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RESEARCH ARTICLE

# Enrichment of denitrifying methanotrophic bacteria from Taihu sediments by a membrane biofilm bioreactor at ambient temperature

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**Abstract** Denitrification coupled to anaerobic methane oxidation is a recently discovered process performed by bacteria affiliated to the NC10 phylum. These microorganisms could play important roles in the energy-efficient way of anaerobic wastewater treatment where residual dissolved methane might be removed at the expense of nitrate or nitrite. The difficulty to enrich these microorganisms due to a slow growth rate, especially at low temperatures, limited its application in engineering field. In this study, an NC10 bacteria community was enriched from Taihu sediments by a membrane biofilm bioreactor at ambient temperature of 10–25 °C. After 13 months enrichment, the maximum denitrification rate of the enriched culture reached 0.54 mM $day^{-1}$ for nitrate and 1.06 mM $day^{-1}$ for nitrite. Anaerobic methane oxidation coupled denitrification was estimated from the $^{13}C$-labeled $CO_2$ ($^{13}CO_2$) production during batch incubations with $^{13}CH_4$. Furthermore, analysis of 16S rRNA genes clone library confirmed the presence of NC10 phylum bacteria and fluorescence in situ hybridization showed that NC10 bacteria dominated the reactor. All of the results indicated the NC10 bacteria community was competitive in terms of treating nitrate-contaminated water or wastewater under natural conditions.

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## Introduction

Cost-effective denitrification is a major technical challenge in treatment of low C/N ratio wastewater. The studies regarding on cheap carbon and electron sources will help to reduce the cost of wastewater treatment. Methane is one of the major fermentation products during anaerobic digestion of sludge and organic waste. If this part of methane generated on site can be used as the electron donor for denitrification, it will greatly reduce the processing costs (Thalasso et al. 1997; Park and Yoo 2009) and provide a new way for greenhouse gas utilization (Bogner et al. 2008). Denitrification coupled to anaerobic methane oxidation (DAMO) is a recently discovered process performed by bacteria belonging to the NC10 phylum, which can directly use methane as electron donor for denitrification in the absence of oxygen (Eqs. 1 and 2) (Raghoebarsing et al. 2006).

$$5\ CH_4 + 8\ NO_3^- + 8H^+ \rightarrow 4N_2 + 14H_2O + 5CO_2 \quad (1)$$

$$3\ CH_4 + 8\ NO_2^- + 8H^+ \rightarrow 4N_2 + 10H_2O + 3CO_2 \quad (2)$$

The NC10 phylum bacteria were reported to play a key role in the DAMO process. The first DAMO culture was obtained from the freshwater systems in the Netherlands after 16 months enrichment, and two groups of microorganisms were found to dominate the culture, a bacterium affiliated with the NC10 phylum and an archaea distantly related to anaerobic methanotrophic archaea (ANME-II) (Raghoebarsing et al. 2006). Further research showed that this process can be

achieved by NC10 bacteria, designated "Candidatus *Methylomirabilis oxyfera*", without the involvement of archaea (Ettwig et al. 2008; Hu et al. 2009). Pure cultures have not yet been isolated, but it has been detected in a variety of water bodies, such as lake ecosystems (Deutzmann and Schink 2011; Kojima et al. 2012), river ecosystems (Shen et al. 2013), wastewater (Luesken et al. 2011a, b), and paddy field (Wang et al. 2012; Shen et al. 2014; Zhou et al. 2014), while only a few enrichment cultures have been obtained using eutrophic freshwater canal sediments (Raghoebarsing et al. 2006), wastewater treatment sludge (Luesken et al. 2011a, b), a mixture including sediment from a freshwater lake, anaerobic digester sludge and return activated sludge (Hu et al. 2009, 2011), minerotrophic peatland soil (Zhu et al. 2012) as the inoculums, and they were enriched at temperature of 25–35 °C. To date, only several DAMO enrichment cultures have been obtained due to a slow growth rate and a long time to enrich.

Methane is thought to be the key element on NC10 bacteria enrichment due to its low mass transfer in the water (Thauer and Shima 2008). The membrane biofilm bioreactor is a novel system that can enhance the mass transfer (Syron and Casey 2008). The core component of membrane biofilm bioreactor is the hydrophobic hollow-fiber microporous membranes which can be operated at high gas pressures for dissolution, and the surface of membranes could be used as a support layer which improves the retention of microorganisms. Recently, the membrane technology has also been used to enhance methane mass transfer in water and improve its utilization efficiency by NC10 bacteria (Shi et al. 2013; Chen et al. 2014). In addition, temperature is thought to be another important factor which may affect its application in engineering field.

The research objectives were (1) to enrich a DAMO culture at ambient temperature range of 10–25 °C by a membrane biofilm bioreactor, which was different from previous studies, (2) to investigate the nitrate and nitrite removal performance of the DAMO culture using methane as the sole electron donor, and (3) to analyze the biodiversity and abundance of NC10 bacteria in the DAMO culture.

## Materials and methods

### Reactor and operation

A 3.6-L cylindrical membrane biofilm bioreactor with an inside diameter of 7 cm and a height of 94 cm was set up (Fig. 1). It consisted of a cylindrical membrane module which contained 50 hollow fibers of microporous polyvinylidene fluoride (PVDF) membrane with a length 60 cm, an outside diameter 0.11 cm, a pore size 0.16 μm, and a gas permeability coefficient 0.71 mL $(m^2\ s\ Pa)^{-1}$. The total surface area of membrane was 0.05 $m^2$. Sponge cubes (1–1.5 cm on each side) were packed around the membrane to enhance biomass attachment. Methane diffused into the liquid through the microporous membrane under the partial pressures ranging from 10 to 24 kPa.

The reactor was inoculated with sediments sampled from Taihu (31° 01.265′ N, 120° 27.044′ E), the biggest freshwater lake in China. The samples were collected from a depth of 94 cm sediments in June 2011, named TH4, and then kept at 4 °C in the dark. The synthetic medium used to enrich NC10 bacteria contained (g $L^{-1}$): 0.722 $KNO_3$, 1.0 $MgSO_4{\cdot}7H_2O$, 0.27 $CaCl_2{\cdot}2H_2O$, 0.0091 $FeSO_4{\cdot}7H_2O$, 2.0 mL of phosphate buffer, and 1.0 mL of trace mineral solution. The phosphate buffer contained (g $L^{-1}$): 24.4 $KH_2PO_4$ and 10.2 $Na_2HPO_4$. The trace mineral solution contained (g $L^{-1}$): 2.486 $FeSO_4{\cdot}7H_2O$, 0.5 $MnCl_2{\cdot}4H_2O$, 0.05 $ZnCl_2$, 0.101 $NiSO_4{\cdot}6H_2O$, 0.05 $CoCl_2{\cdot}6H_2O$, 0.026 $Na_2MoO_4{\cdot}2H_2O$, 0.05 $H_3BO_3$, 0.31 $CuSO_4{\cdot}5H_2O$, and 35 % HCl 5 mL (Modin et al. 2008), pH 7.0. The medium was boiled to remove dissolved oxygen and continuously sparged with $N_2$ (99.999 % purity) to maintain anoxic condition before added to the reactor in upflow mode with a pump (BT300M, Baoding Longer Precision Pump Co. Ltd., China). The membrane biofilm bioreactor was cultured in batch mode for 13 months at ambient temperature of 10–25 °C through 4 stages (Table 1). To promote the mass transfer in the reactor, the medium was circulated through the reactor since stage II with a peristaltic pump (BT100M, Baoding Longer Precision Pump Co. Ltd., China) at a flow rate of 54 mL $min^{-1}$. During stage IV, nitrite was used as electron acceptor instead of nitrate by changing $NaNO_2$ with $KNO_3$ in the medium. The nitrate (nitrite) concentration in the medium was measured 3–5 times per week, and when it was lower than 1 mg $L^{-1}$, the medium was replaced. During each stage, enriched culture was sampled from the sponge carriers and the denitrification activity was measured as described below.

### Denitrification activity measurement of enriched culture

Anaerobic batch tests were conducted to evaluate the denitrification activity change coupled to anaerobic methane oxidation of enriched culture during the different culture stages. Methane consumption and nitrate (nitrite) conversion rates of enriched culture were determined with 120 mL serum bottles (Meulepas et al. 2009). The boiled and cooled fresh medium was continuously sparged with $N_2$ (99.999 % purity) until 20 mL enriched culture and 60 mL fresh medium were transferred to the serum bottles. After the addition of nitrate (3 mM) or nitrite (1 mM), these bottles were sealed with black butyl rubber stoppers and aluminum caps. The headspaces of these bottles were replaced six times with $N_2$ (99.999 % purity) and vacuumed under the same conditions, and then they were filled with 20 mL methane using a pressure-lock syringe (Baton Rouge, LA USA) to ensure the equal amounts of

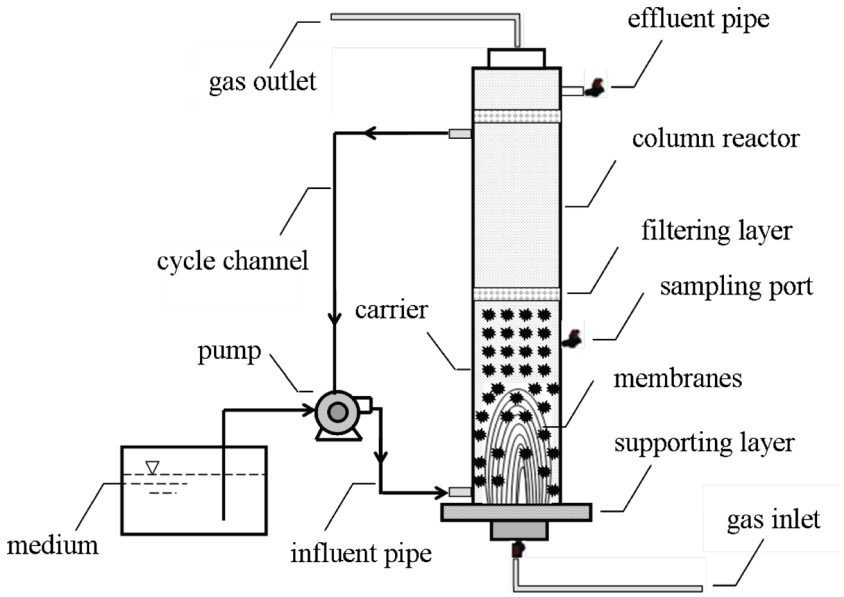


**Fig. 1** Schematic of the constructed membrane-aerated reactor

methane in each bottle. Then, these bottles were incubated in a shaker at 30 °C, 180 rpm. Methane and nitrate (nitrite) consumption in these bottles were determined and each assay was performed in triplicate.

Methane consumption was measured with a gas chromatography (78910A, Agilent, USA) equipped with a thermal conductivity detector. Each sample was measured by manual injection of 100 μL headspace gas with a pressure-lock syringe (Baton Rouge, LA USA) as described previously (Raghoebarsing et al. 2006). Nitrate and nitrite concentrations were measured colorimetrically according to standard methods (WEF 2005).

## Stable isotope tracer

$^{13}$C-labeled $CH_4$ ($^{13}CH_4$) was purchased from Sigma (Aldrich, 490229). The tests were carried out with 120 mL serum bottles using $^{13}CH_4$ as the sole electron donor and nitrate (nitrite) as the sole electron acceptor with three replicates, and the operation was the same as the anaerobic batch test described previously. The batch tests without $^{13}CH_4$ or nitrate (nitrite) were used as controls. The $^{13}C$ contents of $CH_4$ and $CO_2$ were analyzed with gas chromatograph-combustion-isotope ratio mass spectrometry (GC-C-IRMS) system (Thermo Finnigan, Germany).

**Table 1** Operating conditions of the membrane biofilm bioreactor during the enrichment of inoculated sediments

| Stage | I | II | III | IV |
|---|---|---|---|---|
| Internal circulation | No | Yes | Yes | Yes |
| Incubation time (month) | 2 | 2 | 5 | 4 |
| Temperature (°C) | 15-25 | 15-20 | 10-15 | 10-25 |
| N feeding | $NO_3^-$ | $NO_3^-$ | $NO_3^-$ | $NO_2^-$ |
| Methane pressure (kPa) | 24 | 17 | 10 | 20 |

I: from day 0 to day 60; II: from day 61 to day 120; III: from day 121 to day 210; IV: from day 211 to day 410

## Molecular analysis

### *Phylogenetic analysis*

Total genomic DNA in the enriched culture samples was extracted using a PowerSoil DNA isolation kit (Mo Bio Laboratories). The diversity of NC10 phylum bacteria was analyzed by constructing a 16S rRNA gene library. Fragments of 16S rRNA gene were amplified using NC10 specific forward primer 202 F and general bacterial reverse primer 1545R (Ettwig et al. 2009; Juretschko et al. 1998). Then, the obtained PCR products were used as templates for a nested PCR with NC10-specific primers qP1F and qP2R (Ettwig et al. 2009; Luesken et al. 2011a, b). The amplification protocol for both PCR entailed 1 cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min (step 1), 65 °C for 1 min (step 2), and 72 °C for 3 min (step 3). Additional elongation step at 72 °C for 10 min was performed. The PCR products were cloned with the pGEM-T Easy vector (Promega) and JM109 competent cells (Promega). Positive clones were selected and sequenced. Sequences were analyzed in the Genbank database (http://www.ncbi.nlm.nih.gov), and operational taxonomic units (OTUs) were defined with Dotur program at a cutoff value of 0.02.

Phylogenetic tree was performed with ClustalX and MEGA 4.0 by using the neighbor-joining methods and bootstrapping with 1000 repetitions as previously described (Tamura et al. 2007). The partial 16S rRNA gene sequences of

the enrichment culture have been deposited in the Genbank database with accession numbers of JX235723-JX235831.

*Fluorescence in situ hybridization*

The reactor was disassembled at the filtering layer in the middle of the reactor (Fig. 1). The enriched culture samples for fluorescence in situ hybridization (FISH) were obtained from the surface and surrounding of the PVDF membrane, then the reactor was reinstalled and run under anoxic condition as short time as possible. Biomass (2 mL culture liquid) sampled from the enriched culture was collected regularly, fixed, washed, stored, and hybridized as described previously with modifications (Ettwig et al. 2008; Luesken et al. 2011a, b). The pellets were washed with phosphate-buffered saline (PBS; 10 mM $Na_2HPO_4/NaH_2PO_4$ pH 7.2 and 130 mM NaCl) and fixed for 1–3 h with 4 % (*w*/*v*) paraformaldehyde in PBS on ice. After washed with PBS, samples were stored in PBS/ethanol (1:1) at −20 °C. Fixed biomass (5–10 μL) was spotted on gelatin-coated microscopic slide and sequentially dehydrated in 50, 80, and 96 % ethanol for 3 min. Each probe was hybridized for 1.5 h at 46 °C in hybridization buffer (900 mM NaCl, 2 mM Tris/HCl pH 8.0, 0.2‰ sodium dodecyl sulfate, 40 % formamide, and a combination of the following oligonucleotide probes: S-*-DBACT-1027-a-A-18, specific for bacteria affiliated with the NC10 phylum (Raghoebarsing et al. 2006), and S-DBact-0338-a-A-18 (EUB338), specific for most bacteria (Daims et al. 1999). For image acquisition, a Nikon A1Rs confocal laser scanning microscope was used together with the NIS-Elements software package (Nikon, Japan). Percentages of phylogenetic groups were estimated based on visual inspection of at least one complete well.

## Results and discussion

### Denitrification activity change of the enriched culture

Denitrification activity change of enriched culture during the different culture stages by the membrane biofilm bioreactor is shown in Fig. 2. After the first 2 months of culture, the nitrate removal rate of the enriched culture reached 1.27 mM $day^{-1}$, which was, on average, determined as the slope of the curve in Fig. 2 (stage I), but the nitrate could not always be completely consumed. After the medium was circulated since stage II, the nitrate removal rate increased to 2.36 mM $day^{-1}$ (stage II), but decreased to 0.35 mM $day^{-1}$ (stage III) along with depletion of organic residues (chemical oxygen demand of 29.09 mg $L^{-1}$) contained in the inoculums. Then, nitrate removal rate increased again along with the enrichment evolution and reached 0.54 mM $day^{-1}$ (stage IV), and the nitrite removal in the culture also showed a higher rate of 1.06 mM $day^{-1}$. Since during this period, organic residues were depleted, and

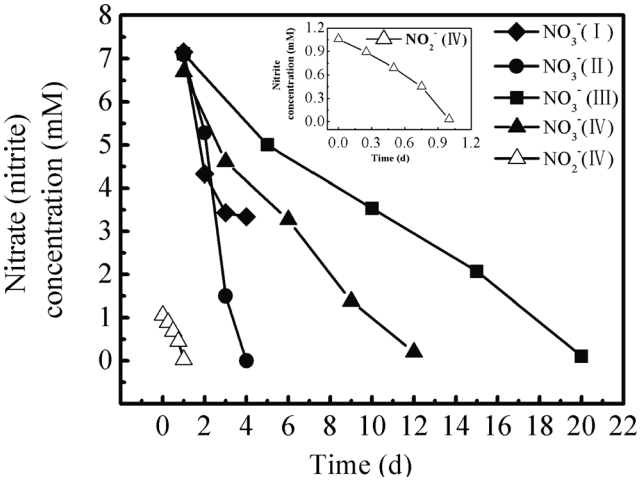


**Fig. 2** Denitrification activity changes of the enriched cultures sampled from the membrane biofilm bioreactor during different culture stages. *I*: from day 0 to day 60; *II*: from day 61 to day 120; *III*: from day 121 to day 210; *IV*: from day 211 to day 410

denitrification was presumed to be mainly contributed by coupling with methane. In addition, the relative abundance of NC10 exhibited a marked increase and reached 73 % at the 13th month during stage IV (data showed below), which was closely related to the performance of the reactor. Previous study showed that the nitrate removal rate was independent of methane partial pressure when superior or equal to 8.8 kPa (Islas-Lima et al. 2004). In this study, methane partial pressure is not the rate-limiting factor for denitrification. Residual organic carbon, operation conditions, and microbial enrichment may affect nitrate removal rate during different stages.

After 13 months of enrichment at ambient temperature of 10–25 °C, the maximum nitrate reducing activity in the membrane biofilm bioreactor was 0.54 mM $day^{-1}$ and nitrite reducing activity was 1.06 mM $day^{-1}$ (equivalent to 14 mg $NO_2$–N $(Ld)^{-1}$), indicating that nitrite was preferred over nitrate as an electron acceptor, consistent with previous studies (Raghoebarsing et al. 2006; Ettwig et al. 2008). The nitrate removal rate 0.54 mM $day^{-1}$ was almost the reported maximum value achieved at shorter enrichment time without temperature control, except for 2.0 mM $day^{-1}$ obtained at temperature of 35 °C (Raghoebarsing et al. 2006; Hu et al. 2009, 2011). Although the nitrite removal rate 14 mg$NO_2$–N $(Ld)^{-1}$ (equivalent to 1.06 mM $day^{-1}$) was lower than the maximum nitrite removal rate of 37.8 mg$NO_2$–N $(Ld)^{-1}$, which was achieved after 22 months of enrichment at temperature of 30 °C reported by Kampman et al. (Kampman et al. 2012), it was similar to those reported by other studies (Raghoebarsing et al. 2006; Luesken et al. 2011a, b; Zhu et al. 2012), even though our DAMO culture was enriched at ambient temperature (10–25 °C). Generally, nitrate was the major component of TN in wastewater. Our DAMO culture had more advantages to treat nitrate-contaminated water

or wastewater under natural conditions compared to other DAMO cultures.

### Coupling denitrification and methane consumption

After 13 months enrichment, coupling denitrification and methane consumption of the enriched biomass were investigated. The measured anaerobic methane oxidizing activity was 0.6 μmol $day^{-1}$ and nitrite removal activity was 2.14 μmol $day^{-1}$ (Fig. 3a). The observed stoichiometry of methane consumption versus nitrite conversion was 3 $CH_4$:10.6 $NO_2^-$, which slightly deviated from the theoretical values 3 $CH_4$: 8$NO_2^-$ (Raghoebarsing et al. 2006) due to the residual electron donors like organics or ammonium may also be involved in the denitrification. Simultaneously, the similar result was obtained in nitrate-depended methane anaerobic oxidation (Fig. 3b), Similar results were reported in previous studies (Hu et al. 2009; Luesken et al. 2011a, b).

To further demonstrate the methane anaerobic oxidation coupled denitrification, anaerobic methane oxidation activity was estimated from the $^{13}$C-labeled $CO_2$ ($^{13}CO_2$) production during batch incubations with $^{13}CH_4$. The natural abundance of $^{13}$C was 1.1 %. The addition of nitrite (nitrate) caused a clear stimulation of $^{13}CO_2$ compared to untreated controls in all inoculated samples (Fig. 4). The abundance of $^{13}CO_2$ in the samples gradually increased and reached a maximum of 19.58 %

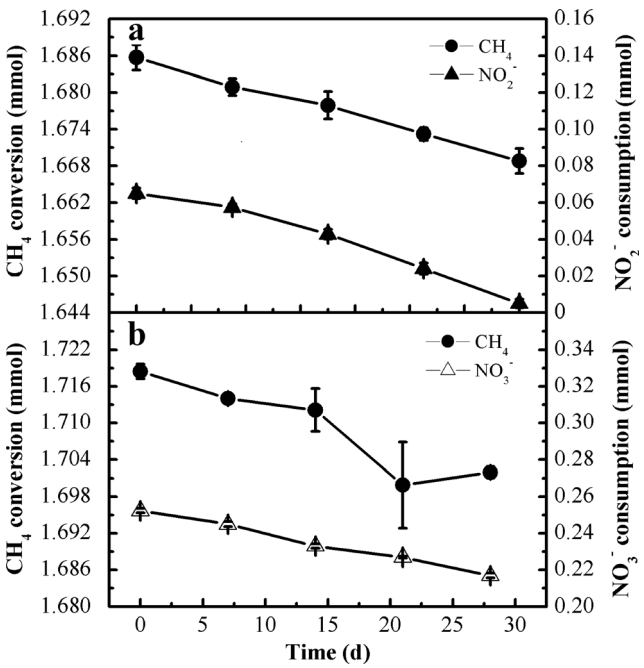


**Fig. 3** Methane and nitrite (nitrate) conversion of the enrichment culture on day 387. **a** $NO_2^-$ as the electron acceptor; **b** $NO_3^-$ as the electron acceptor

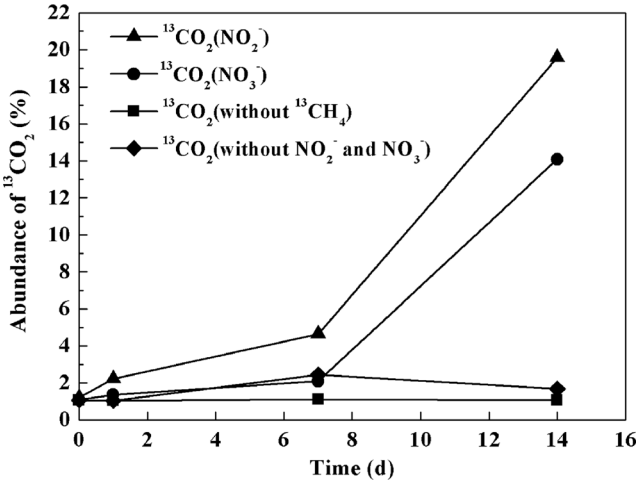


**Fig. 4** Tracing the formation of $^{13}CO_2$ from $^{13}CH_4$ in incubations of the enrichment culture by isotope labeling batch test

(nitrite added) and 14.08 % (nitrate added) on day 14. Without the addition of nitrite (nitrate), $^{13}CO_2$ value was slightly higher than the natural abundance, but it was much lower than those in the nitrite (nitrate) added samples. This may be attributed to a small amount of residual nitrite which was not depleted in the initial inoculums. The abundance of $^{13}CH_4$ in inoculated samples above was all remained unchanged (data not shown). Without the addition of $^{13}CH_4$, $^{13}CO_2$ values remained within the background level, suggesting that no other electron donor was involved in the denitrification.

### Identification of NC10 phylum bacteria

The enriched culture was analyzed with clone library targeting the 16S rRNA genes of bacteria affiliated to NC10 phylum after enrichment of 13 months. A total of 37 positive clones were selected and sequenced. Sequence analysis revealed that the coverage of biodiversity was up to 89 %. These sequences were related to NC10 phylum bacteria and fell into two distinct groups (*a* and *b*) of the NC10 phylum (Fig. 5), and group *a* was reported to perform nitrite-dependent anaerobic methane oxidation (Ettwig et al. 2009). Phylogenetic analysis showed that all the 37 clones were grouped into six OTUs, and 34 clones belonging to the three dominant OTUs were clustering in group *a* of the NC10 phylum, such as TH4-13, TH4-30, and TH4-45 which exhibited highest sequence similarity to the NC10 bacteria obtained in Twentekanaal and Ooijpolder eutrophic ditch sediment clones (Raghoebarsing et al. 2006; Ettwig et al. 2009). Other three OTUs which were represented by only one clone each clustered in group *b*, and they were closely related with *Acidobacteria* phylum. Moreover, clone library of archaea was also analyzed (data not

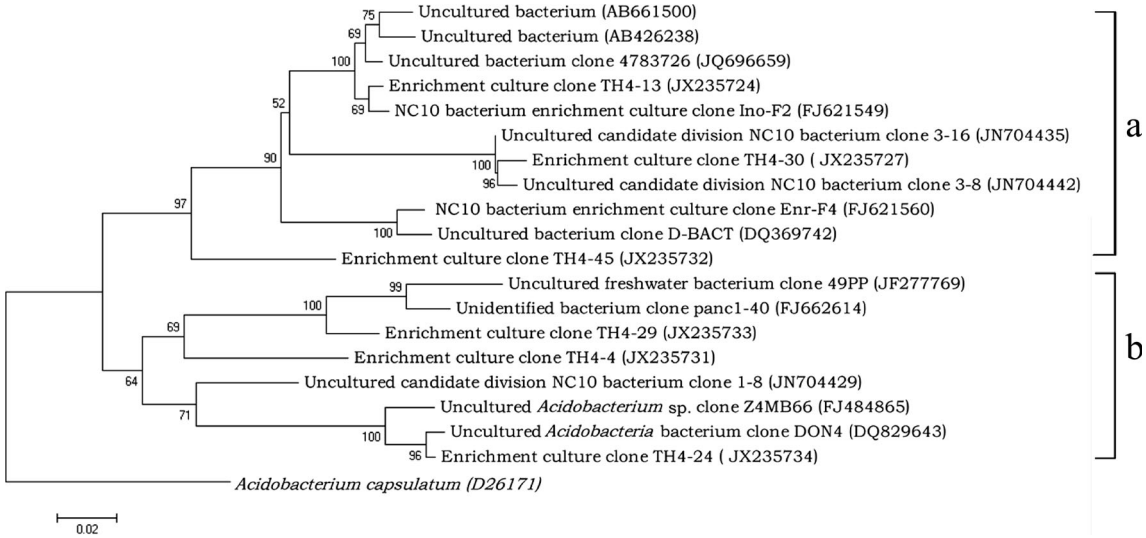


**Fig. 5** Phylogenetic tree of the NC10 bacteria 16S rRNA gene sequences obtained from the enrichment culture. The tree was constructed using the neighbor-joining methods. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. *Acidobacterium capsulatum* (D26171) was used as an outgroup to root the tree. The scale bar (0.05) indicates the number of changes per one site

shown). Phylogenetic analysis of archaea showed that all the 46 clones were grouped into six OTUs, and 5 clones belonging to a group of *Methanosarcinales* which has several times been enriched under nitrate and methane conditions (Raghoebarsing et al. 2006; Hu et al. 2009; Chen et al. 2014). Other clones were closely related with *Methanobacteriales*. These results showed that the specific group of *Methanosarcinales* was not enriched or disappeared in the culture after enrichment of 13 months under nitrite and methane conditions, and the enriched NC10 was responsible for nitrite removal in the culture. These results were in consistent with previous studies (Kampman et al. 2012; Ettwig et al. 2009).

## FISH analysis of the enriched culture

To further confirm and obtain the relative abundance of "Candidatus *M. oxyfera*"-like bacteria in the enriched culture, FISH was applied using previously described specific probes targeting denitrifying methanotrophic bacteria of the NC10 phylum (Ettwig et al. 2008, 2009). There was no clear hybridization signal with NC10 specific probes in the initial inoculums (Fig. 6a). After 9 months culture, NC10 bacteria could be detected and the relative abundance reached 55 % (Fig. 6b). The NC10 bacteria had become the dominant with the enrichment level of about 73 % at the 13th month (Fig. 6c), indicating that denitrification coupled to anaerobic methane

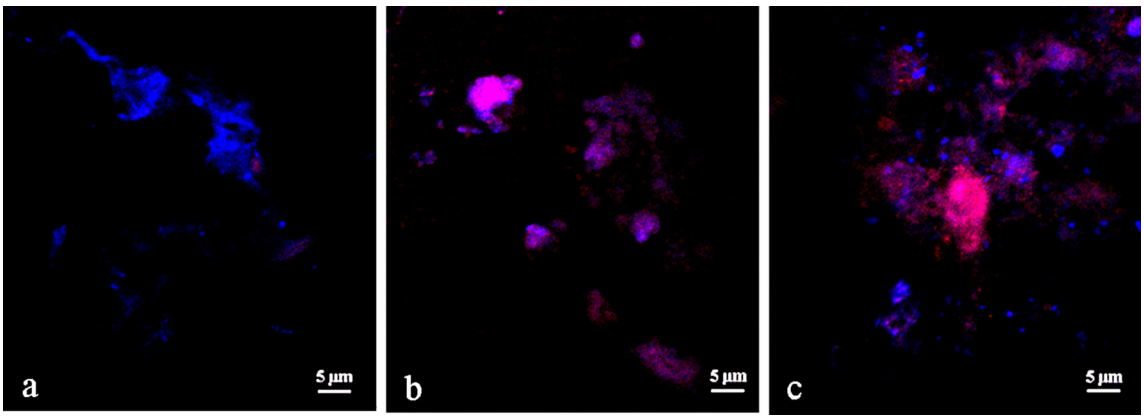


**Fig. 6** Fluorescence in situ hybridization of the enriched culture samples from the membrane biofilm bioreactor at different times: **a** initial sample, **b** after 9 months enrichment, **c**: after 13 months enrichment. The cells were hybridized with the probes S-*-DBACT-1027-a-A-18 specific for the NC10 phylum (Cy3, *red*) and S-D-Bact-0338-a-A-18 that target most bacteria (Cy5, *blue*). "NC10" bacteria appear pink due to double hybridization with both specific and general bacterial probes. Scale bar=5 μm

oxidation was the major denitrification process in the reactor, consistent with denitrification results of the reactor (data showed previously).

## Conclusions

A DAMO culture was enriched from Chinese Taihu sediments by a membrane biofilm bioreactor after 13 months culture at ambient temperature of 10–25 °C. The maximum denitrification rate of the enriched culture reached 0.54 mM $day^{-1}$ for nitrate and 1.06 mM $day^{-1}$ for nitrite. The $^{13}C$-labeled $CO_2$ ($^{13}CO_2$) production during batch incubations with $^{13}CH_4$ indicated that anaerobic methane oxidation could be coupled to denitrification in the DAMO culture. Microbial community analysis of 16S rRNA genes clone library confirmed the presence of NC10 phylum bacteria and fluorescence in situ hybridization showed that NC10 bacteria dominated the membrane biofilm bioreactor.

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