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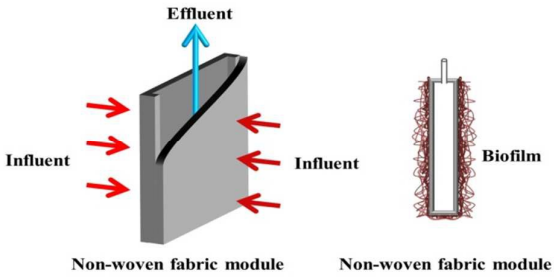
Graphical abstract



Apart from membrane separator, non-woven fabric module could be employed as biomass carrier to enhance microorganism proliferation and nitrogen removal.

Enhancement of anammox performance in a novel non-woven fabric membrane bioreactor (nMBR)

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Abstract

To reduce operating cost and membrane fouling of conventional membrane bioreactors (cMBR), a novel MBR using non-woven fabric membrane (nMBR) was constructed and two MBRs' performance was compared for anaerobic ammonium oxidation (anammox) cultivation. The results showed that the start-up period for nMBR (44 days) was notably shorter than that for cMBR (56 days), meanwhile nMBR achieved 2-time higher nitrogen removal rate (231.5 mg N/L/d) compared to cMBR (112.3 mg N/L/d). Illumina MiSeq sequencing showed that *Candidatus* Kuenenia and *Candidatus* Jettenia were main distinguished anammox bacteria. FISH analysis revealed anammox bacteria had predominated in both reactors, especially in nMBR (58%) corresponding to qPCR analysis of 1.07×10^9 copies/ml (day 120). The N_2O emission analysis confirmed the advantage of nMBR in N_2O reduction to reduce the influence of greenhouse gas emission while treating identical nitrogen. These results clearly demonstrated that nMBR would be a prospective choice for anammox start-up and performance enhancement.

Keywords: Anaerobic ammonium oxidation (anammox); Illumina MiSeq sequencing; MBR; Non-woven fabric; FISH.

1. Introduction

Anaerobic ammonium oxidation (anammox) is a promising biotechnology to treat ammonium-containing wastewater especially with low biological degradable carbon [1, 2]. In this process, anammox bacteria, namely *Planctomycetales*, convert ammonium to dinitrogen by using nitrite as electron acceptor under strict anaerobic condition (Equation 1). Anammox has been implemented in various full-scale applications for treating ammonium-rich wastewater, such as sludge digestion water and industrial wastewater [2, 3]. As an efficient and economical alternative to the traditional nitrification and denitrification process, anammox process avoids the addition of external carbon and the aeration, resulting in a remarkable saving of operation cost associated with higher nitrogen removal rates (NRR) [4-6].

$$NH_4^+ + 1.32NO_2^- + 0.06HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

However, the start-up of anammox process is mainly affected by the availability of anammox biomass due to their slow-growing rate (0.072/d measured at 32 °C) [7, 8]. Even a quite unimpressive biomass loss via the effluent or other approach may impede the start-up of anammox process severely because of the deficient biomass. Moreover, the severe sludge washout caused by granule floatation could lead to instability or even system collapses, particularly at high nitrogen loading [9]. Thus, an efficient system or operation strategy is required in order to minimize biomass run off with effluent.

Massive efforts on reducing biomass loss via choosing suitable reactors configurations have been attempted by researchers. The conventional membrane

bioreactor (cMBR) using hollow fiber module has been regarded as a suitable reactor to start up anammox process as it can achieve high biomass retention [10, 11]. Several studies have proved that submerged MBRs are excellent tools for enriching slow-growing microorganisms [12], especially anammox bacteria [11]. In MBRs, membrane modules can not only function as biofilter, but also as biofilm carriers [13], which is beneficial to the formation of biomass aggregates, attached biofilms and granular sludge. With higher biomass density, these sludge forms are more efficient to deal with high load nitrogen pollutants than flocculated sludge [10, 14, 15].

Considering the cost of hollow fiber membrane, MBR using non-woven fabric (nMBR) has attracted increasing interest in biofilm formation and biomass retention. The non-woven fabric membrane is porous with abundant small hollow areas, which is efficient in attaching anammox biomass, leading to enhanced anammox reactors performance [13, 16, 17]. Holding larger pore size compared to hollow fiber membrane, the separation mode of nMBR is broadly categorized as macro-filtration, while cMBR is categorized as micro-filtration. Therefore, the operating transmembrane pressure (TMP) of cMBR (the TMP of micro-filtration < 100-200 kPa) is generally higher than that of nMBR (the TMP of macro-filtration < 25 kPa) [18-20]. Based on the low TMP which is employed for monitoring membrane fouling, the membrane fouling of non-woven fabric including membrane pore blocking, cake formation and biofouling could also be alleviated to some extent [19].

Many researchers have explored the feasibility of anammox start-up with submerged anaerobic cMBR [10, 11, 14, 21]. However, there were few studies devoting to

using nMBR to cultivate anammox bacteria. Meng et al. [13] employed a nMBR to evaluate the performance of a novel anammox biofilm process using anammox sludge as seed sludge. The results showed that NRR of about 1.6 kg $N/m^3/d$ could be achieved. The non-woven modules as well as the attached biofilm also prevented the loss of free bacteria effectively. Although they analyzed the composition of bacterial community in the reactor, the definite enumeration of anammox bacteria had not been reported. Therefore, the further investigation of the components and amount of biocenosis during anammox start-up period using mixed sludge is needed. Another aspect, Meng et al. [13] laid emphasis on investigating nitrogen removal limit of non-woven modules with seeding mature anammox bacteria, while more attention was paid to explore a more potential program for municipal and industrial application using mixed sludge with little anammox bacteria in this research. The concentration and load of nitrogen pollutant also were set to a scale nearing to reality.

This study focused on comparing anammox performance of two submerged MBRs, namely cMBR and nMBR. Both reactors were inoculated with the same type of seed sludge for anammox start-up. Start-up time and nitrogen removal performance were compared between these two reactors. Furthermore, high-throughput sequencing (Illumina MiSeq sequencing), fluorescence in situ hybridization (FISH) and real-time quantitative PCR (qPCR) were employed to investigate the composition and quantities of bacterial community in reactors. The variation of N_2O emission was also examined during the entire anammox cultivation process. Since the analysis of the bacterial community in nMBR has not been explored in previous research, it could

provide valuable information for quick start-up of anammox process and its application.

2. Materials and Methods

2.1. Experimental set-up

The configuration of submerged MBR in this study is depicted in Fig.1, which had a double-walled cylindrical column with inner diameter of 14 cm and height of 50 cm. The only difference between two MBRs was the selection of membrane modules (Fig.1 (5), cMBR: hollow fiber membrane and nMBR: non-woven fabric). The hollow fiber membrane module (absolute pore size: 0.1 μm) consisted of 100 tubes (diameter ca. 1 mm, length ca. 300 mm) and the non-woven fabric module (a pore size of 0.1 mm approximately) was composed of two same rectangular modules (24 cm×10 cm). The filtration area of both membrane modules were around 0.01 m^2.

The peristaltic influent pump (BT100-2J, Longerpump, China) was used to adjust influent rate for controlling the hydraulic retention time (HRT). Liquid level sensor was placed at 39 cm height of the reactor to maintain an effective volume of 6 L. Level-controlled peristaltic effluent pump (BT300-2J, Longerpump, China) was used for drainage. TMP of membrane module was monitored by a vacuum gauge connecting with effluent pump. Thermostatic bath was connected to the double wall of the reactor to maintain the temperature at 33^{o}C. Both MBR reactors were also covered with tinfoil for avoiding light.

2.2. Operational strategy

The seed sludge were a mixture of 1/4 anammox sludge and 3/4 anaerobic

129 granular sludge. Anammox sludge was taken from a lab-scale UASB reactor which
130 was used for anammox process start-up [22] and the anaerobic sludge was obtained from
131 a running UASB reactor. 4L mixed sludge was divided equally to control the initial
132 mixed liquor suspended solids (MLSS) of 4000±50 mg/L in cMBR and nMBR.

133 The composition of synthetic wastewater for anammox bacteria enrichment is
134 showed in Table 1 and the trace element was composed of EDTA: 20.0 g/L, $FeSO_4$:
135 5.0 g/L, $ZnSO_4 \cdot 7H_2O$: 0.43 g/L, $CoCl_2 \cdot 6H_2O$: 0.24 g/L, $MnCl_2 \cdot 4H_2O$: 0.99 g/L,
136 $CuSO_4 \cdot 5H_2O$: 0.25 g/L, $Na_2MoO_4 \cdot 2H_2O$: 0.22 g/L, $NiCl_2 \cdot 6H_2O$: 0.19 g/L,
137 $Na_2SeO_4 \cdot 10H_2O$: 0.21 g /L and H_3BO_4: 0.014 g/L. The ratio of ammonium to nitrite
138 concentration in synthetic wastewater was set to 1:1.20. In order to maintain
139 anaerobic condition in each reactor, the synthetic wastewater was fed to the reactor
140 after deoxygenation by flushing with argon gas. The pH of influent varied in the range
141 of 7.5-8.0 without intended control. The inject rate was adjusted to maintain the initial
142 HRT at 48 h (the first 10 days was set to 96 h for anammox bacteria in seed sludge to
143 adapt to the new environment), and then the HRT was shortened by increasing inject
144 rate when the removal of ammonium and nitrite was effective (>90%) and stable. The
145 membrane module was replaced or cleaned chemically when the TMP reached up to
146 45 kPa to prevent the membrane fouling.

147 *2.3. Chemical analyses*

148 The concentrations of ammonium, nitrite and nitrate were measured according to
149 standard APHA method after filtering sample through 0.45 μm syringe filter. The
150 MLSS of sludge and suspended solids (SS) of effluent were also determined

according to standard methods [23]. A digital portable DO meter (HQ40d, Hach, America) and a microelectrode (5307, Unisense, Denmark) were employed to detect DO level and N_2O emission in liquid, respectively.

2.4. DNA extraction and Illumina MiSeq sequencing

1 mL biomass sample from each reactor was collected and harvested by centrifugation for DNA extraction on day 60 and 120 using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's protocol. Based on the main existence form of sludge, the biomass sample in cMBR was collected from suspended sludge while cake layer biofilm was employed in nMBR.

Agarose gel electrophoresis (1%) stained with ethidium bromide solution was adopted to assess extracted DNA quality. The following PCR reactions were processed on an ABI GeneAmp® 9700 (Applied Biosystems, USA) and the PCR product was gel purified. Afterwards, the Illumina MiSeq sequencing was carried out by Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) using DNA samples on day 120.

2.5. Fluorescence in situ hybridization (FISH)

FISH analysis was applied to verify the existence and distribution of anammox bacteria in mature sludge. The sludge samples were collected from both reactors on day 120. The EUB338Mix (EUB338, EUB338-II and EUB338-III) probe (FITC-labeled) was used to monitor almost all bacteria (green) while anammox bacteria (red) were targeted using AMX820 probe (Cy3-labeled). An Olympus BX53 confocal scanning laser microscope (Olympus, Japan) was employed for image

acquisitions. Fluorescence images obtained were also processed a semi-quantitative analyze using the software Image-Pro Plus 6.0.The concrete steps of FISH were conducted as described elsewhere [24].

2.6. Real-time quantitative PCR (qPCR)

Amx809F and Amx1066R (Table 2) were used as the primer set to target 16S rRNA gene for quantifying anammox bacteria [25]. The concrete steps of qPCR were according to the previous study [22] and the relevant curves were added as supporting information (Fig. S1 and S2).

3. Results and Discussions

3.1. Enrichment and reactor performance

The initial nitrogen loading rate (NLR) which was applied in cMBR was 67.5 mg N/L/d and the influent ammonium and nitrite concentrations were maintained around 123.3±4.0 and 152.4±3.7 mg/L respectively as shown in Fig. 2. During day 0 to 12, the input ammonium was barely consumed while much more nitrite was consumed, indicating no significant anammox phenomenon was detected. The effluent nitrate concentration ranged from 0 to 5 mg/L, which might attribute to endogenous denitrification since no organics were fed as other relevant researches [26, 27]. After 30 days, the removal efficiencies of ammonium and nitrite were about 44.8±0.7% and 52.8±1.0%, leading to the NRR about 63 mg N/L/d. The happening of anammox process could be confirmed by ammonium (removal efficiency varied between 44.8 and 87.6%) and nitrite (removal efficiency varied between 52.7 and 91.0%) were consumed synchronously in conjunction with nitrate was produced in the range of

9.0-35.8 mg N/L. The increasing ammonium and nitrite removal efficiencies reflected a tendency of gradually increased anammox activity.

During day 56 to 60, both ammonium and nitrite removal efficiencies were above 90% suggesting a successful anammox start-up. However when the NLR was further increased to 203.2 mg N/L/d from day 64 to 72, the performance of nitrogen removal suddenly decreased to 66.0±4.8% and 60.4±9.1% for ammonium and nitrite respectively. Literature showed that residual nitrite concentration of 50 mg/L could inhibit anammox bacteria partially while 100 mg/L concentration could inactivate anammox bacteria completely [28]. As cMBR did not present to the capacity of accommodating higher nitrogen concentration as nMBR, the NLR was adjusted back to 137.2 mg N/L/d again through regulating the HRT to initial 48 h for avoiding the damage by nitrite. After that, the performance of cMBR improved gradually again. On day 108, the ammonium and nitrite removal efficiencies steadily maintained at 94.0±1.5% indicating the success of anammox activity recovery.

The initial nitrogen compounds concentrations together with NLR of nMBR were set to the same value of cMBR while endogenous denitrification was also found during day 0 to 12 which led to a low ammonium and high nitrite removal efficiencies. From day 16 to 28, the removals of ammonium and nitrite improved rapidly to 64.8±20.2% and 79.6±12.0% respectively, revealing a significant evidence of anammox activity, and then nMBR achieved stable ammonium and nitrite nitrogen removal above 90% on day 44. Similarly, in order to supply efficient nutrients and increase anammox growth capacity, the NLR was also increased to 203.2 mg N/L/d

on day 64. On the following day, nitrite concentration of the effluent increased to 38.4 mg N/L and total nitrogen removal efficiency dropped to 67.7%, which was consistent with to the phenomenon observed by Isaka et al. [29]. However, unlike the inferior performance of cMBR, after accommodation for a few days, anammox removal in nMBR rose significantly again. During day 84 to 100, the nitrite removal efficiency remained close to 100%, while ammonium removal efficiency varied from 88% to 95%.

No inhibition of nitrogen removal was observed in nMBR until the NLR was increased by 2 times (from around 141.7 to the maximum 283.3mg N/L/d) on day 104, when the ammonium and nitrite removal efficiencies speedily declined to 29.9±1.2% and 39.7±0.8% respectively. Afterwards, the anammox activity in the system was recovered within 4 days. On day 128, the nMBR exhibited 2-time NRR (nMBR: 245.4 mg N/L/d, cMBR: 122.6 mg N/L/d), showing enormous advantage of adopting non-woven fabric for improving NRR. Compared with other relevant research demonstrated in Table 3 (start up from activated or anoxic sludge), the nMBR could accomplish the start-up of anammox process successfully in shorter time with higher NRR and nitrogen removal efficiency. In addition, inoculation with mixed sludge rather than total anammox sludge in many other researches would decrease the financial pressure during start-up and operation due to the scarcity and rarity of anammox sludge.

Fig. 3 illustrates the values of consumed nitrite/ammonium ratio and produced nitrate/consumed ammonium ratio. The values of consumed nitrite/ammonium ratio in

two reactors during anammox stage varied from 1.20 to 1.30, which was a little lower than the theoretical value of 1.32 as described in Equation 1. This could partly attribute to the influent nitrite/ammonium ratio of 1.20 aiming at avoiding the damage of high residual nitrite on anammox bacteria. The existence of nitrification and denitrification also contributed to this phenomenon. The values of produced nitrate/consumed ammonium ratio in two reactors during anammox stage also varied in a normal range of 0.24 to 0.29 approaching the theoretical value of 0.26. Corresponding with the lower nitrogen removal efficiency in cMBR from day 64, the values of nitrite removal/ammonium removal in cMBR also were quite lower than that in nMBR particularly during day 64 to 92. Such long-term different nitrogen loading might further lead to an effect in biomass or microorganism.

3.2. Biomass growth and retention

The main purpose for adopting MBR configuration was to minimize the loss of biomass through effluent flow. During the whole experiment, the effluent SS from the cMBR was less than 15 mg/L, whereas nMBR showed higher biomass loss (25-35 mg/L). On day 60, the effluent SS in nMBR dropped below 19 mg/L because the suspended sludge turned to attached biofilm moderately for preserving more precious anammox bacteria, and maintained less than 13mg/L from day 90 (Fig. S3).

Apart from the function of membrane-like separation, the non-woven fabric modules also served as biofilm carriers. Thus, anammox bacteria in the suspended sludge were attached to non-woven fabric due to the significant attachment propensity of anammox bacteria [13]. The shear stress, which generated from water flow crossing

the flat non-woven membrane, also directed biomass to approach the fabric and finally attached on the membrane loosely [30]. With the extension of this process, the loose biofilm structure became denser. The suction from the effluent pump might also facilitate this phenomenon.

After 130 days of operation, nearly all biomass in cMBR existed in the form of suspended sludge with little sludge adhering on the membrane surface, and the MLSS in cMBR gradually climbed up to over 6650±50 mg/L. On the contrary, the biomass in nMBR mainly presented as attached biofilm on the non-woven fabric while a small number of residual free living bacteria were observed, resulting in a difficulty of MLSS evaluation. At the end of the operating stage, a dry weight 46.48 g of dominant attached growth biomass and 254±20 mg/L of MLSS were detected, representing approximate 8000±20 mg/L of total MLSS in nMBR. In addition, the attached biomass on non-woven membrane could act as a permeable reactive barrier for nitrogen removal and ammonium-rich wastewater would be disposed again by the microorganisms in cake layer before been discharged. The analysis of nitrogen concentration showed that around half of the ammonium (ca. 49%) and nitrite (ca. 53%) in the sludge mixture of nMBR were removed during their transport to the effluent through the biofilm.

3.3. Illumina MiSeq sequencing analysis

The pyrosequencing-based analysis of 16S rRNA genes was used to assess the composition of bacterial community. Good's coverage values were 91.34% and 91.60% for cMBR and nMBR, respectively, which indicated that the number of sequences was

sufficient to characterize the microbial communities. The composition of bacterial communities at the phylum level in both reactors is described in Fig. 4 (a). The top 9 phyla observed in cMBR and nMBR were *Actinobacteria, Armatimonadetes, Bacteroidetes, Chlorobi, Chloroflexi, GOUTA4, Nitrospirae, Planctomycetes* and *Proteobacteria*.

Planctomycetes was the most important division (Fig. 4 (b)), comprising approximately 14.5% (4189 reads) in cMBR and 11.8% (4755 reads) in nMBR. Anammox bacteria are mainly identified as *Planctomycetes* [31-33]. However, regarding to species, Illumina MiSeq sequencing analysis cannot indicate specifically what these microorganisms are, as most of them were unclassified *Planctomycetes*. Till now, only two of the 102 detected *Planctomycetes* operational taxonomic units (OUTs) were identified to be the recognized anammox bacteria species (*Candidatus* Kuenenia and *Candidatus* Jettenia). In addition, not all the detected *Planctomycetes* could be defined to anammox bacteria, and thus anammox bacteria as *Planctomycetes* had a quite lower abundance than expected.

Similar to previous findings [32, 34], there were some other phyla in the reactors coexisting with anammox bacteria (i.e. *Planctomycetes*). The most abundant phylum was *Proteobacteria* whose relative abundances were 18.0% (5147 reads) in cMBR and 21.5% (8971 reads) in nMBR. Previous studies indicated that several species of *β-Proteobacteria* (38 OTUs, 3233 reads in cMBR; 54 OTUs, 5530 reads in nMBR) could embody low anammox activity to convert ammonium and nitrite to N_2 using nitrogen dioxide as an electron acceptor, for instance autotrophic aerobic

ammonium-oxidizing bacteria (AAOB), especially *Nitrosomonas eutropha* and *N. europaea* [32, 34]. Another abundant phylum was *Chloroflexi* bacteria (4416, 9641 reads in cMBR, nMBR respectively), which was found frequently in anammox reactors playing an important role in sludge granulation and biofilm formation [13, 34, 35]. The numbers of *Chloroflexi* bacteria coincided well with experimental results of significant biofilm formation in nMBR while such phenomenon in cMBR was not clear.

Fig. 5 depicts the phylogenetic tree based on 16srRNA gene fragments of almost all bacteria in cMBR and nMBR. As part of the Illumina MiSeq sequencing analysis, the phylogenetic tree reflected the main bacteria communities and their relatives in public databases. Bootstrap values (>50%) was indicated at branch points. The number 0.01 was the scale bar, which represented 0.01 nucleotide substitutions per nucleotide position. *Candidatus* Kuenenia (7, 2 reads in cMBR, nMBR respectively) and *Candidatus* Jettenia (9, 86 reads in cMBR, nMBR respectively) were the only definitely detected anammox bacteria genera, which showed a relative high genetic similarity with *Blastocatella* genus, which affiliated to *Actinobacteria* as one of dominant bacterial communities in soil. Overall, these results suggested that Illumina MiSeq sequencing, which aimed to detect the categories and quantities of universal bacteria based on 16S rRNA, was not effective or sensitive enough for this anammox culture [32, 36]. It might be attributed to sensitive anammox genes are mainly 250 bp whilst Illumina MiSeq sequencing mainly focus on genes about 300-400 bp. Therefore, FISH and qPCR analysis were adopted for further detection the proportion

and quantity of anammox bacteria to improve the sensitivity and specificity.

3.4. FISH analysis

Sludge samples were collected from cMBR (suspended growth sludge) and nMBR (attached-growth biofilm) on day 120 for FISH analysis to investigate the spatial distribution and proportion of anammox bacteria. As shown in Fig. S4, the red color, which represented anammox bacteria, was observed in both two sludge samples. Through relative abundance analysis using Image-Pro Plus 6.0, the results also revealed that anammox bacteria accounted for about 58% of total bacteria in nMBR which was significantly higher than those (51%) in cMBR. Although the results were estimated values with certain limitation, they elucidated that anammox bacteria had been the dominant community in the reactors and non-woven fabric was more adaptable for proliferation of anammox bacteria. The observations together with the findings of qPCR analysis corresponded well to the performance of nitrogen removal in reactors: the more anammox bacteria, and the higher nitrogen removal efficiency.

3.5. qPCR analysis

To further investigate the benefit of non-woven fabric in terms of proliferating anammox bacteria, qPCR analysis was performed to enumerate anammox bacteria precisely. The qPCR enumeration results on day 60 demonstrated improved proliferation for nMBR with more preferable anammox cells enumeration of 1.24×10^{7} copies/mL compared to cMBR (6.79×10^{6} copies/ml). In case of assuming all nitrogen was removed by anammox bacteria, the estimated per cell nitrogen removal rate in cMBR was also much higher than that in nMBR due to the orders of magnitude

difference in cells enumeration. In other words, the low per cell nitrogen removal rate in nMBR indicated that nMBR had higher potential for improving nitrogen removal by anammox bacteria, which could be contributed to the use of non-woven fabric membrane.

Afterwards, the numbers of anammox bacteria continued to ascend speedily and reached to 5.32×10^8 copies/mL in cMBR and 1.07×10^9 copies/mL in nMBR on day 120. Nevertheless, the estimated per cell nitrogen removal rate declined again considering the change in the orders of magnitude. Variation in the activity of anammox bacteria was a common phenomenon in stationary phase of bacteria community growth [37], meaning that insufficient substrate supply could become the main restriction factor. Once higher nitrogen concentration was employed to satisfy the need of anammox bacteria, higher NRR and more anammox bacteria could be obtained.

3.6. N_2O emission

Considering the existence of nitrifying bacteria and denitrifying bacteria in the seed sludge, N_2O, which is a typical by-product of nitrification and denitrification, could still be produced in the reactors during the cultivation of anammox bacteria. The initial liquid N_2O emissions from cMBR and nMBR were measured about 56.8 and 58.0 μmol/L (Fig. 6 (a)), respectively, which could be attributed to endogenous denitrification consuming the bacteria itself as carbon source as well as using the nitrite/nitrate existing in the influent or transforming by anammox process [26, 27]. Many literatures also found that anammox bacteria (affiliated to *Planctomycetes*, 4189 and

4755 reads in cMBR and nMBR respectively), nitrifying and denitrifying bacteria (affiliated to *Proteobacteria*, 5147 and 8971 reads in cMBR and nMBR respectively) coexisted in anammox reactors [32, 34]. Hence, the occurrence of denitrification was entirely possible.

During the 60-day period the emission of liquid N_2O in nMBR fell from 58.0 to 41.6 μmol/L gradually. Meanwhile, the emission in cMBR also declined, but significantly less (46.1 μmol/L), suggesting that anammox bacteria replaced the nitrifying and denitrifying bacteria to become the dominant bacteria in two MBRs with more significant tendency in nMBR. From day 70, the HRT of nMBR moderately was shortened to 12 h, resulting in NLR doubled to 283.3 mg N/L/d approximately. As a result, liquid N_2O emission in nMBR increased dramatically to 60.5 μmol/L on day 120 associated with transient decrease of anammox activity to accommodate the new conditions, as excess nitrogen was provided to residual nitrifying bacteria and denitrifying bacteria. The results could also be proved by the presence of *Proteobacteria*, 18.0% of relative abundance (5147 reads) in cMBR and 21.5% (8971 reads) in nMBR, such as *Nitrosococus oceani* and *Nitrosococus halophilus (γ-Proteobacteria), Nitrosomonas* and *Nitrosospira (β-Proteobacteria)* [38].

If the arithmetical unit was changed to μmol •d/ g N (N_2O emission/NRR) (Fig. 6 (b)) to compare the N_2O emission in cMBR and nMBR for treating commensurate nitrogen. The downward trend in both two reactors during the whole cultivation demonstrated the gradual purified anammox process and strengthened anammox activity. Being consistent with the performance of nitrogen removal, the N_2O

emission indexes of nMBR were lower than those of cMBR, reflecting the diminishment of N_2O emission was superior to cMBR.

3.7. Membrane fouling

During the entire operation period, the hollow fiber module in cMBR was replaced once on day 80 when the TMP reached 45 kPa (Fig. 7), whereas the TMP development of the non-woven module in nMBR was only 25 kPa and the module was not replaced or cleaned during the 128 days of operation indicating less serious membrane fouling. Some researchers reported that a bigger pore size filter could cause more membrane fouling issue particularly pore blocking in micro-filtration in term of same biomass concentration in MBRs [19, 39, 40]. Nevertheless, the different separation modes of hollow fiber membrane (micro-filtration) and non-woven fabric (macro-filtration) made the discipline not appropriate in this situation. Such distinction was also the main reason for the large gap of TMPs. As the practical pore sizes of hollow fiber membrane and non-woven fabric decreased gradually with the formation of loose cake layer especially in nMBR, the increasing trend of membranes fouling corresponding with TMPs became lower and lower (Fig. 7). With a lower MLSS of suspended sludge (e.g. cMBR: 6650±50 mg/L, nMBR: 254±20 mg/L on day 130), the TMP in nMBR was lower than that in cMBR during the whole cultivation. The TMP growth rate of nMBR also slowed down gradually while the decrease rate of suspended sludge slowed down. Thus, the use of non-woven module could mitigate membrane fouling, indicating notable lower operation cost and competitive advantage compared to polymer membrane.

4. Conclusions

This study compared the performance of two MBRs during anammox bacteria cultivation. Compared to cMBR, nMBR exhibited advantage of accelerating the start-up of anammox process corresponding to higher NRR of approximate 245.3 mg N/L/d. The non-woven membrane was beneficial to the formation of biofilm, which contributed to anammox bacteria proliferation, leading to a qPCR enumeration result of 1.07×10^{9} copies/ml. The reduction of N_2O emission was also observed during the period of operation. Moreover, nMBR could lead to less serious membrane fouling due to less TMP development. Overall, nMBR could be a promising, labor-saving and money-saving technology for anammox process start-up. In view of start-up time and engineering cost comprehensively, the method of this paper might be a more suitable choice for the further municipal and industrial applications of anammox technology.

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Appendix supporting information

Supporting information is available with this manuscript.

References

1. M. S. Jetten, I. Cirpus, B. Kartal, L. van Niftrik, K. T. van de Pas-Schoonen, O. Sliekers, S. Haaijer, W. van der Star, M. Schmid, J. van de Vossenberg, I. Schmidt, H. Harhangi, M. van Loosdrecht, J. Gijs Kuenen, H. Op den Camp and M. Strous, *Biochem Soc Trans*, 2005, **33**, 119-123.
2. A. Joss, D. Salzgeber, J. Eugster, R. Konig, K. Rottermann, S. Burger, P. Fabijan, S. Leumann, J. Mohn and H. Siegrist, *Environ Sci Technol*, 2009, **43**, 5301-5306.
3. W. R. van der Star, W. R. Abma, D. Blommers, J. W. Mulder, T. Tokutomi, M. Strous, C. Picioreanu and M. C. van Loosdrecht, *Water Res*, 2007, **41**, 4149-4163.
4. W. R. Abma, C. E. Schultz, J. W. Mulder, W. R. van der Star, M. Strous, T. Tokutomi and M. C. van Loosdrecht, *Water Sci Technol*, 2007, **55**, 27-33.
5. N. Chamchoi, S. Nitisoravut and J. E. Schmidt, *Bioresource Technology*, 2008, **99**, 3331-3336.
6. U. Imajo, T. Tokutomi and K. Furukawa, *Water Sci Technol*, 2004, **49**, 155-163.
7. M. S. M. Jetten, M. Strous, K. T. van de Pas-Schoonen, J. Schalk, U. G. J. M. van Dongen, A. A. van de Graaf, S. Logemann, G. Muyzer, M. C. M. van Loosdrecht and J. G. Kuenen, *FEMS Microbiology Reviews*, 1998, **22**, 421-437.
8. M. Strous, J. J. Heijnen, J. G. Kuenen and M. S. M. Jetten, *Appl Microbiol Biotechnol*, 1998, **50**, 589-596.
9. Z. Li, X. Xu, B. Shao, S. Zhang and F. Yang, *Chemical Engineering Journal*, 2014, **254**, 9-16.
10. T. Wang, H. Zhang, F. Yang, S. Liu, Z. Fu and H. Chen, *Bioresource Technology*, 2009, **100**, 2501-2506.
11. W. R. L. van der Star, A. I. Miclea, U. G. J. M. van Dongen, G. Muyzer, C. Picioreanu and M. C. M. van Loosdrecht, *Biotechnology and Bioengineering*, 2008, **101**, 286-294.
12. R. J. W. Meulepas, C. G. Jagersma, J. Gieteling, C. J. N. Buisman, A. J. M. Stams and P. N. L. Lens, *Biotechnology and Bioengineering*, 2009, **104**, 458-470.
13. F. Meng, G. Su, Y. Hu, H. Lu, L.-N. Huang and G.-H. Chen, *Water Research*, 2014, **58**, 82-91.
14. C. Trigo, J. L. Campos, J. M. Garrido and R. Méndez, *Journal of Biotechnology*, 2006, **126**, 475-487.
15. G. Gonzalez-Gil, L. Seghezzo, G. Lettinga and R. Kleerebezem, *Biotechnology and Bioengineering*, 2001, **73**, 125-134.
16. T. Fujii, H. Sugino, J. D. Rouse and K. Furukawa, *Journal of Bioscience and Bioengineering*, 2002, **94**, 412-418.
17. S.-Q. Ni, P.-H. Lee, A. Fessehaie, B.-Y. Gao and S. Sung, *Bioresource Technology*, 2010, **101**, 1792-1799.

18. N. Lee, G. Amy, J.-P. Croue and H. Buisson, *Water research*, 2004, **38**, 4511-4523.
19. W. Guo, H.-H. Ngo and J. Li, *Bioresource Technology*, 2012, **122**, 27-34.
20. P. Bai, X. Chen, H. Yin, J. Wang and G.-H. Chen, *Desalination and water treatment*, 2010, **18**, 297-301.
21. Y. Tao, D.-W. Gao, Y. Fu, W.-M. Wu and N.-Q. Ren, *Bioresource Technology*, 2012, **104**, 73-80.
22. L.-F. Ren, S.-Q. Ni, C. Liu, S. Liang, B. Zhang, Q. Kong and N. Guo, *Environmental Science and Pollution Research*, 2015, **22**, 2925-2934.
23. W. E. Federation and A. P. H. Association, *American Public Health Association (APHA): Washington, DC, USA*, 2005.
24. M. Schmid, U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K.-H. Schleifer and M. Wagner, *Systematic and applied microbiology*, 2000, **23**, 93-106.
25. I. Tsushima, T. Kindaichi and S. Okabe, *Water Research*, 2007, **41**, 785-794.
26. N. Chamchoi and S. Nitisoravut, *Chemosphere*, 2007, **66**, 2225-2232.
27. A. Dapena-Mora, S. W. H. Van Hulle, J. Luis Campos, R. Méndez, P. A. Vanrolleghem and M. Jetten, *Journal of Chemical Technology & Biotechnology*, 2004, **79**, 1421-1428.
28. M. S. M. Jetten, M. Wagner, J. Fuerst, M. van Loosdrecht, G. Kuenen and M. Strous, *Current Opinion in Biotechnology*, 2001, **12**, 283-288.
29. K. Isaka, T. Sumino and S. Tsuneda, *Journal of Bioscience and Bioengineering*, 2007, **103**, 486-490.
30. Y. Liu and J.-H. Tay, *Water Research*, 2002, **36**, 1653-1665.
31. B.-l. Hu, P. Zheng, C.-j. Tang, J.-w. Chen, E. van der Biezen, L. Zhang, B.-j. Ni, M. S. Jetten, J. Yan and H.-Q. Yu, *Water research*, 2010, **44**, 5014-5020.
32. X.-R. Li, B. Du, H.-X. Fu, R.-F. Wang, J.-H. Shi, Y. Wang, M. S. Jetten and Z.-X. Quan, *Systematic and applied microbiology*, 2009, **32**, 278-289.
33. M. Strous, E. Pelletier, S. Mangenot, T. Rattei, A. Lehner, M. W. Taylor, M. Horn, H. Daims, D. Bartol-Mavel, P. Wincker, V. Barbe, N. Fonknechten, D. Vallenet, B. Segurens, C. Schenowitz-Truong, C. Médigue, A. Collingro, B. Snel, B. E. Dutilh, H. J. M. Op den Camp, C. van der Drift, I. Cirpus, K. T. van de Pas-Schoonen, H. R. Harhangi, L. van Niftrik, M. Schmid, J. Keltjens, J. van de Vossenberg, B. Kartal, H. Meier, D. Frishman, M. A. Huynen, H.-W. Mewes, J. Weissenbach, M. S. M. Jetten, M. Wagner and D. Le Paslier, *Nature*, 2006, **440**, 790-794.
34. S. Qiao, Y. Kawakubo, Y. Cheng, T. Nishiyama, T. Fujii and K. Furukawa, *Biodegradation*, 2009, **20**, 117-124.
35. S. Cho, Y. Takahashi, N. Fujii, Y. Yamada, H. Satoh and S. Okabe, *Chemosphere*, 2010, **78**, 1129-1135.
36. M. C. Schmid, A. B. Hooper, M. G. Klotz, D. Woebken, P. Lam, M. M. Kuypers, A. Pommerening-Roeser, H. J. Op Den Camp and M. S. Jetten, *Environmental microbiology*, 2008, **10**, 3140-3149.
37. T.-F. C. Mah and G. A. O'Toole, *Trends in microbiology*, 2001, **9**, 34-39.

38. S. Avrahami, R. Conrad and G. Braker, *Applied and Environmental Microbiology*, 2002, **68**, 5685-5692.
39. A. Lim and R. Bai, *Journal of membrane science*, 2003, **216**, 279-290.
40. S. Hong, T. Bae, T. Tak, S. Hong and A. Randall, *Desalination*, 2002, **143**, 219-228.

Table and Figure captions

Table 1 Composition of synthetic wastewater for enrichment.

Table 2 List of PCR primers used in this study.

Table 3 Comparison of different MBR anammox reactors.

Fig. 1. Schematic diagram of the reactor system for anammox enrichment. (1): Influent tank, (2): Influent pump, (3): Stirrer, (4): Argon, (5): Membrane module, (6): Vacuum gauge, (7): Gas outlet, (8): Level sensor, (9): Effluent pump, (10): Effluent tank.

Fig. 2. The nitrogen removal performance of cMBR and nMBR.

Fig. 3. The values of nitrite removal/ammonium removal in cMBR (■) and nMBR (●) and the values of nitrate production/ammonium removal in cMBR (□) and nMBR (○) from day 32 to 130.

Fig. 4. (a) Composition of different communities at phylum level in cMBR and nMBR; **(b)** Relative abundance of different communities at phylum level in cMBR and nMBR.

Fig. 5. Phylogenetic tree of clones obtained from DNA samples of cMBR and nMBR used Illumina MiSeq sequencing analysis.

Fig. 6. (a) The total liquid N_2O emission in cMBR and nMBR; **(b)** The liquid N2O emission per consumed NRR (1 mg N/L/d) in cMBR and nMBR.

Fig. 7. The variation of TMP in cMBR and nMBR.

Table 1 Composition of synthetic wastewater for enrichment.

Substance	Concentration	Unit
$(NH_4)_2SO_4$	594	mg/L
$NaNO_2$	746	mg/L
$KHCO_3$	500	mg/L
$CaCl_2 \cdot 2H_2O$	180	mg/L
$MgSO_4 \cdot 7H_2O$	120	mg/L
KH_2PO_4	27	mg/L
T. element	1	mL/L

Table 2 List of PCR primers used in this study.

Primer name	Target	Sequence(5'-3')	Target site	Annealing temperatures (^{o}C)
Amx809F	anammox	GCCGTAAACGATGGGCACT	809-826	60
Amx1066R	anammox	AACGTCTCACGACACGAGCTG	1047-1066	60

Table 3 Comparison of different MBR anammox reactors.

Reactor type	Source sludge	Membrane module	Start-up condition		Maximal NRR (mg N/L/d)	Ammonium removal efficiency(%)	Nitrite removal efficiency(%)	Start-up time (day)	References
			Ammonium (mg/L)	Nitrite (mg/L)					
nMBR	activated sludge	non-woven fabric[a]	40	53	1047.5	90.9	95.0	64	Ref. 17
nMBR	anammox sludge	non-woven fabirc	25	25	1600	>80	>90	--	Ref. 13
MBR	activated sludge	hollow fiber membrane[b]	50	50	80	>90	>90	50	Ref. 10
MBR	activated sludge	hollow fiber membrane	50	50	345.2[c]	~100	~100	59	Ref. 10
MBR	anoxic sludge	hollow fiber sheet	25	25	218.5	>95	>95	25[d]	Ref. 21
MBR	anammox sludge	hollow fiber membrane	1680	1680	1600	--	≥99	--	Ref. 11
MSBR	anammox sludge	hollow fiber membrane	75.3	83.7	710	>80	>90[e]	--	Ref. 14
cMBR	mixed sludge	hollow fiber membrane	126	151.3	124.2	>95	>95	56	This study
nMBR	mixed sludge	non-woven fabirc	126	151.3	245.4	>95	>95	44	This study

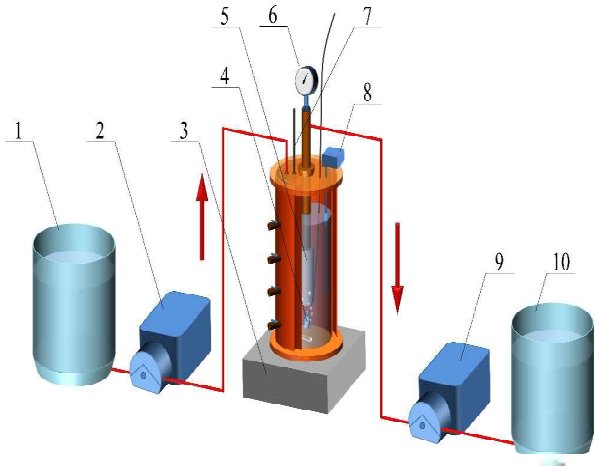
[a]: Non-woven fabric membrane was used as external membrane module in this study.

[b]: Hollow fibre membrane module was empolyed the curtain shape in this study.

[c]: NRR was only considered the removal of ammonium and nitrite in this study.

[d]: Start-up date was defined as the end of unstable phase in this study.

[e]: The average total nitrogen removal efficiency in stable stage was of 73.6% in this study.



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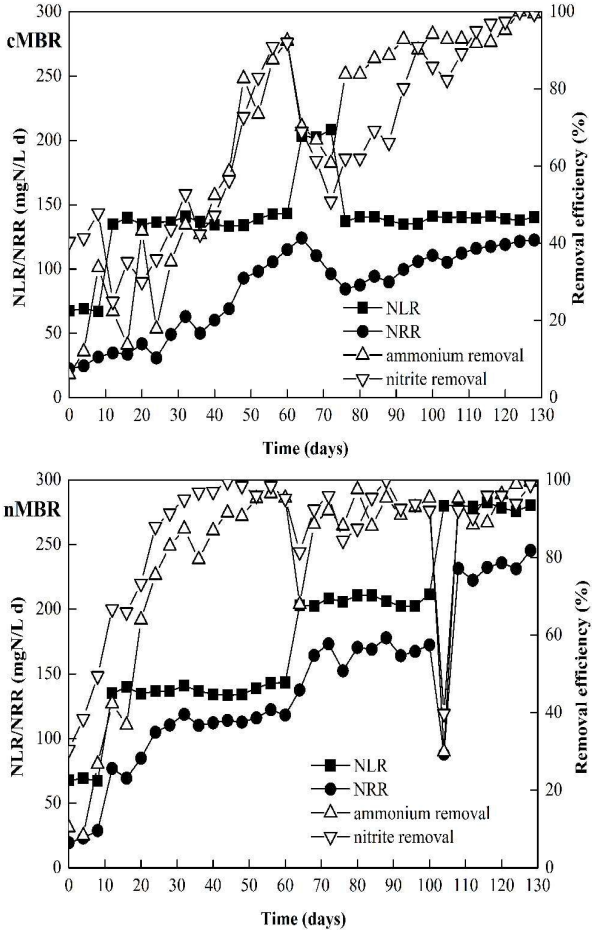

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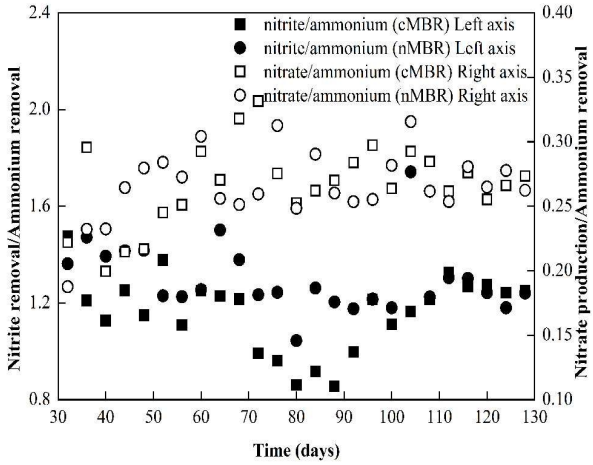

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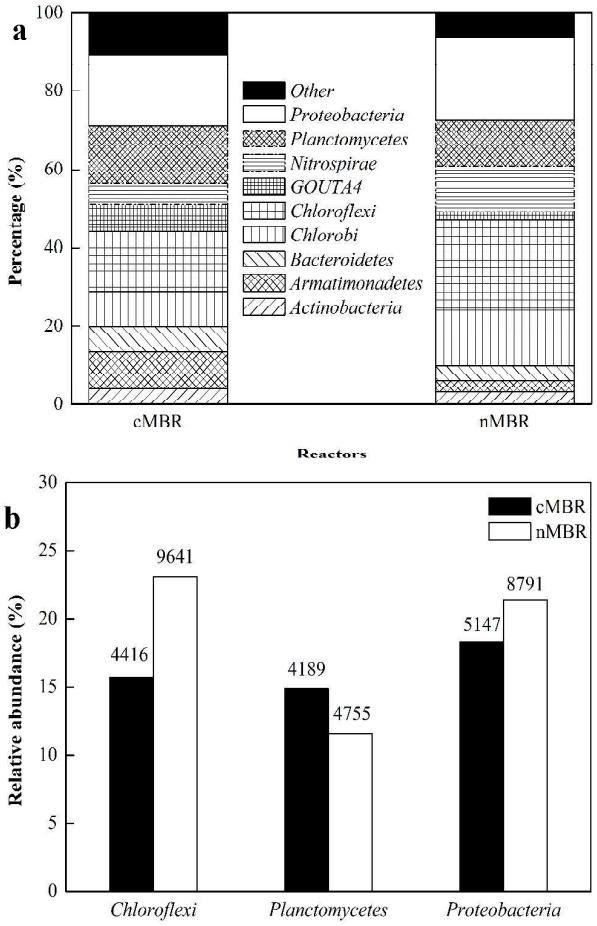

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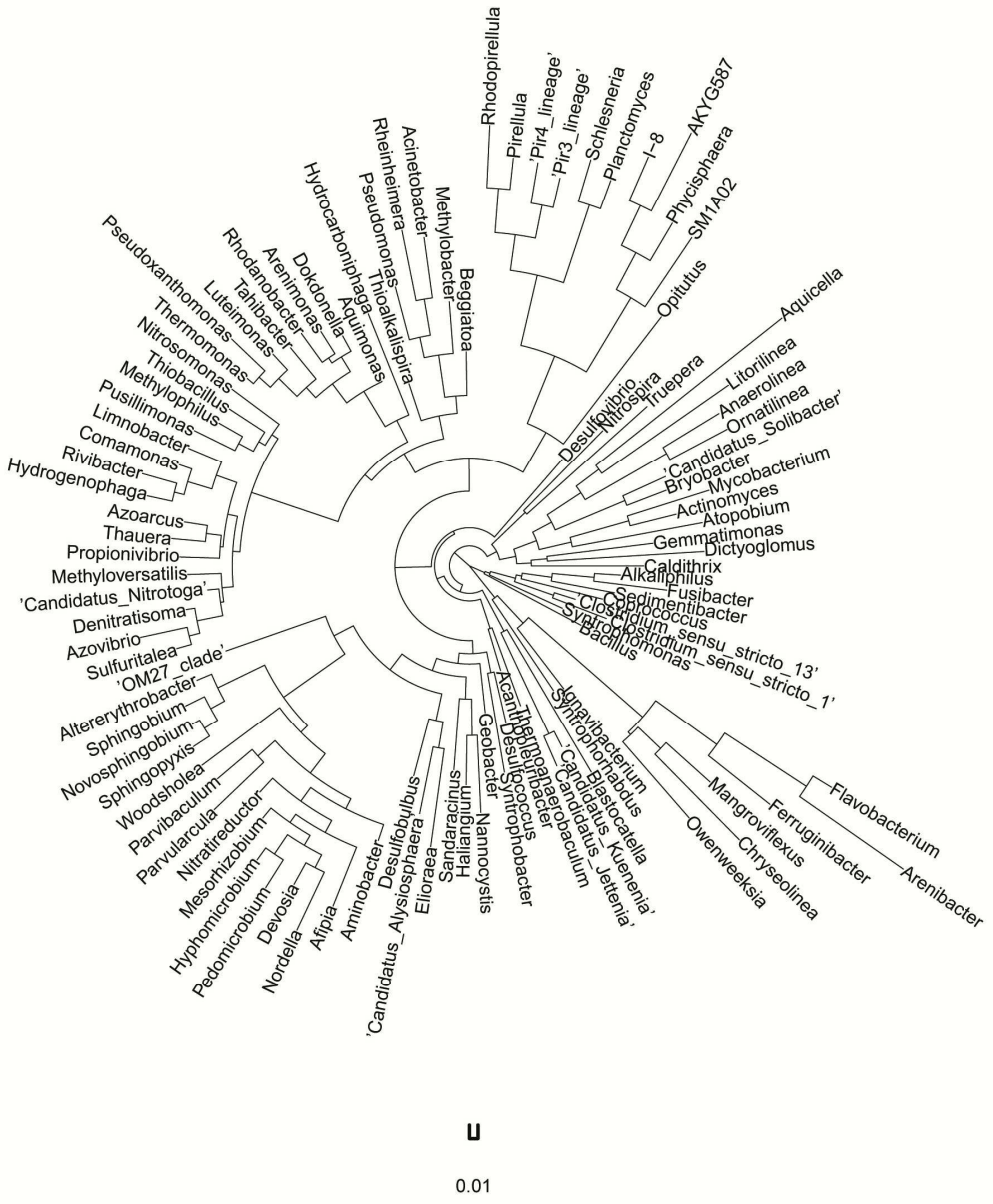

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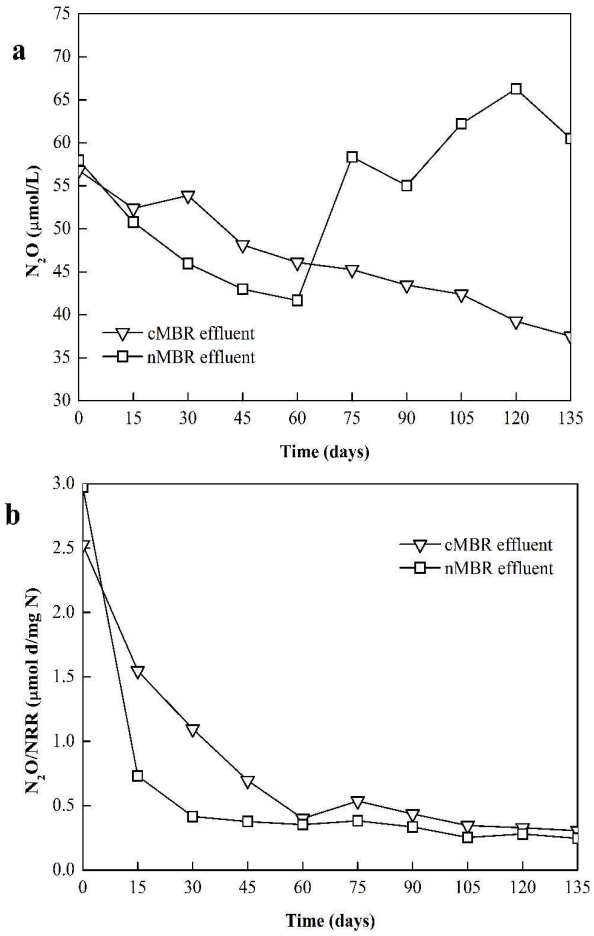

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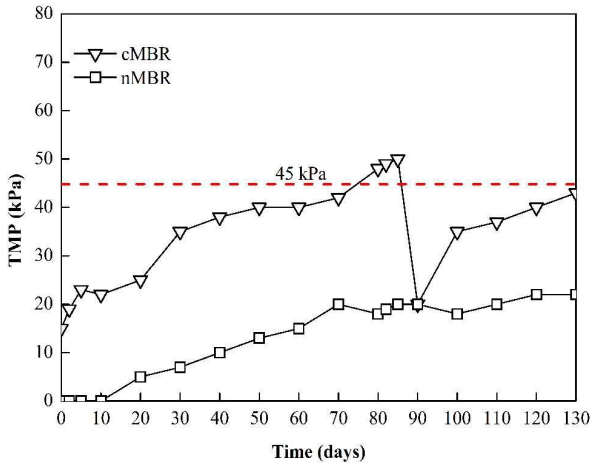

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