Enrichment and application of nitrifying activated sludge in membrane bioreactors
Xiaolin Sheng, Rui Liu, Lujun Chen, Zihua Yin and Jianfeng Zhu

ABSTRACT

In this study, nitrifying bacteria were enriched in a membrane bioreactor (MBR, R1) and their bioaugmentation effectiveness was evaluated in another two MBRs (R2 and R3). Nitrifying activated sludge (NAS) with high nitrification activity of up to 3,000 mg-N/(L·d) was successfully enriched in R1. The results showed that chemical oxygen demand concentration of 100–200 mg/L had no negative effect on NAS enrichment but reduced the ratio of bacterial nitrifiers. Moreover, the cell concentration of nitrifying bacteria in NAS, which was 3.1 × 10¹¹ cells/L, was similar to that of the commercial bacterium agent. For the bioaugmentation test, the reactor inoculated with 14% NAS achieved a 23% higher NH₄⁺-N removal efficiency than that of the uninoculated reactor. Along with the improvement of nitrification performance, the bacterial nitrifiers abundance and microbial richness remarkably increased after bioaugmentation. These results suggested that the MBR system could efficiently enrich nitrifying bacteria using organic carbon containing culture medium, and potentially act as a side-stream reactor to enhance the nitrification function of the wastewater treatment plant.

Key words | bioaugmentation, enrichment, MBR, nitrifying activated sludge

INTRODUCTION

Nitrification is the critical step of the nitrogen removal process because nitrifying bacteria is a group of slow-growing bacteria and can be easily inhibited by unfavourable temperature, pH, dissolved oxygen (DO) concentration, chemical oxygen demand (COD) concentration, ammonium nitrogen (NH₄⁺-N) concentration, free ammonia (FA) concentration, free nitrous acid (FNA) concentration, sludge retention time (SRT) and toxic compounds (Kim et al. 2012; Tang & Chen 2015). There are two main problems for the nitrification system: (1) it is slow to start up and (2) it is prone to break down under conditions of shock loads and/or low temperature. The common solutions to these problems at the wastewater treatment plants (WWTPs) are lengthening the SRT and the hydraulic retention time (HRT) of the aerobic tank, decreasing influent loading rate, or increasing aeration rate and return sludge ratio. However, it takes a long time to recover from the system break-down.

Bioaugmentation through inoculating nitrifying bacteria is an efficient way to improve the removal efficiency of NH₄⁺-N (Herrero & Stucley 2015). Nitrifying activated sludge (NAS) with a low preparation cost but strong adaptability is more efficient in degrading NH₄⁺-N and more compatible with the indigenous microbial communities than the commercial nitrifying bacteria agent (Thompson et al. 2005; Leu & Stenstrom 2010). Some inherent features, such as independent SRT and HRT control, high mixed liquor suspended solids (MLSS) levels and high tolerance ability against contaminants, make membrane bioreactor (MBR) more competitive to enrich NAS compared to aerobic tank and sequencing batch reactor (Radjenović et al. 2008). Previous studies have successfully enriched NAS in MBR using inorganic culture medium (Gao et al. 2004; D’Anteo et al. 2015). The culture medium mixed with a moderate proportion of WWTPs influent is a promising approach to improve the biocompatibility of the NAS and to reduce the preparation cost (Tang & Chen 2015), but the WWTPs influent usually contains high COD concentration of 200–500 mg/L. Despite that NAS could be enriched using organic carbon-containing culture medium (Head & Oleszkiewicz 2004; Toor et al. 2015), the effect of the COD
concentration on the NAS enrichment in MBR and maximum nitrification activity have not been fully understood. Meanwhile, the success of the NAS enrichment primarily depends on the effective bioaugmentation. Thus, a better understanding of the microbial dynamics in bioaugmentation would help to propose an optimize scheme for the NAS enrichment.

This study aims to: (1) establish an effective method to enrich NAS in MBR, and to evaluate the effect of COD concentration and SRT on nitrification activity of NAS; (2) investigate the correlation between population dynamics of nitrifying bacteria and operational conditions; and (3) evaluate the bioaugmentation of NAS against high NH$_4^+$-N concentration shock loads.

**MATERIALS AND METHODS**

**Experimental set-up**

Three MBRs were operated in this study, one (NAS enriched MBR) was used to develop the method for the enrichment of NAS, and the other two were used to evaluate the efficiency of the bioaugmented NAS. Each of the reactors was inoculated with 4 g/L MLSS activated sludge obtained from an aerobic tank at a municipal WWTP (Yixing, China).

**NAS enriched MBR**

The NAS enriched MBR (R1) (10 L) was equipped with hollow-fiber PVDF membrane modules with the pore size of 0.02 μm. The pH was maintained at 7.5 ± 0.5 by adding NaHCO$_3$, DO concentration was controlled above 2 mg/L. The HRT was 4 h. This MBR was operated at three different SRTs: ∞ days (no desludging), 20 days and 15 days. The composition of culture medium was: (NH$_4$)$_2$SO$_4$, KH$_2$PO$_4$, sodium acetate, MgSO$_4$·7H$_2$O (50 mg/L), CaCl$_2$·2H$_2$O (5 mg/L), MnSO$_4$·H$_2$O (2 mg/L), FeSO$_4$·7H$_2$O (0.5 mg/L), ZnSO$_4$·7H$_2$O (0.44 mg/L), NaMoO$_4$·2H$_2$O (0.25 mg/L), CoCl$_2$·6H$_2$O (0.2 mg/L), CuSO$_4$·7H$_2$O (0.1 mg/L) and H$_3$BO$_3$ (0.014 mg/L). The mass ratio of nitrogen to phosphorus was kept at 8:1. Influent COD, which varied between the range of 100–200 mg/L, was close to the influent BOD$_5$ concentration in the real domestic wastewater.

As shown in Table 1, this reactor was continuously operated for 228 days which comprised three running modes: run 1 (day 1–79), rapid enrichment period, in which no sludge was discharged; run 2 (day 80–136), maximum NAS yield period, and run 3 (day 137–228), recovery period.

**Bioaugmentation efficiency in two MBRs**

The bioaugmentation efficiency of enriched NAS was investigated in two 2 L MBRs (R2 and R3). R2 was used as control reactor (was not inoculated with enriched NAS), and R3 was inoculated with enriched NAS. Both R2 and R3 were fed with municipal wastewater (Yixing, China) at the HRT of 5.5 h. In both R2 and R3, DO and pH were maintained at 2.0–3.0 mg/L and 7.0–8.0, respectively. In order to assess the resistance ability of enriched NAS to the NH$_4^+$-N shock load, two intermittent 90 mg/L NH$_4^+$-N shock loads were applied, and each of them lasted for 48 h. The NH$_4^+$-N concentrations of influent and effluent were around 30 mg/L and 1 mg/L in both reactors before the shock loads. NAS was injected to R3 12 h after the first shock load. No NAS was added into R3 during the second shock load period.

<table>
<thead>
<tr>
<th>Operating parameters of R1</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1–42 d</td>
<td>43–63 d</td>
<td>64–79 d</td>
</tr>
<tr>
<td>SRT (Day)</td>
<td>∞</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Inf. NH$_4^+$-N (mg/L)</td>
<td>260 ± 87</td>
<td>374 ± 13</td>
<td>418 ± 30</td>
</tr>
<tr>
<td>NH$_4^+$-N VL (kg-N/(m$^3$d))</td>
<td>1.6 ± 0.5</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>100</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>FA (mg/L)</td>
<td>8.7 ± 6.8</td>
<td>2.0 ± 0.8</td>
<td>1.3 ± 1.5</td>
</tr>
</tbody>
</table>

VL = volumetric loading.
Sludge was discharged twice in Run 3: firstly, discharged 5 vol.% per day during day 176–179; secondly, discharged 5 vol.% per day during days 204–206.
Analytical methods

NH₄⁺-N and NO₂⁻-N were tested following the standard methods (MEPPRC 2002) using a spectrophotometer (SHIMADZU UV-2450, Japan). In situ temperature, pH and DO concentration were measured daily by a portable pH meter (HM-30P, DKK-TOA Corporation, Japan) and a portable DO meter (DO-31P, DKK-TOA Corporation, Japan), respectively. NO₂⁻-N accumulation ratio was calculated as the ratio of NO₂⁻-N to the sum of NO₂⁻-N and NO₃⁻-N.

DNA extraction and real-time polymerase chain reaction (PCR) amplification

NAS samples (1 mL, mixed liquid suspended solids) were taken from R1 on days 45, 78, 116, 136, 186 and 213. One specific activated sludge (SAS) sample was collected from a WWTP in Yixing, China. One commercial bacterium agent (CBA) sample was bought from PROBIOTIC Co., Ltd, China. Total genomic DNA from the above-mentioned eight samples were extracted using the environmental DNA isolation kit (Biocolor, Shanghai, China) and then stored at −20 °C for subsequent real-time PCR amplification.

The quantities of the AOB, NOB and total bacteria (TB) were determined by targeting the AOB amoA genes (Zeng et al. 2014), Nitrospira 16S rRNA genes (Huang et al. 2010), Nitrobacter 16S rRNA genes (Huang et al. 2010) and TB universal 16S rRNA genes (Zeng et al. 2014). All amplifications were conducted using an ABI StepOnePlus (Applied Biosystems, USA). For the amplification, 25 μL of the extracted DNA sample was used, other reagents include: 13 μL reaction mix (SYBR® Premix Ex Taq™, TaKaRa, Dalian, China), 10 μM forward and reverse primers apiece, 2 μL DNA template or standard and 9 μL deionized water. Ten-fold dilution series of plasmid were used to construct the standard curves. The correlation coefficients (R²) of the standard curves were all above 0.99. Calculation of cell numbers were performed assuming two amoA gene copies/AOB cell, one 16S rRNA gene copy/Nitroduct cell, one 16S rRNA gene copy/Nitrospira cell and 3.6 16S rRNA gene copies/bacteria cell (Zeng et al. 2014).

Analysis of microbial community diversity

The dynamics of bacterial communities were investigated by high-throughput sequencing analysis. Three biomass samples were collected, one NAS of R1 on day 141 and two sludge samples of R3 on days 7 and 15. All sludge samples were stored at −70 °C before high-throughput sequencing analysis (Shanghai Sangon Biological Engineering Technology and Services Co. Ltd, China). The sequences achieved were aligned with RDP classifier based on Bergey's taxonomy for taxonomic classification.

Data analysis

Data were visualized through Origin 9.0 (OriginLab, USA). Using SPSS Statistics 20 (IBM, USA), stepwise regression models were built to determine the multiple linear regression equation between nitrification activity and a set of variables (Influent NH₄⁺-N, COD, and sludge mass) related to the operating parameters. FA concentration was calculated according to Equation (1):

\[
FA \text{ as } NH_3-N (mg/L) = \frac{C_{NH_3-N} \times 10^{\text{pH}}}{e^{6344/(273+T)} + 10^{\text{pH}}}
\]

RESULTS AND DISCUSSION

Enrichment and reactor performance

The nitrification activity of NAS and the performance of R1 were shown in Figure 1. NH₄⁺-N volumetric load (NVL) and COD concentration were increased step wisely during run 1. The initial NVL was maintained at around 0.65–0.88 kg-N/(m³·d). In the following 37 days, around 75.4% of the NH₄⁺-N was removed from R1 at the NVL of 2.2 kg-N/(m³·d), while the NO₂⁻-N accumulation ratio was up to 88%. The poor performance of nitrite oxidation process coincided with the reduction of the quantity and activity of NOB. NOB grow more slowly than AOB. During this period, the DO and pH were suitable for NOB growth, but the average FA concentration of 8.5 mg/L exceeded the inhibition concentration of 1.0 mg/L on NOB (Anthonisen et al. 1976). In order to reduce the inhibition of FA and accelerate NOB enrichment, the NVL reduced to maintained around 2.1 kg-N/(m³·d). The effluent NO₂⁻-N concentration dropped down to 30 mg/L within 28 days. During day 64 to day 79, the NVL was increased to about 2.7 kg-N/(m³·d), the nitrification activity increased again along with the increase of influent NH₄⁺-N concentration and finally reached to 2,763 mg-N/(L·d) which was 23 times higher than that of SAS (about 120 mg-N/(L·d)) (Limpiyakorn et al. 2011). Meanwhile, both the effluent NH₄⁺-N and NO₂⁻-N concentrations were below 10 mg/L, suggesting
that NAS was enriched in R1 within 80 days. In a similar study (D’Anteo et al. 2015), the NVL was about 0.65 kg-N/(m³·d) after 380 days of operation in MBR. The correlational analysis indicated that the nitrification activity was significantly (p < 0.05) and positively related to the influent NH₄⁺-N concentration and COD concentration with the contribution ratio of 94% and 41%, respectively. The COD concentration of 100–200 mg/L had no negative effect on NAS, revealing that nitrification could maintain high activity at a low organic carbon concentration (Tang & Chen 2015). Thus, the influent NH₄⁺-N concentration was the main factor in improving the nitrification performance, and the FA concentration was a crucial inhibitor for NOB enrichment.

In Run 2, the maximum NAS yield was achieved by reducing the SRT to 20 days and then 15 days. As shown in Figure 1, the nitrification activity was enhanced at the SRT of 20 days and reached 2,898 mg-N/(L·d) on day 119, while it decreased to 2,608 mg-N/(L·d) at the SRT of 15 days on day 136. These results revealed that moderately discharging sludge mass contributed to maintaining or even improving the nitrification activity, which was coincident with Huang’s study (Huang et al. 2015). The decrease in nitrification activity at SRT of 15 days was speculated to be caused by the loss of nitrifying microorganisms. Thus, SRT of 20 days was suitable for stable NAS production at the NVL of 3.0 kg-N/(m³·d).

Three recovery processes of nitrification function were presented in Run 3. At the SRT of 15 days and COD concentration of 150 mg/L, NH₄⁺-N removal efficiency restored to 95% in 31 days. After discharging 5 vol.% of biomass per day during days 176–179, it took only 5 days to restore the same level of NH₄⁺-N removal efficiency at the same COD concentration. However, at the COD concentration of 120 mg/L, it took 10 days to restore after discharging same amount of biomass per day during days 204–207. It was deduced that the less the nitrifying microorganisms lost, the faster the nitrification function could recover, and that COD concentration of 150 mg/L would speed up the nitrification recovery compared with 120 mg/L.

In general, to improve the nitrification activity of the NAS, the influent NH₄⁺-N was the main factor and the optimal SRT was 20 days. The COD concentrations of 100–200 mg/L have no negative effects on NAS. The nitrification process recovered faster at the COD concentration of 150 mg/L.

Quantification of the nitrifying bacteria by real-time PCR

Population dynamics of nitrifying bacteria in R1, SAS and CBA were illustrated in Figure 2. The number of bacterial nitrifiers in NAS varied in a range of 1.1 × 10¹¹–3.3 × 10¹¹ cells/L throughout the experiment, which was close to that in CBA but was two orders of magnitude higher than that in SAS. An increasing number of AOB and NOB were associated with the increasing of the NVL. The dynamic balance between the growth rate and the loss rate of the bacterial nitrifiers could be maintained at the SRT of 20 days but could not be maintained at the SRT of 15 days. Although the concentration of bacterial nitrifiers increased up to 3.1 × 10¹¹ cells/L, the abundance of nitrifying bacteria decreased to 11% at the COD concentration of 150 mg/L. In order to raise the ratio of bacterial nitrifiers, the COD concentration was reduced to 120 mg/L, but no obvious increasing was observed after 26 days of operation. The ratio of bacterial nitrifiers could not be recovered through decreasing the COD concentration only. The results revealed that the numbers of bacterial nitrifiers related to the NVL and SRT, regardless of COD concentration.

Nitrospira was the dominant NOB in SAS and CBA, despite the existence of another NOB genera Nitrobacter.
This was consistent with previous studies (Cho et al. 2014; Zeng et al. 2014). However, Nitrobacter dominated over Nitrospira in R1. Compared to Nitrospira, Nitrobacter has a higher nitrite half-saturation constant and higher threshold concentrations of FA inhibition, which more tend to dominate nitrite oxidation under conditions of high ammonium and nitrite concentrations (Blackburne et al. 2011). Thus, the Nitrospira was washed out in R1.

**Effectiveness of bioaugmentation in R3**

Profile of NH$_4^+$-N concentration in the R2 and R3 was shown in Figure 5. Before the first shock load (days 1–7), the average NH$_4^+$-N removal efficiencies in R2 and R3 were 96% and 95%, respectively. After being bioaugmented with 14% (volume ratio) NAS from R1, the average removal efficiency of NH$_4^+$-N in R3 during the first shock load period was 81%, which was 23% higher than that in R2. Previous studies have reported that the NH$_4^+$-N removal efficiency was improved by bioaugmentation through adding external nitrifiers (Tang & Chen 2015), but the low adaptability of nitrifiers might reduce the bioaugmentation effect (Herrero & Stuckey 2015). Therefore, the effect of the bioaugmented NAS on the sustainability of nitrification activity in R3 without adding NAS was investigated during the second shock load. The average removal efficiency of NH$_4^+$-N in R3 was 79%, which was close to the NH$_4^+$-N removal efficiency during the first shock load and was 10% higher than that in R2. The effect of single inoculation of bioaugmented NAS could last at least 10 days. Therefore, the bioaugmentation through inoculating NAS could improve the NH$_4^+$-N removal efficiency, distinctly shorten the time for nitrification restoration and reinforce the sustainability of the biosystem.

**Microbial community of activated sludge in R3**

The microbial community structure in R3 and NAS were depicted in Figure 4. As expected, the abundance of *Nitrosomonas* in R3 increased from 1.49% to 3.91% 7 days after bioaugmentation. Interestingly, the abundance of *Comamonas* and *Arenimonas* in R3 were 12.6% and 4.7% higher than the initial values of 2.8% and 3.1%, respectively. Most of *Comamonas* participated in the aerobic nitrification–heterotrophic denitrification process, for example, *Comamonas* sp. GAD4, *Comamonas* sp. strain SGLY2, and *Arenimonas* were reported as heterotrophic denitrifiers (Patureau et al. 1997; Chen & Ni 2010; Liu et al. 2015). In addition, the content of *Nitrosococcus* in R3 still increased even though NAS did not contain *Nitrosococcus* (Table 2). The richness in R3 was improved after bioaugmentation (Figure 5). Several studies have suggested that the stability and NH$_4^+$-N removal performance of a reactor related to the level of bacteria diversity and quantity of nitrifying bacteria in the reactor (Rowan et al. 2005; Pal et al. 2012). It is the reason that the R3 had a better NH$_4^+$-N removal performance than R2 during the second shock loading. Hence, most bacteria in
NAS could survive a new environment and increase the resistance ability of the reactor. Consequently, the NH$_4^+$-N removal efficiency could be improved because of the increase of bacterial nitrifiers abundance.

**CONCLUSIONS**

NAS with nitrification activity of 3,000 mg-N/(L·d)$^{-1}$ was enriched in MBR at the SRT of 20 days. The concentration of nitrifying bacteria in NAS of up to 3.1 × 10$^{11}$ cells/L was similar to that in CBA but was two orders of magnitude higher than that in SAS. The COD concentration of 100–200 mg/L had no negative effect on NAS enrichment but reduced the ratio of bacterial nitrifiers. The enriched NAS significantly improved the nitrification function of a simulating general aerobic biological reactor, achieving a 23% increase in the NH$_4^+$-N removal, a 2.4% increase of the bacterial nitrifiers abundance and a shortened recovery time of nitrification function. The results indicated that MBR could be used as an efficient NAS enrichment reactor as well as a side-stream reactor for enhancing biological nitrification at WWTPs.

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