Effect of wastewater COD/N ratio on aerobic nitrifying sludge granulation and microbial population shift

Lei Wu, Chengyao Peng, Yongzhen Peng*, Lingyun Li,
Shuying Wang, Yong Ma

*Key Laboratory of Beijing for Water Quality Science and Water Environment Recovery Engineering, Beijing University of Technology,
Beijing 100124, China. E-mail: wulei2008@emails.bjut.edu.cn*

Abstract

The effect of COD/N ratio on the granulation process and microbial population succession was investigated. Four identical sequencing batch reactors, R1, R2, R3 and R4, were operated with various initial COD/N ratios ranging from 0/200 to 800/200 (*m/m*). Ethanol was fed as the source of COD. Aerobic granules were successfully cultivated in R2 and R3, operating with the COD/N ratio of 200/200 and 400/200, respectively. Scanning electron microscope observations indicated that short rod-shaped and spherical bacteria were dominant in R2, while granules produced in R3 were surrounded with a large amount of filamentous bacteria. The average specific nitritation rate in R2 and R3 were 0.019 and 0.008 mg N/(mg MLVSS·hr), respectively. Fluorescence *in situ* hybridization results demonstrated that nitrifying bacteria population was enriched remarkably in R2. It indicated that nitrification ability and nitrifying bacteria population were enriched remarkably at low COD/N ratio. However, no granules were formed in R1 and R4 which might attribute to either limited or excessive extracellular polymeric substances production. This study contributed to a better understanding of the role of COD/N ratio in nitrifying sludge granulation.

Key words: aerobic granules; COD/N ratio; sequencing batch reactor; nitrification performance; microbial population shift

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Introduction

Aerobic granules have characteristics as better settling ability, high density, strong microbial structure, high biomass retention and good nutrient removing capability. The ability to form granules depends on various environmental and operational factors, such as substrate composition, organic loading rate (Thanh et al., 2009), feeding strategy (McSwain et al., 2003), reactor design (Beun et al., 1999; Liu and Tay, 2002), settling time (Qin et al., 2004) and aeration intensity (Adav et al., 2007). Studies have been proposed to examine the extracellular polymeric substances (EPS) and filamentous bacteria that may relate to granulation mechanisms (Liu et al., 2004b). EPS can accelerate the granulation by altering the physicochemical characteristics of cellular surface such as charge, hydrophobicity and other properties while filamentous bacteria probably aid the structural stability of the granules by serving as a binding material (Liu et al., 2004b; Liu and Liu, 2006; Wang et al., 2006).

The nitrifying granules, which consist of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) as well as other heterotrophic bacteria, are stable and can withstand fluctuations of environmental conditions (Kim and Seo, 2006). Due to different growth rates of nitrifying bacteria (the highest growth rate 0.014–0.064 hr^{-1}) and heterotrophs (1.0–1.44 hr^{-1}) in conventional activated sludge (Prosser, 1990), organic loading rate is an important operational parameter influencing biodiversity, microbial community distribution and physicochemical characteristics of nitrifying granules, which will in turn affect the performance of system. However, it is reported that nitrifying granules obtained with and without organic substrate exhibited different granular size, granulation time, settling ability, nitrification performance and microbial community by selecting and enriching different bacterial species under aerobic conditions (Moy et al., 2002; Kim and Seo, 2006; Liu et al., 2007). Nitrifying bacteria which dominate the microbial structure and distribution in the granules play an important role for granules stability compared with heterotrophs (Liu et al., 2004a). Nitrifying granules cultivated with inorganic artificial wastewater showed robust nitrification performance but needed a long start-up period (Kim and Seo, 2006). Oppositely, a high organic loading rate may lead to instability or even wash-out of aerobic granules and system performance deterioration. Therefore, an appropriate COD/N ratio should be chosen to favor the cultivation and retention of well-settling granules which can enhance the nitrification ability.

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* Corresponding author. E-mail: pyz@bjut.edu.cn

The main objective of this study was to investigate the effect of different COD/N ratios on the granulation process, physical characteristics, nitrification ability and microbial structure of aerobic granules. It is expected that the information derived from this study would be valuable for enhancement of the nitrification performance and stability of aerobic granules, and application of full-scale aerobic granules-based bioreactor for wastewater treatment.

1 Materials and methods

1.1 Experimental set-up

Four cylindrical reactors of identical geometrical design with a working volume of 10 L (total height: 45 cm; riser diameter: 18 cm) were employed in this study, namely R1, R2, R3 and R4, respectively. Oxygen was supplied by an air compressor through an air diffuser at the bottom of the reactor and air flow rate was set at 0.2 m^3/hr by flow meter. Mechanical stirrer and fine air bubbles were used to provide liquid mixing and shear force for promoting the granulation. The pH and DO sensors were installed for monitoring pH and DO in the reactors. The reactors were housed under a controlled temperature 28°C.

Different COD/N ratios (*m/m*) of 0/200, 200/200, 400/200, 800/200 were applied to reactors R1, R2, R3, and R4, respectively. Except for COD/N, the other operational parameters were identical. Operation cycle consisted of feeding (8 min), aeration (210 min), settling (2 min), discharging (4 min) and idling time (16 min), which were controlled by programmable logic circuit. Six cycles were operated per day. Six liters of supernatant was withdrawn at the end of settling phase and 6 L of fresh wastewater was introduced through the bottom of each reactor at a volume exchange ratio of 60%. Hydraulic retention time (HRT) was maintained at 10 hr. Sodium bicarbonate requirement for nitrification was supplied by pumping during the nitrification to control the pH at a range of 7.0–7.8. DO levels inside reactors were controlled at 4–5 mg/L. The operating parameters, reactor performance and granular growth course were closely monitored during the cultivation.

1.2 Seed sludge

The nitrifying sludge, obtained from a lab-scale sequencing batch reactors (SBR) system treating real domestic wastewater, was seeded directly into four reactors without any acclimation. The mixed liquor volatile suspended solid (MLVSS) was 3000 mg/L.

1.3 Medium composition

Compositions (per liter) of the synthetic wastewater in this study were shown as follows: 0.08 g KH_2PO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.575 mL trace metals solution. The trace metals solution was as described by Smolders et al. (1994) and consisted of (g/L): 1.5 $FeCl_3 \cdot 6H_2O$, 0.15 H_3BO_3, 0.03 $CuSO_4 \cdot 5H_2O$, 0.18 KI, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 2H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 6 \cdot H_2O$ and 10 ethylenediamine

tetra-acetic acid. Reactors R2–R4 were fed with ethanol as the sole COD at 200, 400 and 800 mg/L, while R1 was fed with the same synthetic inorganic wastewater without the ethanol addition. Ammonium chloride used as the source of NH_4^+-N and NH_4^+-N concentration was fixed at 200 mg/L.

1.4 Analytical methods

COD, NH_4^+-N, NO_2^--N, NO_3^--N, sludge volume index (SVI) and MLVSS were measured according to standard methods (APHA, 1999). DO and pH were continuously monitored by pH/oxi340i meter with DO and pH probes (WTW Multi340i, Germany).

1.5 Physicochemical characteristics of granule

Particle size distribution of the granules were analyzed by manual humid sieve method (Laguna et al., 1999) The specific oxygen uptake rate (SOUR) was calculated according to the method described by Ochoa et al. (2002). The respective respirometric activity of AOB and NOB were described by respective $SOUR_{AOB}$ and $SOUR_{NOB}$, whereas the respirometric activity of heterotrophic bacteria was quantified by $SOUR_H$. The whole respirometric activity of nitrifying bacteria entitled $SOUR_N$ was calculated as the sum of $SOUR_{AOB}$ and $SOUR_{NOB}$. Microscopic examination was performed using an Olympus-BX52 (Japan). The morphology of the bacteria was examined with high resolution scanning electron microscopic (SEM) (HITACHI S-4300, Japan). The samples were pretreated for observation according to the methods described by Guo et al. (2009). Extracellular polysaccharides (PS) and proteins (PN) of sludge were extracted by formaldehyde-NaOH method (Liu et al., 2002). Integrity coefficient (IC) (%) is the ratio of residual volatile suspended solid after defined amount of agitation to total volatile suspended solid of the intact granules rior to agitation (Ghangrekar et al., 1996). The gravity of the bioparticles was determined using standard methods (Amann, 1995)

1.6 Fluorescence *in situ* hybridization (FISH)

Sample fixation and hybridization steps were carried out according to methods previously described by Amann (1995). Sludge samples were taken from the SBR at different time intervals and were analyzed for AOB and NOB. The probes applied include EUB_{Mix} (EUB338, EUB338-II and EUB338-III, specific for all bacteria), NSO190 (specific for Betaproteobacterial AOB), NIT3 (specific for *Nitrobacter* sp.) and Ntspa662 (specific for *Nitrospira* genera). All probes were commercially synthesised with 5′ FITC (fluorosceinisothiocyanate), or one of the sulfoindocyanine dyes, indocarboncyanine (Cy3) or indodicarbocyanine (Cy5) by ThermoHybaid (Interactiva Division, Ulm, Germany). Sample images were collected using OLYMPUSBX52 fluorescence microscope. FISH quantification was performed by Image-pro plus 6.0 Software, where the relative abundance of the interested bacteria was determined as the mean percentage of all bacteria.

2 Results and discussion

2.1 Effect of COD/N ratio on nitrifying sludge granulation process

Variation of MLVSS and SVI in reactors during the cultivation is shown in Fig. 1. Nitrifying granules were successfully cultivated in R2 and R3 under three operational phases: acclimation phase, multiplication phase and maturation phase. The acclimation phase lasted from day 1 to day 9, during which the MLVSS and SVI both decreased along with the formation of granules, followed by the multiplication phase, which was characterized by a significant increase in the concentration of MLVSS from 891 to 1700 mL/g in R2. SVI value stabilized between 40–45 mL/g during the maturation phase from day 20 to the end of reactor operation on day 30 (Fig. 1). Reactor R3 showed similar phenomena which exhibited a higher concentration of MLVSS of 3000 mg/L and a lower SVI of 30–35 mL/g (Fig. 1). The granules showed better settling ability than seed sludge.

The phase contrast microscopy and SEM images of the sludge in the granulation process under different COD/N ratios are shown in Fig. 2. Initially, the seed sludge shared the same floc structure as shown in Fig. 2a. Tiny aerobic granules appeared in R2 on day 10 (Fig. 2e) and in R3 on day 6, and then color of activated sludge gradually changed from grey to yellow on day 10 (Fig. 2i). During the next 20 days, the mean diameter of the granules increased rapidly in both reactors and showed more regular morphology. In R2, granules cultivated under the COD/N ratio of 200/200 had a compact, smooth regular morphology and limited quantity of granules consisted of filamentous bacteria (Fig. 2f–h) while granules in R3 cultivated under the COD/N ratio of 400/200 had a hairy appearance with a relative loose microbial structure dominated by filamentous bacteria (Fig. 2j–l). SEM images showed that rod-like species in R2 tightly clustered together were found to be predominant and some cavities were also present.

In contrast, granules were not formed in R1 and R4 during 30-day operation. Reactor R1 was operated without any organic substrate. The biomass in R1 was dominated by loosely structured bioflocs (Fig. 2b–d), which contained very low MLVSS about 150 mg/L. Oppositely, some

studies reported that aerobic nitrifying granules had been cultivated with inorganic carbon source, and these granules showed an excellent nitrification ability (Tsuneda et al., 2003; Kim and Seo, 2006; Belmonte et al., 2009). Tsuneda et al. (2003) produced nitrifying granules with a diameter of 346 mm in an aerobic upflow fluidized bed reactor within 300 days. Kim and Seo (2006) also showed that sludge flocs became granulated in sequencing batch airlift reactor within 50 days. Belmonte et al. (2009) reported that nitrifying granules with a mean diameter of 0.9 mm were accumulated in a pulsing SBR after 400 days of operation. However, the results from the present study did not support the above mentioned conclusions, which might attributed to the short operation time (30 days) and the configuration of reactors.

Reactor R4 was operated under an initial COD/N of 800/200, the highest organic loading used in this study. As seen from Fig. 1, the biomass concentration dropped sharply to 500 mg/L while SVI increased to 298 mL/g within 10-day operation as a result of the biomass washout from the reactor. R4 was unable to biologically remove NH_4^+-N and failed to cultivate aerobic granules due to a rapid system failure (Fig. 2m). The reactor had to be ceased compulsively on day 11. Restart of R4 also showed the same results. Similar phenomena were detected in other publications. Moy et al. (2002) reported that acetate-fed granules could not sustain high organic loading rate of 9 kg COD/(m³·day) and disintegrated. Zheng et al. (2006) pointed out that compact aerobic granules formed under a COD loading rate of 6.0 kg COD/(m³·day) began to disintegrate and be washed out of the reactor after 30 days. These results clearly indicated that high organic loading deteriorated the stability of aerobic granules. In this study, organic substrate-excess, equivalent to 5.48 kg COD/(m³·day) led to deteriorated settling behavior of sludge and operational disorders.

2.2 Effect of COD/N ratio on extracellular polymeric substances production

EPS is believed to play a positive role on the adhesion among bacteria (Liu et al., 2004b). Traditionally, PS and PN are regarded as the predominant components of EPS and PS could play a so-called bioglue role in the gran-
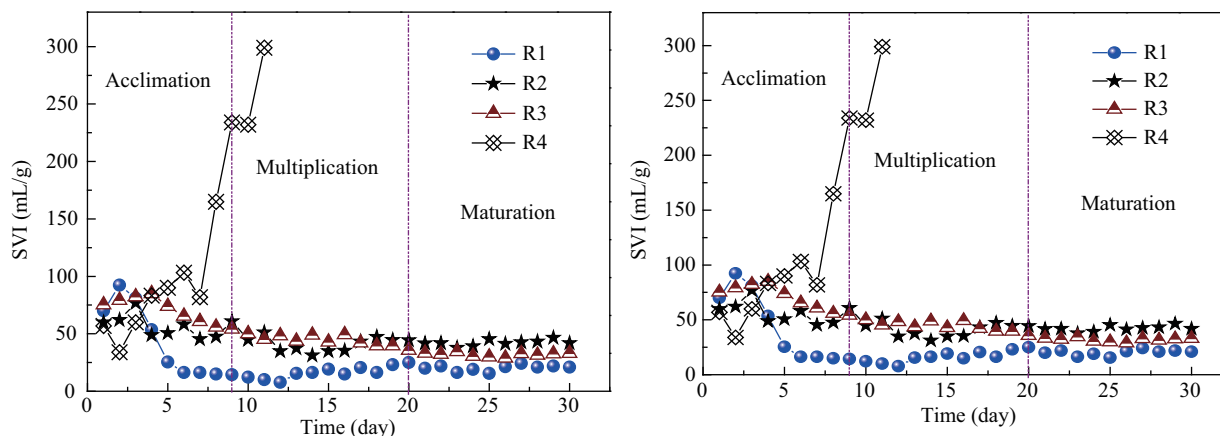

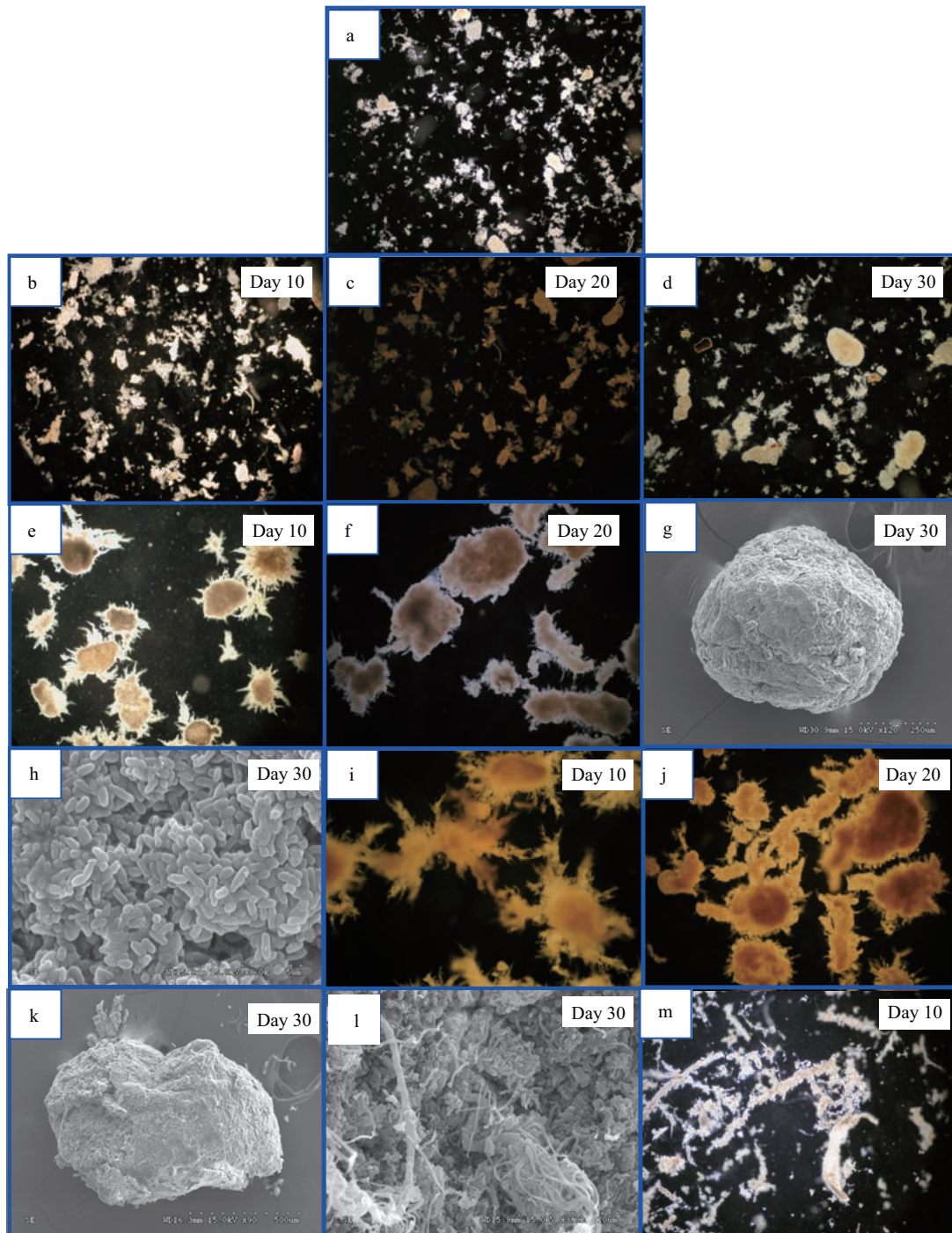**Fig. 1** Variation of MLVSS and SVI in reactors during the cultivation.

Fig. 2 Phase contrast microscopy and scanning electron microscopic pictures of the granules formed in four reactors. (a) seed sludge; (b–d) R1; (e–h) R2; (i–l) R3; (m) R4. Magnification = 40× in phase contrast.

ulation process to mediate the cohesion and adhesion of microbial cells (Liu et al., 2004b). PS/PN ratios in the four reactors during the entire operation are calculated in Fig. 3a. Under COD/N of 0/200 in R1, the PS/PN ratio was around 0.5 during the whole operation time, which was lower than the appropriate PS/PN ratio of 0.6 for aerobic granulation in the literature (Li et al., 2008). Initially, EPS increased rapidly from 0.6 to 1.3 and 1.9 mg PS per mg PN from day 1 to day 12 respectively in R2 and R3, and then stabilized. PS/PN ratio of R2 and R3 was compromised 3–4 times higher than that of R1. This rapid increase was linked directly to the appearance of granules. It has been

reported that autotrophic nitrifying bacteria hardly adhere to carriers as a biofilm due to their low growth rate and lack of production of EPS (Wang et al., 2007). In R1, the phenomenon of less PS synthesis of metabolic product resulting in a low PS/PN ratio was probably found to prevent microbial aggregation.

Furthermore, there is strong evidence that EPS is involved in microbial physiology and improvement of long-term stability of granules (Liu et al., 2004b). Since the cell wall of bacteria in the granules is surrounded by an EPS layer, this may imply that cell surface hydrophobicity might be related to EPS. Liu et al. (2004b) suggested that
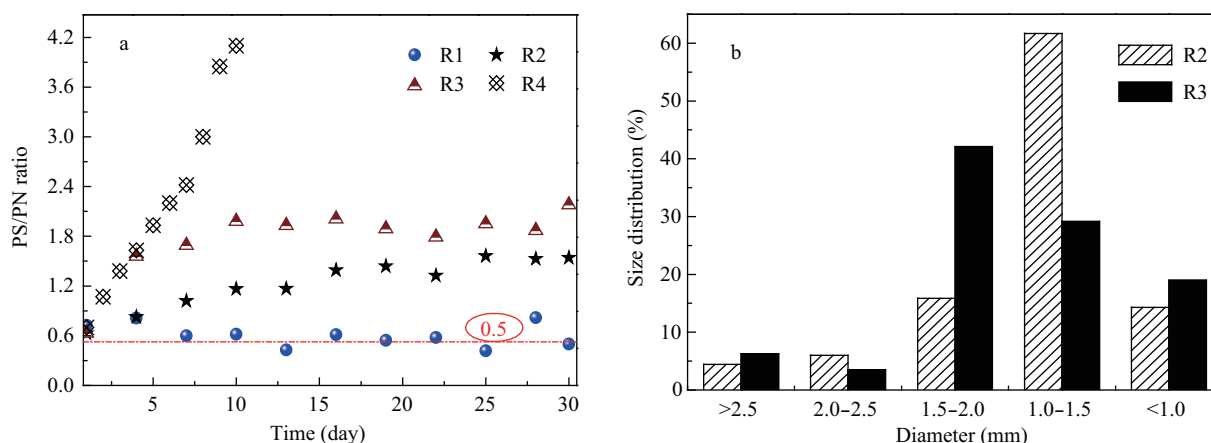
Fig. 3 Variations of PS/PN ratio during the cultivation in R1–R4 (a) and granule size distribution of R2 and R3 on day 30 (b).

PN was the hydrophobic components of the EPS, while PS are hydrophilic. Ghigo et al. (2003) reported that very few bacteria is actually in contact with the hydrophilic surface, but rather with the hydrophobic surfaces of other cells. Therefore, excessive PS synthesis is found to destroy the balance of hydrophobic and hydrophilic amount which are present in EPS, and indeed reflects the average hydrophobicity of the granules (Liu et al., 2004b). Under the COD/N ratio of 800/200 in R4, the PS/PN ratio increased to peak at 4.1 mg PS per mg PN on day 10 during the short period, which was almost 3–4 times higher than that in the other three reactors. It had been reported that an increased substrate COD/N ratio stimulated the production of extracellular PS, thereby resulting in improved bacterial attachment to solid surfaces. However, excessive PS secreted resulted in a lower cell surface hydrophobicity, which might lead to the failure of the granule formation in R4.

2.3 Effect of COD/N ratio on physical characteristic of granules

In order to investigate the effect of COD/N ratio on microbial aggregated size, the granule size distribution was measured (Fig. 3b). The average diameter in R2 was 0.41 mm, as opposed to 0.92 mm under high COD/N ratio in R3. No clear correlation between COD/N ratio and average granule diameter was found and there was only a trend towards larger granules under higher COD/N ratio. Similarly, the settling velocity of granules was accelerated with increasing organic loading which is much higher than that of activated sludge (Table 1). The high variation in settling velocity was due to the presence of granules without any carrier support. Specific gravity (SG) values in R2 and R3 were 1.045 and 1.074, respectively, respectively, which were higher than that of the seed sludge (1.002). It indicated that the appearance of granules in R2 and R3 was accompanied by significant increases of SG in the reactor and granules could retain more biomass than conventional sludge.

As can be seen from Table 1, the strength results showed that the granules in R2 possessed higher strength, with an IC of 97% while granules in R3 displayed weaker strength, with an IC of 92%. The average $SOUR_N/SOUR_H$ were 0.87 and 0.31 on day 30 in R2 and R3, respectively, which

Table 1 Characteristic of granules produced in R2 and R3 on day 30

Parameters	R2	R3
Mean diameter (mm)	0.41	0.92
Specific gravity	1.045	1.074
Average SVI (mL/g)	40	32
Average integrity coefficient (%)	97	92
Average settling velocity (cm/sec)	1.2	2.4
$SOUR_N/SOUR_H$	0.87	0.31

indicated that the nitrification activity was significantly enhanced, because nitrifying bacteria has been suggested to outcompete heterotrophic bacteria at low COD/N ratio. Results present above clearly demonstrated that the aerobic granules developed under the COD/N ratio of 200/200 had a compact structure which improved stability and high-rate nitrification performance compared to those under the COD/N ratio of 400/200.

2.4 Effect of COD/N ratio on nitrification performance of granules

Figure 4 shows the performance of R1, R2, R3 and R4 regarding COD and NH_4^+-N removal. COD was not considered in R1 due to no organic substrate addition. During the 30-day cultivation, the average COD removal efficiencies were 59%, 80% and 82% in R2, R3 and R4, respectively (Fig. 4b–d). In R1, the NH_4^+-N removal efficiency remained low at average 39%. In R2 and R3, granules appeared on day 10 and 7 and the maximum NH_4^+-N removal efficiency was achieved with 97% and 92% on day 14 and 15, respectively. It could be observed that nitrification performances were enhanced with different extent during aerobic granulation process, which attributed to high biomass retention in granules. Unfortunately, this satisfactory state did not last further in R3. The NH_4^+-N removal efficiency decreased significantly to 88.62% on day 15 and then dropped to as low as 68% on day 30 in R3 (Fig. 4c) while the NH_4^+-N removal ability were stabilized from day 16 to 30 in R2. Under the same initial ammonia loading and aeration time, R1 shows a better NH_4^+-N removal efficiency than R2 and R3. An opposite phenomenon was observed as discussed above. The possible reason was the sticky EPS accumulated on the surface of bacterial cells for adhesion during the
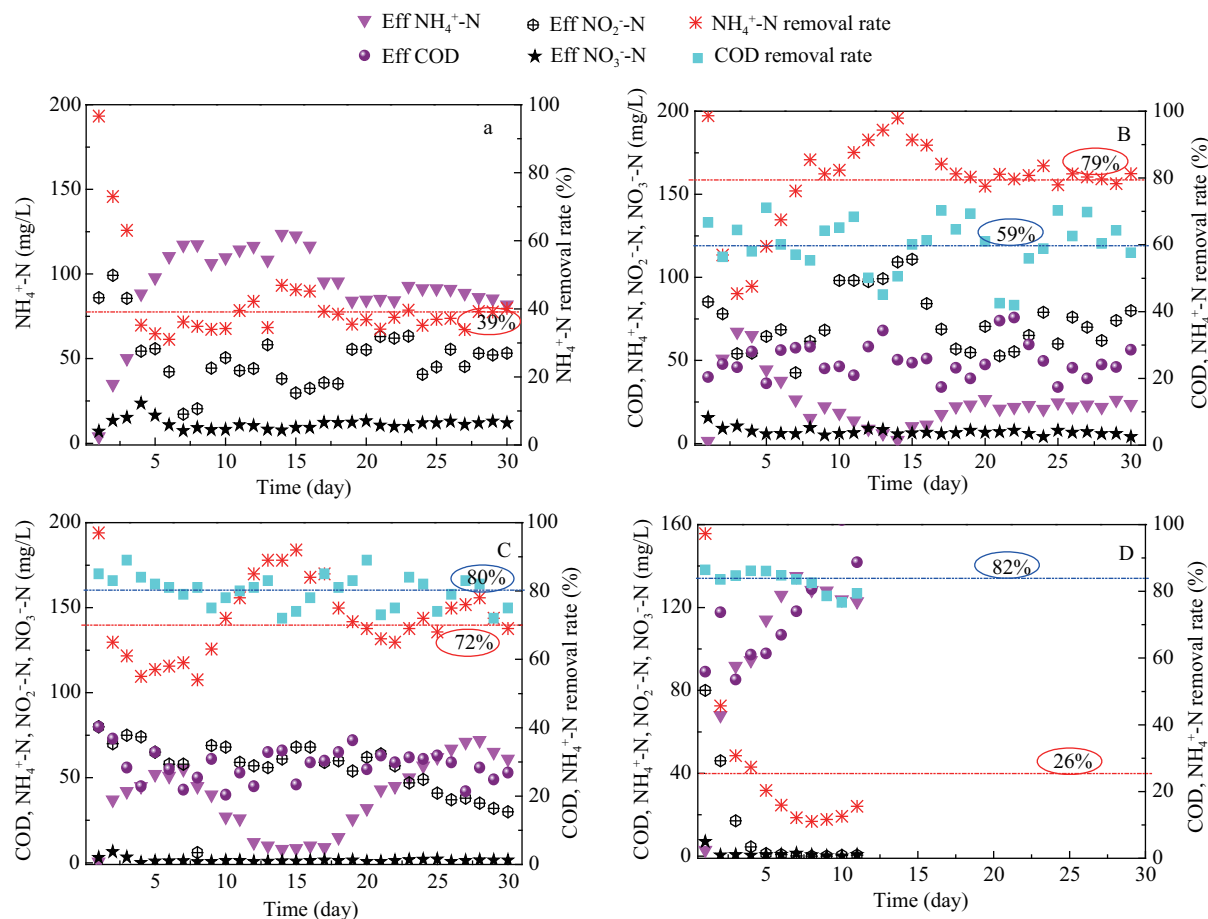
Fig. 4   Nutrient concentration profiles of R1 (a), R2 (b), R3 (c) and R4 (d) during the cultivation.

granulation process in R2 and R3. However, bacteria cells in R1 were not affected by adhesion phenomena and washed out with the effluent constantly given the shorter settling time (2 min) and higher volume exchange ratio (60%). Regretfully, the NH_4^+-N removal efficiency was found as low as 26% in R4. After wash-out of microbial cells with poor settling properties, nitrifying bacteria had no chance to grow in a high organic substrate condition given the same aerobic duration, which resulted in a poor nitrification performance.

To compare the NH_4^+-N oxidization ability distinctly, the specific nitration rate (SNR) is illustrated in Fig. 5. The SNR of R2 was similar to that of R3 around 0.015 mg NH_4^+-N/(mg MLVSS·hr) in the first five days. Afterwards, nitrification activity in R2 was much higher than that in R3 during the remaining of study period. The average SNR of R2 was 0.019 mg NH_4^+-N/(mg MLVSS·hr), 2.5 times higher than that of R3 with 0.009 mg NH_4^+-N/(mg MLVSS·hr) during the whole operation. The differences between SNRs were mainly attributed to different COD/N ratios. It was widely acknowledged that a higher organic loading resulted in a higher growth rate of heterotrophic population in aerobic granules (Liu et al., 2004a), since the relative activity of nitrifying population over heterotrophic population was lower under a high substrate COD/N ratio. It is suggested that an optimal organic loading should be chosen to cultivate the nitrifying granules with short granulation time and enhance performance by selecting
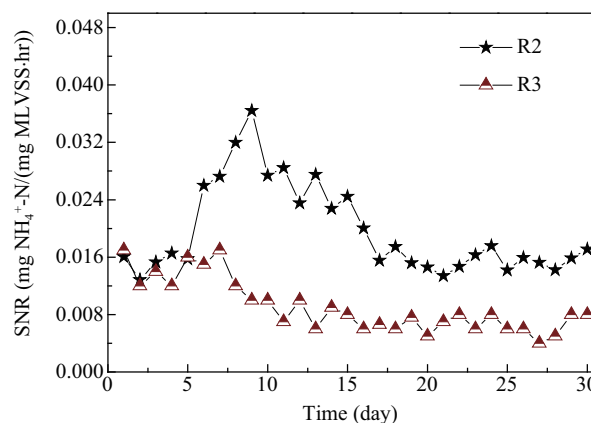

Fig. 5   Specific nitration rate (SNR) of R2 and R3 during the cultivation.

and enriching nitrifying bacteria.

2.5 Effect of COD/N ratio on microbial community structure and succession of granules

To determine the influence of COD/N ratio on bacteria community structure, the composition of microbial population was characterized using FISH method. According to FISH results, the nitrifying bacteria population of granules proliferated in R1, R2 and R3. Biomass of R1 was mainly composed of AOB and NOB owing to no organic substance addition. The AOB quantification increased from 3.9% (seed sludge) to 19% in 30-day granulation operation in R2, while the NOB quantification increased from 2.6% to

Table 2   Shift of nitrifying population of seed sludge and granules in reactors on day 30

Nitrifying bacteria composition	Seed sludge (%)	Flocs in R1 (%)	Granules in R2 (%)	Granules in R3 (%)	Flocs in R4 (%)
Ammonia-oxidizing β-proteobacteria (NSO190)	3.9	25.2	19	11.2	2.3
Nitrospira (Ntspa662)	2.1	3.1	2.5	0.9	0.5
Nitrobacter (Nit3)	0.5	1.9	1.0	0.6	0.2
Total	6.5	30.2	22.5	12.7	3.0

3.5% based on the total bacteria. In R3, AOB and NOB accounted for 11.2% and 1.5%, respectively. FISH results indicated that the population of AOB and NOB were both enhanced to a certain extent during the aerobic granulation process. Differently, the nitrifying population of aerobic granules in R2 was enriched markedly under low COD/N ratio (Table 2). It was indicated that the relative abundance of nitrifying population over heterotrophic population in the aerobic granules in R2 was clearly higher as compared to the granules in R3. However, a small quantity of nitrifying bacteria was detected in the sample sludge taken from R4. The competition on nutrients between nitrifying and heterotrophic populations in granules would be more significant under high substrate COD/N ratio, where bacteria distribution adapts to suit particular condition. It indicates that slow-growing nitrifying bacteria could survive best under a low COD/N ratio. Some studies reported similar results (Liu et al., 2004a; Kim and Seo, 2006).

As can be seen from above, COD/N ratio is a microbial selection pressure which can select and enrich heterotrophic or nitrifying bacteria in granules at different ranges. Organics functioned as a granulation catalyst that accelerates the granulation process because heterotrophic bacteria can produce larger amounts of EPS than autotrophic bacteria which is unable to metabolize organic compounds. However, either high or low organic loading may lead to microorganisms maladjusted and system failure. High organic concentration would favor the selection and enrichment of heterotrophic bacteria which had a strong competition for substrate utility (Liu et al., 2004a; Wang et al., 2007). Therefore, the nitrification ability and microbial community structure were determined by the amount of organic substrate in the culture. From this study, it can be concluded that choosing a moderate substrate concentration as initial selection pressure is also crucial for aerobic granulation. Aerobic granules with high nitrification activities and good-settling ability have been demonstrated only under proper COD/N ratio.

3 Conclusions

COD/N ratio play a significant role in nitrifying sludge granulation. Granulation failed in R1 and R4 under the improper COD/N ratio of 0/200 and 800/200. In contrast, nitrifying granules were successfully cultivated in R2 and R3 under the COD/N ratio of 200/200 and 400/200, but exhibited better physical characteristics and nitrifying microbial population under low COD/N ratio. The average specific nitration rate in R2 and R3 were 0.019 and 0.009 mg N/(mg MLVSS·hr), respectively. This study provided and demonstrated a new cultivation strategy of stepwise

changed selection pressure for development of aerobic granules based bioreactors with improved stability and performance.

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