Constance (p ≤ 0.001) were related to more unique branch than expected when compared with their randomly distribution. But 16S rRNA gene amplified sequences from E401 (p ≤ 0.001) and E704S (p ≤ 0.001) were significant, indicating that n-damo bacteria specific to these locations would be rarely found in others.

Principal coordinate analyses were performed for n-damo bacteria in the above four habitats based on 16S rRNA and pmoA gene sequences, respectively (Figure 3 and S4). Results are in agreement with the significance tests using qualitative measurements as well as quantitative analyses. For analyses of both 16S rRNA and pmoA genes-amplified products, communities from marine and freshwater sediments were separated clearly by the first principle using qualitative measurement (p1 > 47.14%) as well as quantitative analysis (p1 > 67.64%).

The n-damo community structures in the SCS sediments were distinguished from those in lake sediments (p1 in

**Table 2. Alpha diversity of n-damo bacteria in sediment samples**

| Sample | Selected clone 16S rRNA | Selected clone pmoA | n-damo related clone 16S rRNA | n-damo related clone pmoA | OTU 16S rRNA | OTU pmoA | Shannon-Wiener 16S rRNA | Shannon-Wiener pmoA | Chao1 16S rRNA | Chao1 pmoA |
|--------|------------------------|---------------------|-------------------------------|----------------------------|--------------|---------|------------------------|-------------------|-------------|----------|
| E401   | 16S rRNA: 77, 28        | 16S rRNA: 13        | 24                            | 3                          | 2.9922       | 0.9110  | 56                     | 1                 | 10          |
| E704S  | 62                     | 28                  | 29                            | 24                         | 3.1580       | 1.0055  | 146                    | 1                 | 10          |
| Biwa   | 126                    | 21                  | 39                            | 21                         | 5            | 1       | 1.1789                 | 0.0000            | 1           |
| Constance | 65                  | NA                  | 64                            | 15                         | 8            | 6       | 1.4440                 | 1.4878            | 10          |

*Based on percentage sequence identity of 97% similarity for 16S rRNA gene and 95% for pmoA gene.*
Figure 3). For lake sediments, Biwa and Constance harbored similar community structures based on 16S rRNA gene. In contrast, marine sediment E401 and E704S shared identical pmoA gene structure when analyzed without abundance weights (Figure 3b), while their community structures based on 16S rRNA gene were affected largely by the first and second environmental variables with taxonomic abundance (Figure 3c).

Discussion

Although the relationship between anammox and n-damo bacteria seems to be competitive for the electron acceptor (Haroon et al. 2013), two co-cultures of n-damo and anammox bacteria were obtained from freshwater habitats (Luesken et al. 2011a; Zhu et al. 2011) and their co-occurrence was also reported in paddy soil recently (Wang et al. 2012). The concentrations of NO$_x$ in the selected four sample sites ranged from 2.15 to 12.88 $\mu$M, and may not be limited to allow denitrification process, suggested by the residence of detected anammox community responsible for removal of inorganic N in the South China Sea sediments (Hong et al. 2011; Li et al. 2013).

We recovered 16S rRNA and pmoA gene sequences of denitrifying anaerobic methanotrophs from two sediments E401 and E704S using primer sets specific for $M$. oxyfera-like bacteria (Ettwig et al. 2009; Luesken et al. 2011c). Since the discovery of n-damo bacteria by enriching them from freshwater sediments (Raghoebarsing et al. 2006), it is unclear whether AOM coupling to denitrification can actually occur in marine environments (Luesken 2011). It was assumed that n-damo process could occur in ocean habitats, e.g., cold seep sediments, but would be largely limited by the depletion of methane by sulfate reduction before it reached to nitrite-containing sediments (Orcutt et al. 2011). This could be an explanation for the fact there is no publication about the presence of $M$. oxyfera-like bacteria in marine environments. The above findings in this study can be considered as the first evidence of the existence of n-damo bacteria in marine sediments as far as we know.

The majority of n-damo bacteria from South China Sea sediments (Figure 2a) formed a novel Group e based on 16S rRNA gene phylogenetic tree other than the previous named groups (Ettwig et al. 2009), and clustered with some new uncultured clone sequences from some hydrothermal fields or gas-hydrate potential sediments, suggesting that n-damo bacteria in marine sediments shared the same ancestor with Candidatus $M$.oxyfera (Ettwig et al. 2010), but had little variation in evolution history. Phylogenetic analysis of pmoA gene-amplified sequences was consistent with the results obtained with 16S rRNA gene. Besides, topology of pmoA gene phylogenetic tree based translated amino acids (Figure S5) was in agreement with that based on the nucleotides (Figure 2b).

The combination of primer 202F and general primer for 16S rRNA gene resulted in an acceptable amplification in enrichment culture (Ettwig et al. 2009) and natural freshwater sediments (Deutzmann and Schink 2011; Kojima et al. 2012). Results in our work showed that the detection of $M$.oxyfera-like bacteria based on pmoA gene primers was more specific and more efficient than that using 16S rRNA gene-based PCR primers (Han and Gu 2013). Our results here strongly suggested that South China Sea sediment E401 and E704S harbored nitrite-dependent AMO bacteria, which would expand the understanding of the distribution of n-damo bacteria greatly and imply that they might be involved in the marine C and N cycles.

However, it is difficult to conclude that no n-damo sequence was retrieved in the SCS sediments CF5 and E702S due to the lack of electron donor CH$_4$ since methane is known to release from deep ocean sediment. In addition, it is not easy to amplify the 16S rRNA gene of $M$.oxyfera-like bacteria in wastewater sludge samples using a direct PCR, and nine out of ten samples were proven to harbor n-damo bacteria based on a nested PCR (Luesken et al. 2011b).

Meanwhile, the primers used in this study were developed from limited n-damo sequences in freshwater habitats or enrichments, which might result in an apparent under-estimation of the diversity and distribution of n-damo bacteria in marine environments. In our previous study focusing on South China Sea sediments, genus “Scalindua” were the dominant anammox bacteria in the deep-ocean sediment of SCS (Li et al. 2010). It would be of interest and important to prove some marine dominant species responsible for the n-damo process other than $M$.oxyfera-like bacteria in SCS sediments.

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**Table 3. Significance tests of microbial communities for each pair of samples with 1000 tree permutations and no abundance weights**

| Sample  | Unifrac significance | p-test significance |
|---------|----------------------|---------------------|
|         | 16S rRNA             | pmoA                | 16S rRNA             | pmoA                |
|         | Constance E401 E704S | Constance E401 E704S| Constance E401 E704S| Constance E401 E704S|
| Biwa    | 1                    | +**                 | +                    | +                    |
| Constance| +                    | +                   | +                    | +                    |
| E401    | 0.096                | +                   | +                    | +                    |
| E704S   | +                    | +                   | +                    | +                    |

* p-values have been corrected for multiple comparisons using the Bonferroni correction. (Each p-value was multiplied by the number of pairwise comparisons performed.
** + means ≤0.001 highly significant.
There is a significant difference between n-damo community structures in South China Sea sediments and those in lake sediments (Table 3, Figure 3). Due to the limited physicochemical characteristics (Deutzmann and Schink 2011; Kojima et al. 2012), high salinity, pressure and low temperature in the ocean should be the most important environmental factors to shape the community structures of n-damo bacteria between marine and freshwater habitats. Interestingly, the second environmental variable had a great impact on the community structures from marine sediments (E401 and E704S) based on 16S rRNA gene, in contrast they were almost identical based on pmoA gene without taxonomic abundance weights (Figure 3b).

![Figure 3. PCoA based on the UniFrac metric of n-damo bacteria diversity using 16S rRNA (a, c) and pmoA (b, d) gene sequences. The effects of the first and second variations were shown. Red dot: Lake Constance; Blue square: Lake Biwa; Yellow triangle: SCS-E704S; Green triangle: SCS-E401. A and b were qualitative measurement, while c and d were quantitative analyses based on weighted UniFrac with a nonnormalized abundance.](image-url)
An adverse effect of the third principle was observed. Community structures of freshwater sediments (Biwa and Constance) showed an opposite distribution pattern, suggesting that n-damo bacteria display high niche specificity. Using molecular detection method, n-damo sequences were also recovered in the sediments of three freshwater wetlands (Hu et al. 2014). After comparing their community structures with those of SCS, results further suggested that n-damo communities untraveled in the sediments of the South China Sea were clearly different from those in the freshwater habitats (Figure S3). The consistency of the qualitative and quantitative PCoA indicated that these community differences would not be sensitive to the relative taxon abundance.

*M. oxyfera* evolved a newly discovered pathway of intra-aerobic denitrification coupling to AOM, producing oxygen without photosynthesis, and might have supported microorganisms to thrive on the abundant methane in the Archaean atmosphere (Ettwig et al. 2010; Wu et al. 2011b). Under anaerobic condition, *M. oxyfera* could transcribe and express the entire pathway of aerobic methane oxidation by starting with pMMO (Luesken et al. 2012). Therefore, the presence of *M. oxyfera*-like bacteria in marine sediments is important for understanding the evolution of aerobic pathways, i.e., the emergency of oxidative nitrite production (Vlaeminck et al. 2011) in marine microbial communities on early Earth (Holland 2006).

Furthermore, we detected n-damo sequences in both shallow ocean habitat (E704S, 175 m bellow seawater) and deep-sea sediment (E401, 3300 m bellow seawater), which implied that the aerobic metabolism in microbial communities with n-damo bacteria might have continuously played impact on the decomposing of Archaean deposits in deeper and dark oceans even during the first known ice age (2.9 Ga). On the other hand, the discovery of four sets of genes encoding terminal respiratory oxidases in its assembled genome suggested that *M. oxyfera* produces O$_2$ mainly for methane oxidation, and may use the residual oxygen for other purposes (Ettwig et al. 2010; Wu et al. 2011a). Thus, the oxygenic degradation of organic matter in Archaean deep oceans might not be limited by photosynthetic organisms in surface oceans, and therefore, should be greater than previously predicted (Holland 2006).

In conclusion, *M. oxyfera*-like sequences were retrieved from both surface and subsurface sediments of the South China Sea using 16S rRNA and *pmoA* genes as biomarkers. The existence of new denitrifying anaerobic methanotrophs in ocean sediments suggests that marine phyotypes are very different from the freshwater ones. The n-damo bacteria in the ocean ecosystems may contribute to the marine microbial cycling of both carbon and nitrogen.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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