Effect of reflux ratio on nitrogen removal in a novel upflow microaerobic sludge reactor treating piggery wastewater with high ammonium and low COD/TN ratio: Efficiency and quantitative molecular mechanism

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HIGHLIGHTS

• To treat piggery wastewater with high NH4+ density and low C/N ratio.
• An innovative upflow microaerobic sludge reactor was constructed.
• Effect of reflux ratio (RR) and mechanism of nitrogen removal were investigated.
• RR 34 was favorable for NH4+ and TN removal with anammox as the dominant mechanism.
• Decrease in RR stimulated ammonia-oxidation but inhibited anammox.

ABSTRACT

A novel upflow microaerobic sludge reactor (UMSR) was constructed to treat manure-free piggery wastewater with high NH4+-N and low COD/TN ratio. In the light of the potential effect of effluent reflux ratio (RR) on nitrogen removal, performance of the UMSR was evaluated at 35°C and hydraulic retention time 8 h with RR decreased from 45 to 25 by stages. A COD, NH4+-N and TN removal of above 77.1%, 80.0% and 86.6%, respectively, was kept with a RR over 35. To get an effluent of TN not more than 80 mg/L with a TN load removal above 0.88 kg/(m3 d), the RR should be at least 34. Real-time quantitative polymerase chain reaction of functional bacteria revealed that the RR of less than 34 stimulated ammonium oxidation but badly inhibited anammox, the dominant nitrogen removal pathway, resulting in the remarkable decrease of nitrogen removal in the reactor.

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1. Introduction

The increasing demand for pork has stimulated a continual expansion of pig-breeding, resulting in increasing discharge of piggery wastewater from large-scale pig farms, especially in developing countries such as China. If not disposed properly, it would result in significant health and environmental consequences (Zhao et al., 2014). The strength of piggery wastewater varies greatly due to the manure collection methods (Wang and Guo, 2009). As a traditional method, urine-free manure collection is widely used and the flushed wastewater of piggery is defined as manure-free piggery wastewater (MFPW) (Zhao et al., 2014). This MFPW is characterized by high ammonium (NH4+-N) and low ratio of chemical oxygen demand (COD) to total nitrogen (TN), posing a challenge to traditional nitrification-denitrification processes for nitrogen removal (Zhang et al., 2016). Anaerobic-aerobic combined process has been developed for nitrogen removal from sewage and proved to be robust (Bernet et al., 2000; Canals et al., 2013). The combined process could also be used for piggery wastewater treatment, despite of the costly requisite for physical chemistry pre-treatment to get a feasible COD/TN ratio for biological nitrogen removal (Bernet et al., 2000). It is essential to develop a novel treatment processes that is more efficient and economical for treating MFPW.

Microaerobic condition is regarded as a transitional state between aerobic and anaerobic with a dissolved oxygen (DO) ranging from 0.3 to 1.0 mg/L (Zheng and Cui, 2012; Zitomer, 1998). Previously, a novel upflow microaerobic sludge reactor (UMSR) was developed to treat MFPW (Meng et al., 2015). Ammonia-
oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), hetero-
trophic denitrifiers, autotrophic denitrifiers and phosphate accum-
ulating organisms were found to coexist in the UMSR, and their
synergistic action improved microaerobic process in the syn-
chronous removal of COD, NH4+–N, TN and total phosphorus (TP)
(Meng et al., 2016). The microaerobic condition in the UMSR was
achieved by recirculating part of the effluent that was aerated to
a DO of about 3 mg/L (Meng et al., 2015). Obviously, reflux ratio
(RR) plays a crucial role in controlling the microaerobic condition
in the reactor. In fact, the microbial community structure can be
remarkably affected by the change of RR, which would result in a
variation in pollutant removal efficiency (Jin et al., 2012). An exces-
sive RR would not only restrict anaerobic denitrifiers due to the
increased DO, but also increase the treatment cost (Yuan et al.,
2002). On the other hand, a RR too low would lead to an insuffi-
cient DO for NH4+–N oxidation, also resulting in a bad TN removal
in biological nitrogen removal processes (He et al., 2006). There-
fore, a feasible RR is essential for the microaerobic treatment sys-
tem to enhance TN removal and cut down the treatment cost.
However, few researches can be found to investigate the effect of
RR on microaerobic processes.

In the present research, the UMSR was continually performed
for another 176 days, by stages, to evaluate the effect of RR on
the synchronous removal of COD, NH4+–N, TN and TP. To understand
the mechanism for biological nitrogen removal in the reactor, func-
tional microbial populations under different RR were investigated
using Illumina Miseq platform, while the nitrogen removal path-
ways were also analyzed based on the absolute abundances of
functional bacteria identified by real-time quantitative polymerase
chain reaction (qPCR).

2. Materials and methods

2.1. Microaerobic treatment system

The lab-scale UMSR process, a microaerobic treatment system,
was illustrated in Fig. 1. The UMSR was a 0.5-meter-high plexiglass
column with a working volume of 4.9 L, while with a 0.5 L circular
cone attached to the bottom. A 3 L solid-liquid-gas separator was
designed on the top of the column with the off-gas discharge after
a water lock. Four sampling ports at 10 cm interval from each other
were allocated along the vertical height of the column. The temper-

ature in the reactor was kept at 35 ± 1 °C by a temperature con-
troller (Jetten et al., 2001). The effluent was collected by a 10 L
tank, in which part of the effluent was aerated to a DO of about
3 mg/L and then recirculated into the reactor from the bottom.

2.2. Wastewater and inoculum

The raw MFPW was collected from a local pig breeding farm in
Harbin, China. The wastewater quality fluctuated following the
changes in breeding seasonality with a COD, NH4+–N, nitrite (NO2-
N), nitrate (NO3–N), TN, TP and pH ranging from 241 to 459,
237.4 to 343.3, 0.0 to 2.2, 0.0 to 5.8, 285.4 to 458.2, 6.4 to 21.4
mg/L and 7.0 to 8.4, respectively. Obviously, the high concentration
of NH4+–N contributed mainly to the TN in the wastewater.

The seed sludge inoculated in the UMSR was anaerobic sludge
collected from an upflow anaerobic sludge blanket (UASB) treating
swine wastewater in the State Key Laboratory of Urban Water
Resource and Environment, Harbin Institute of Technology, China.
The initial biomass in the UMSR was 5.68 g/L in terms of mixed
liquor suspended solids (MLSS), i.e. 1.95 g/L in terms of mixed
liquor volatile suspended solids (MLVSS). The UMSR previously
started up and performed for 200 days (Meng et al., 2015). At the
start of the present research, MLVSS in the UMSR was 3.5 g/L
with a MLVSS/MLSS ratio of 0.66. The sludge was dominated by flocs,
among which some small-sized granular sludge (<2 mm) could
also be observed.

2.3. Operation of the UMSR

In the present research, the UMSR was continued to be operated
for another 176 days in a 4-stage procedure with a consistent
hydraulic retention time (HRT) of 8 h. Quality of the raw MFPW
and the other controlling parameters in the 4 stages are illustrated
in Table 1. The influent and effluent were sampled daily for analy-
sis. Activated sludge in the reactor was sampled at the end of each
stage for measurement of biomass (MLVSS and MLSS) and analysis
of high-throughput pyrosequencing and qPCR.

2.4. Analytical methods

COD, NH4+–N, NO2–N, NO3–N, TP and biomass (MLSS and MLVSS)
were detected by closed reflux titrimetric method, Nessler’s reagent
colorimetric method, colorimetric method, ultraviolet spectrophotometric screening method, vanadomolybdophosphoric
acid colorimetric method and gravimetric method, respectively,
according to the Standard Methods (APHA, 2005). DO and pH were
measured with a dissolved oxygen meter (Taiwan Hengxin, AZ
8403) and a pH meter (Switzerland Mettler Toledo, DELTA320),
respectively. TN was measured on a total nitrogen analyzer (Ger-
many Analytikjena Multi, N/C 2100S).

2.5. High-throughput pyrosequencing

Sludge sampled from the UMSR at each end of the 4 stages
(Table 1) was named S1, S2, S3, and S4 in sequence, and the total
DNA of the samples were extracted, respectively, using the Bacteria
DNA Isolation Kit Components (MOBIO) according to the manufac-
turer’s instruction. Bacterial 16S rRNA genes for the V3-V4 regions
were amplified with primer pairs 341f (5′-CCTACGGGAGGCAG
CAG-3′) and 805r (5′-GACTACHVGGGTATC TAATCC-3′)
(Herlemann et al., 2011; Hugenholtz et al., 2014). Composition of the PCR products of V4 region were determined by pyrosequencing
on the Illumina Miseq sequencer platform (Shanghai Sango,
China).
Nitrospira spp., Nitrobacter the 16S rRNA of total bacteria, anammox bacteria, sludge samples, seven independent qPCR assays were conducted.

95\degree C for 3 min at 95\degree C and 7.2\degree C for 20 cycles each including 15 s at 95\degree C, 20 s at 55\degree C and 30 s at 72\degree C in sequence. The PCR program was as follows: 3 min at 95\degree C followed by 40 cycles each including 15 s at 95\degree C, 20 s at 55\degree C and 30 s at 72\degree C in sequence. The PCR was controlled by a reaction without template and each of the qPCR was performed in triplicate.

All of the plasmid standards of the 16S rRNAs and the functional genes were constructed by Sangon Biotechnology Incorporated Company (Shanghai, China). The standard curves were prepared using the three replicate measurements and plotted as error bars for analyzing data variations and measurement errors.

2.6. qPCR analysis

To determine the amount of nitrifiers and denitrifiers in the sludge samples, seven independent qPCR assays were conducted in a real-time PCR system (USA Foster, ABI 7500) by quantifying the 16S rRNA of total bacteria, anammox bacteria, Nitrobacter spp., Nitrospira spp., and gene of amoA, nirS and narG, respectively. The primers used are illustrated in Table 2. Each of the PCR reactions was performed in a 20 l PCR-grade sterile water. The PCR program was as follows: 3 min at 95\degree C and 7.2\degree C for 20 cycles each including 15 s at 95\degree C, 20 s at 55\degree C and 30 s at 72\degree C in sequence. The PCR amplification efficiency for the genes was between 90\% and 110\%.

The absolute abundance of the seven functional genes (i.e. total bacterial, anammox, Nitrobacter spp., Nitrospira spp., amoA, nirS and narG) obtained from each of the triplicate tests were averaged, respectively, and then used as the basic candidate variables in non-linear regression analyses (SPSS 22, USA) for the correlations with nitrogen transformation rates. Standard deviations were calculated using the three replicate measurements and plotted as error bars for analyzing data variations and measurement errors.

3. Results and discussion

3.1. Pollutant removal in the UMSR

After the previous 200-day performance (Meng et al., 2015), the UMSR was continued to be operated for another 176 days following a progressive decrease in RR (Table 1) with a consistent HRT of 8 h at 35 ± 1\degree C. The performance in pollutant removal within the 176 days was illustrated in Fig. 2. The results showed that, in stage 1 with the RR 45, NH₃-N removal in the reactor exhibited a rapid decrease phase within the first 6 days, and then increased gradually in fluctuation thereafter and reached a steady phase within the last 13 days (55th–67th day) (Fig. 2A). The average NH₃-N removal in the steady phase reached 86.2\% with a residual amount of about 40.2 mg/L in effluent. The NH₄-N removal was remarkably affected and decreased sharply to 37.7\% when the RR decreased to 35 in the first day of stage 2, but rebounded and reached a new steady state since the 96th day with the removal and effluent concentration averaging 77.1\% and 67.4 mg/L, respectively. When RR was further decreased to 30 in stage 3 and 25 in stage 4, the same variation pattern of NH₄-N removal with time were also observed. The NH₄-N removal in the steady phase of stage 3 (130th–141th days) and stage 4 (164th–176th days) was about 73.1\% and 53.3\%, with a NH₄-N of about 81.3 and 137.8 mg/L in effluent, respectively. Obviously, the decrease of RR had badly affected the ammonium oxidation in the reactor.

Variation of TN removal (Fig. 2B) in the UMSR found the same trend as that of NH₄-N removal, with the steady states obtained within the last 13 days (55th–67th day) and stage 4 (164th–176th days) was about 73.1\% and 53.3\%, with a NH₄-N of about 81.3 and 137.8 mg/L in effluent, respectively. Obviously, the decrease of RR had badly affected the ammonium oxidation in the reactor.
concentration of about 45.8, 73.2, 89.2 and 140.6 mg/L, respectively. The average TN load removal (TNLR) in the four steady phases was calculated to 0.94, 0.88, 0.82 and 0.66 kg/(m$^3$/d), respectively. The inhibited NH$_4^+$-N oxidization by the decreased RR (Fig. 2A) was considered as the main reason for the decrease of TN removal because no obvious NO$_x$-N (including NO$_2^-$-N and NO$_3^-$-N) was detected.
NO\textsubscript{3}-N) acclimation was found in the four steady phases. However, NO\textsubscript{3}-N accumulation could be found in the first days of all the stages. In stage 1, for example, NO\textsubscript{3}-N accumulated to 50.4 mg/L on the 31th day, but decreased rapidly with time and no observable accumulation was detected in the steady phase. The same phenomenon was also observed in the next 3 stages, though the accumulation of NO\textsubscript{3}-N decreased slightly with the stages. This performance accorded with the cultural characteristics of anammox bacteria (van Dongen et al., 2001), indicating anammox was an important approach to nitrogen removal in the UMSR.

All through the 176-day operation, COD removal in the UMSR was above 65% with an effluent COD less than 125 mg/L (Fig. 2C). It was found that COD removal in the steady phases of stage 1 and stage 3 were almost the same (77.9% and 74.5%, respectively), though the RR (45 and 30, respectively) was the remarkable difference. The COD removal in the steady phases of stage 2 (RR 35) and stage 4 (RR 25) were also similar (86.6% and 83.8%, respectively). It was noticed that COD/TN ratio in stage 1 (0.84) and 3 (0.82) were almost the same, as well as those in stage 2 (0.95) and 4 (0.94). The results suggested that COD/TN ratio had a stronger effect than RR on the COD removal in the UMSR. Within the 176-day operation of the UMSR, no excess sludge was discharged and the biomass in terms of MLVSS at each end of the four stages was 5.20, 7.15, 4.11, and 6.26 g/L, respectively. Evidently, the higher COD/TN ratio in stage 2 and stage 4 had stimulated the growth of chemo-heterotrophic bacteria, resulting in the better COD removal in the reactor (Li et al., 2016).

The stimulated growth of biomass was favorable for TP removal in the UMSR. As shown in Fig. 2D, TP removal in the second steady phase reached 83.5% with the highest biomass (7.15 g/L). With a lower biomass in stage 1 (5.20 g/L), stage 3 (4.11 g/L) and stage 4 (6.26 g/L), the TP removal decreased to 46.8%, 52.5% and 66.6% in their steady phases, respectively. The effluent TP in the four steady phases averaged 5.1, 2.1, 4.4 and 5.0 mg/L, respectively. Observably, TP removal in the UMSR was positively correlated with the biomass, indicating sludge growth was the most important approach for TP removal in the microaerobic treatment system.

Discharge standards for various wastewater have been promulgated in many countries. According to the Discharge Standard of Pollutants for Livestock and Poultry Breeding issued by the Ministry Environmental Protection of China (MEPPRC, 2001), the discharge standard for NH\textsubscript{4}-N should not be more than 80 mg/L. All through the 176-day operation of the UMSR, the effluent COD and TP could meet the standard required very well, while the effluent NH\textsubscript{4}-N and TN could meet the standard only with a RR not less than 35 (Fig. 2). To get an economical RR for treating manure-free piggery wastewater, the influence of RR on nitrogen removal (NH\textsubscript{4}-N and TN) and TNLR in the four steady phases were analyzed as illustrated in Fig. 3, in which an inhibitory threshold for nitrogen removal was defined when TN removal dropped to around 78.0%, with an effluent TN more than 80 mg/L. Based on the quadratic polynomial regression equations (Fig. 3), the RR threshold should be 34, at which a TNLR of 0.88 kg/(m\textsuperscript{3}·d) could be obtained in the UMSR.

### 3.2. Diversity of microbial community and synchronous pollutant removal

To understand the biological mechanism of synchronous removal of COD, NH\textsubscript{4}-N, TN and TP in the UMSR, DNA extraction and PCR amplification of the sludge sampled at the end of each stage (Table 1) were carried out. As shown in Table 3, 41,342, 36,383, 37,906 and 19,515 reads were obtained from the sludge sampled from stage 1 (S1), stage 2 (S2), stage 3 (S3) and stage 4 (S4), respectively. The identified operational taxonomic units (OTUs) in the four sludge samples reached 5632, 5211, 6542 and 1459, respectively. The results indicated that better species diversity and richness had been kept in the UMSR, though the Chao1 and ACE index, as well as the Shannon index, varied with the decrease of RR.

The microbial community structure and composition under different RR were characterized by phylogenetic classification. As shown in Fig. 4A, Proteobacteria was the most predominant phylum, followed by Firmicutes and Bacteroidetes, all the time in the UMSR. Fig. 4B indicated that the main subgroups of phyla Proteobacteria in S1, S2 and S4 were the same β-Proteobacteria, while γ-Proteobacteria dominated the S3. It is reported that β- and γ-Proteobacteria play a critical part in the biological removal of nitrogen and phosphorus (Yang et al., 2014).

Phylogenetic classification of the four sludge samples at genus level (Fig. 5) showed that the microbial community structure in the UMSR was distinguished by the difference of RR. Even so, heterotrophic bacteria (including aerobic and anaerobic heterotrophic bacteria), nitrifiers (including AOB and NOB), and denitrifiers (including heterotrophic and autotrophic denitrifiers) were found to coexist and flourish in the microaerobic system, resulting in the synchronous removal of COD, NH\textsubscript{4}-N and TN. However, no genera related to anammox had been identified from the activated sludge samples by high-throughput pyrosequencing (Fig. 5). Thus, the nitrogen removal mechanism had to be further investigated.

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**Fig. 3.** Effect of reflux ratio on nitrogen removal in the UMSR.
3.3. Nitrogen removal pathways

Nitrification-denitrification, shortcut nitrification-denitrification and anammox are well known as the approaches to the TN removal in biological denitrification processes (Xie et al., 2016). The reduction of nitrate to nitrogen gas (N₂ or N₂O) needs more carbon source to supply electron than that of nitrite reduction, and nitrous oxide (N₂O) production would need less carbon source than the
Fig. 5. Bacterial community structure at genus level of the activated sludge sampled from the UMSR at the end of the first stage (A), the second stage (B), the third stage (C) and the fourth stage (D).
production of N₂. Theoretically, without assimilation, 2.86 g or 1.71 g COD is needed to reduce 1 g NO₃⁻-N or 1 g NO₂⁻-N to N₂, respectively (Li et al., 2012; Saggar et al., 2013). For the reduction of 1 g NO₃⁻-N or 1 g NO₂⁻-N to N₂O, 2.29 g or 1.14 g COD would be consumed respectively. Based on the cooperation of the theoretical COD/TN ratio with the measured CODremoved/TNremoved ratio in the UMSR, the theoretical TN removal efficiencies via various pathways in the microaerobic system were calculated and illustrated in Table 4. The results showed that the TN removal in the UMSR was remarkably lower than that of the actual efficiency even all of the removed COD were used for the denitrification via nitrite reduction or nitrate reduction to N₂. Even for nitrite reduction to N₂O, the least carbon-source-consumption denitrification, the theoretical TN removal was also observably lower (stage 1, stage 2 and stage 3) or similar (stage 4) to that of the actual efficiency in the UMSR. In the steady phase of stage 1, for example, the theoretical TN removal efficiency via NO₂ production by nitrite reduction, N₂ production by nitrite and nitrate reduction were 59.0%, 39.1% and 23.5%, respectively, considerably lower than the actual 87.2%. Hence, the autotrophic anammox must be one of the most important pathways to nitrogen removal in the UMSR.

To confirm the existence of anammox and understand the nitrogen removal mechanism in depth, the absolute abundances of amoA, nirS, narG and Nitrobacter 16S rRNA, Nitrospira 16S rRNA, anammox 16S rRNA and total bacteria 16S rRNA in the sampled sludge (S₁, S₂, S₃ and S₄) were quantified using qPCR assays and the results were illustrated in Fig. 5. Among the seven functional genes, amoA was responsible for NH₄⁺-N conversion and NO₂⁻-N production, while nirS for NO₂⁻-N reduction, narG for NO₃⁻-N reduction, both Nitrobacter 16S rRNA and Nitrospira 16S rRNA for NO₂⁻-N oxidation, and anammox 16S rRNA for NH₄⁺-N and NO₃⁻-N consumption. Absolute abundances of the seven functional genes were used as basic candidate variables for nonlinear regression analyses (SPSS 22, USA) to get the correlation with nitrogen conversion rates. Then the following equations are obtained.

$$NH_4^+ - N = -4.706(amoA/(anammox + Nitrobacter + Nitrospira)) + 0.780$$

$$TN = -2.881(amoA/(anammox + Nitrobacter + Nitrospira)) + 23.625(nirS + narG)/Bacterial + 0.831$$

The R² in Eqs. (1) and (2) was 0.977 and 0.989, respectively. It was found that the NH₄⁺-N removal had a negative correlation with amoA/(anammox + Nitrobacter + Nitrospira). The NH₄⁺-N regression model suggested that NH₄⁺-N removal in the UMSR was not only influenced by amoA gene but also by anammox, Nitrobacter and Nitrospira 16S rRNA, and the increased amoA would reduce the NH₄⁺-N removal in the UMSR. TN removal in the UMSR was related to denitrification, too (Li et al., 2016). Both nirS/(total bacteria) and narG/(total bacteria), involved in NO₂⁻-N and NO₃⁻-N reduction respectively, were positively correlated with TN removal in the UMSR.

**Table 4**

Actual TN removal efficiency in the UMSR comparing with the theoretical values.

<table>
<thead>
<tr>
<th>Stage</th>
<th>COD removal (kg/(m³·d))</th>
<th>TN removal (kg/(m³·d))</th>
<th>CODremoved/TNremoval</th>
<th>Actual TN removal (%)</th>
<th>TN removal via NO₂ production from NO₃⁻-N (%)</th>
<th>TN removal via nitrite reduction to NO₂ (%)</th>
<th>TN removal via nitrate reduction to N₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>0.72</td>
<td>0.94</td>
<td>0.76</td>
<td>87.2</td>
<td>59.0</td>
<td>39.1</td>
<td>23.5</td>
</tr>
<tr>
<td>Stage 2</td>
<td>0.89</td>
<td>0.88</td>
<td>1.01</td>
<td>80.0</td>
<td>70.9</td>
<td>47.0</td>
<td>28.3</td>
</tr>
<tr>
<td>Stage 3</td>
<td>0.63</td>
<td>0.82</td>
<td>0.77</td>
<td>75.4</td>
<td>50.9</td>
<td>33.8</td>
<td>20.3</td>
</tr>
<tr>
<td>Stage 4</td>
<td>0.86</td>
<td>0.66</td>
<td>1.30</td>
<td>60.9</td>
<td>69.4</td>
<td>46.0</td>
<td>27.7</td>
</tr>
</tbody>
</table>

* The average during steady phase of the stage.

**Fig. 6.** Copies per ngDNA of nitrogen functional genes with the reflux ratios in the UMSR.

UMSR (Eq. [2]). Above all, the qPCR assays indicated that shortcut nitrification, anammox, and denitrification coexisted in the UMSR, and their combined action resulted in the better TN removal without supply of extra carbon source.

3.4. Quantitative molecular mechanism of pollutant removal

As illustrated in Fig. 5, though the RR decreased from 45 to 25 by stages, the heterotrophic bacteria were the foremost populations all along the 176-day performance of the UMSR. The flourishing heterotrophic population resulted in the better COD removal in the UMSR all through the four operation stages (Fig. 2C). Among the heterotrophic bacteria, Variovorax (Prasad and Suresh, 2012), Novosphingobium (Kertesz and Kawasaki, 2010), Stenotrophomonas (Binks et al., 1995) and Altererythrobacter (Park et al., 2011) are strict aerobic bacteria, and the total proportion decreased from 4.66% in S₁ to 2.27% in S₄ with the decreased RR from 45 to 25 stage by stage. Obviously, it was the decreased RR that had reduced the DO concentration and resulted in the decline of aerobic heterotrophic bacteria in the UMSR.

It was interesting to find that the decrease of RR enriched AOB and NOB in the reactor. With the RR 45, genera related to AOB in S₁ were Sphingomonas (Zheng et al., 2005), unclassified_Nitrosomonadaceae (Prosser et al., 2014), Nitrosococcus (Stein et al., 2013), Nitrosomonas (Vock et al., 1995) and Nitrosospira (Hollibaugh et al., 2002), with a total proportion of 1.07% (Fig. 5A). When the RR was decreased to 35 and then to 30, the total proportion of AOB in S₂ (Fig. 5B) and S₃ (Fig. 5C) was enhanced to 1.53% and 1.43%, respectively. Meanwhile, the total proportion of NOB was also improved from 0.17% in S₁ to 0.25% in S₄ and then to 0.43% in S₄. The results showed that the microaerobic condition in the UMSR was feasible for the growth of aerobic autotrophic bacteria. However, the total proportion of NOB was remarkably decreased to 0.08% with the reduction of RR to 25 in stage 4, though the
proportion of AOB was further increased to 2.91% at the same time (Fig. 5D).

As aerobic polyphosphate accumulating bacteria (Barker and Dold, 1996), *Acinetobacter* was also found in the UMSR. The proportion in total bacteria was remarkably decreased from 0.84% to 0.05% by stages, indicating *Acinetobacter* was more sensitive to DO. However, as shown as Fig. 2D, TP removal in the four steady phases of the UMSR was 46.8%, 83.5%, 52.5% and 66.6%, respectively. Obviously, the TP removal in the reactor was not positively correlated with the abundance of *Acinetobacter*, but with the biomass as expressed in Section 3.1.

In fact, aerobic bacteria in the UMSR had played an important role in consuming the limited DO in forming an anoxic environment that allowed anaerobic bacteria located deep in the activated sludge flocs (Meng et al., 2016). As illustrated in Fig. 5, heterotrophic denitrification was one of the most important approaches to nitrogen removal in the UMSR. In stage 1 with the RR 45, the proportion of heterotrophic denitrifiers in *S* 1 was 10.44% (Fig. 5A). With the RR decreased to 35, 30 and 25 by stages (Table 1), the total oxygen supplement to the microaerobic system decreased and resulted in an enhancement of heterotrophic denitrifiers to 16.01%, 10.76% and 15.54%, respectively (Fig. 5B, C and D). In addition to the heterotrophic denitrifiers, *Thiobacillus* was also observed in the microaerobic system. Its proportion decreased from 0.83% to 0.68% stage by stage, positively correlated with the decrease of TN removal (Fig. 2B). qPCR analysis of the four sampled activated sludge (Fig. 6) showed that: 1) the total bacteria in the UMSR decreased gradually from 7.87 × 10^9 copies/ngDNA in *S* 1 to 2.35 × 10^9 copies/ngDNA in *S* 4 with the decreased RR from 45 to 25 by stages, 2) the *amoA* gene in *S* 1 was 5.6 times as that of 5.28 × 10^1 copies/ngDNA in *S* 1, 3) *Nitrospira* 16S rRNA decreased obviously from 2.10 × 10^2 copies/ngDNA in *S* 1 to only 1.10 copies/ngDNA in *S* 4, 4) *Nitrobacter* 16S rRNA also showed a decreased trend with 10 copies/ngDNA in *S* 4. With the decrease of RR in the UMSR, both absolute abundance variation of *amoA* and the total 16S rRNA of *Nitrobacter* and *Nitrospira* were consistent with the change of AOB and NOB revealed by the high throughput (Fig. 5). It is known that AOB has a better oxygen affinity than NOB (Blackburne et al., 2008). Thus, it could be understood that the ratio of *amoA*/*Nitrotracer* + *Nitrospira* increased with the decrease of RR. It was found that anammox 16N RNA was the most abundant nitrogen functional gene (Fig. 6) with the copies of 6.00 × 10^2, 4.19 × 10^2, 5.81 × 10^1 and 4.44 × 10^1 in *S* 1, *S* 2, *S* 3, *S* 4, respectively. This result indicated that anammox was the most important approach for NH4^+ and TN removal in the UMSR.

Since *nirS* gene was more abundant than counterpart *nirK* gene in wastewater treatment system (Kim et al., 2011; Zhi et al., 2015), *nirS* along with *nirG* were selected as the denitrifying genes in the present study. Because the dominant anammox would consume the most NH4^+ in the MFWW, only a small amount of NOX^-N was produced (Fig. 2B). Without enough NOX^-N as substrate for denitrifiers, both *nirG* and *nirS* genes exhibited a downward trend in the UMSR with the decrease of RR by stages (Fig. 6). Therefore, anammox was identified as the main mechanism for nitrogen removal in the UMSR, and the decrease of RR had a significant negative effect on anammox bacteria, resulting in a less removal for both NH4^-N and TN.

4. Conclusion

RR had a remarkable effect on the nitrogen removal in the UMSR. The TN removal presented a decreasing trend with the decrease of RR from 45 to 25 by stages. To get an efficient TN of not more than 80 mg/L with a TN load removal above 0.88 kg/m^3·d^, the RR should be at least 34. The RR of less than 34 badly inhibited the growth of anammox bacteria. Though the lower RR enriched AOB, the nitrogen removal remarkably decreased in the reactor due to the inhibited anammox that was the dominant nitrogen removal pathway.

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