Formation and Stability of Nitrifying Granules under High Loading Rates

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Abstract. Nitrifying granules are generally believed to have high nitrification ability due to the immobilization of large quantities of nitrifying bacteria with low growth rate. However, high loading rate is normally not recommended for nitrifying granules due to the high inhibition status. In this study, nitrifying granules, able to treat ammonia nitrogen as high as 1000 mg N/L (2 kg N/m3·d), were cultivated in the laboratory scale sequencing batch reactor. During around 200-day operation, the granules exhibited good performance with 99% ammonia removal efficiency. In the meantime, MLSS increased from initial 6 g/L to the final 10 g/L, and the value of SVI30 decreased from around 100 to 15 ml/g. However, it was observed that the nitrifying granules gradually disintegrated into small aggregates with mean size decreased from 286 to 138 μm from the operation day 50 to 120, after which they gradually recovered by themselves with increased mean size of 235 μm at the end of the operation. Notably, this disintegration did not compromise performance and characteristics of the nitrifying granules. These results demonstrated that nitrifying granules may have the capability to undertake ammonia loading rates as high as 1000 mg N/L with good self-healing ability, which make the process more stable and promising in practice.

Key words: nitrifying granules, ammonia oxidation, self-healing, stability, sequencing batch reactor (SBR)

1. Introduction

Aerobic granulation has been widely reported to be effective in the field of wastewater treatment. Under selection pressure, normally believed as settling time [1], [2] and exchange ratio [3]-[5], activated sludge could further aggregate and form compact granules with a certain size, dense structure, diverse microbial species, good settling ability and high tolerance to shock load and toxins. So far, aerobic granules have been proved effective in the treatment of wastewater with carbon, nutrients and many toxic materials. In traditional biological nitrogen removal system, nitrifying bacteria was characterized by slow growth rate and high sensitivity to both toxic compounds and unfavorable environmental conditions. However, nitrifying granules, as aggregates of nitrifying bacteria, have been proved effective in enhancing nitrification rate and system stability by the high retention of nitrifying bacteria [6]-[9].

Information on the nitrifying granules cultivated with inorganic wastewater rich in ammonium nitrogen has been reported. Tsuneda proved that nitrifying bacteria able to treat ammonia nitrogen as high as 500 mg N/L could be self-immobilized in an aerobic upflow fluidized reactor, with nitrifying granules of 350 μm in diameter produced after 300 days of operation [6]. Liu cultivated nitrifying granules with size of 240 μm and SVI of 40 mg/L on day 21 in SBR, which was able to treat 450 mg N/L of ammonia [8]. Shi reported that the compact nitrifying granules were formed after 120 days of operation, and an NH4+-N of 100 to 250 mg/L was found to be appropriate for the operation of the nitrifying granules [9]. It could be seen that ammonia nitrogen concentration in the reported literature is generally below 500 mg N/L for nitrifying granular system. However, in practice, high ammonia concentration, e.g. as high as 1000 mg N/L, is

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common in high strength nitrogen-containing wastewater, such as leachate and feedlot wastewater etc.[10]-[13]. Therefore, granules with high nitrogen removal efficiency are still highly desired.

High loading rate has been reported to be one of the main causes of the failure of granular system during long-term operation [14], [15]. The cultivated aerobic granules could keep their structural integrity and high COD degradation rates at certain COD level and started to disintegrate at a high loading pressure [14]. Jin reported that the nitrifying granules began to appear on day 30 and matured in 75 days in continuous-flow airlift reactor (ALR), which went worse upon increasing of the influent ammonia concentration up to 1120 mg N/L [16]. The proposed breakdown mechanism of the granules includes over growth of filamentous microorganisms [17], intracellular protein hydrolysis and degradation at the anaerobic granule core and the reduced protein quantity secreted by cells [18]. For nitrifying granules, free ammonia (FA) and free nitrous acid (FNA), which may become tenser with the increment of loading rate in influent, exert extra inhibition to the nitrifying granules. FA has been reported to hinder the formation of aerobic granules seriously through its inhibition to energy metabolism of microorganisms [19]. In the meantime, FNA has been proved to be more toxic and has a much lower inhibition threshold for nitrifying bacteria [20]. Therefore, high loading rate could exert severer inhibition on nitrifying granules, which could cause bigger perturbations to nitrifying granules and readily failure of the system. However, the acclimation of nitrifying granules to high FA and FNA conditions at high nitrogen loading rates has also been reported, which may facilitate nitrifying granules to withstand higher inhibition conditions [21]. In the meantime, nitrifying granules observed in the present studies mostly have smaller size and higher density and settling ability than heterotrophic granules, which make them more stable in structure. Therefore, there is possibility that nitrifying granules could undertake higher loading rate, at least higher than the applied loading rates in current studies.

In this study, ammonia concentration as high as 1000 mg N/L was loaded in the feed in a stepwise manner to investigate the capability of the nitrifying granules to withstand high loading conditions. In the meantime, long-term stability of the system under the high loading rate was investigated.

2. Material and Methods

2.1. Experiment Set up and Operation

Bubble column with a diameter of 5 cm and H/D (height/diameter) ratio of 20 was employed in this study. It was operated sequentially with a cycle time of 6 hr, which included 3 min of influent filling, 323 to 346 min of aeration, 5 to 30 min of settling and 4 min of effluent discharging. Effluent was discharged from the middle port of the reactor with a volumetric exchange ratio of 50%. Fine air bubbles for aeration were supplied through an air sparger at the reactor bottom with an airflow rate of 1.66 cm s⁻¹.

2.2. Media

A synthetic inorganic wastewater with the following compositions was used as influent for the cultivation of nitrifying granules: (NH₄)₂SO₄, KH₂PO₄, NaHCO₃, and micronutrients. (NH₄)₂SO₄ was nitrogen source and NaHCO₃ was inorganic carbon source as well as pH control material. The NH₄⁺-N concentration in the influent was adjusted during the operation period, but the NaHCO₃: NH₄⁺-N : P ratio was always kept at about 65: 5: 1 (w: w: w). The micronutrients in the influent contained CaCl₂·2H₂O 25 mg L⁻¹, MgSO₄·7H₂O 20 mg L⁻¹, FeSO₄·7H₂O 10 mg L⁻¹, EDTA-2Na 10 mg L⁻¹, MnCl₂·4H₂O 0.12 mg L⁻¹, ZnSO₄·7H₂O 0.12 mg L⁻¹, CuSO₄·5H₂O 0.03 mg L⁻¹, (NH₄)₆Mo₇O₂₄·4H₂O 0.05 mg L⁻¹, NiCl₂·6H₂O 0.1 mg L⁻¹, CoCl₂·6H₂O 0.1 mg L⁻¹, AlCl₃·6H₂O 0.05 mg L⁻¹, H₂BO₃ 0.05 mg L⁻¹.

2.3. Analytical Methods

Commercial photochemical test kits (Hach Lamge GmbH, Dusseldorf, Germany, Test LCK303, LCK304, LCK339, LCK340, LCK341, LCK342; spectrophotometer type LANGE Xion500) were used for the measurement of NH₄⁺-N, NO₂⁻-N and NO₃⁻-N in the reactor. Sludge volume index (SVI), biomass dry weight (MLSS) and mixed liquor volatile suspended solids (MLVSS) were analyzed in accordance to the standard methods (APHA 1998). Average particle size was determined by laser particle size analysis system with a measuring range from 0 to 2000 μm (Malvern MasterSizer Series 2600, Malvern Instruments Ltd, Malvern, UK). Samples for DNA extraction were preserved every one or two weeks. Both polymerase chain reaction
(PCR) amplification of extracted bacterial 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) were conducted based on the methods described by Liu et al [22].

3. Results

3.1. The Cultivation of the Nitrifying Granules

Nitrifying sludge with size of 109 μm and SVI30 of 92 ml/g, which had been stored in 4°C fridge for about 2 months, were seeded in the reactor for the cultivation of nitrifying granules. At the beginning of the reactor operation, the ammonia nitrogen concentration in the feed was set at 200 mg N/L. However, it was found that the nitrifying sludge consumed all the ammonia nitrogen in the influent during one batch cycle. So influent ammonia nitrogen concentration was increased gradually after 2-day operation, till the 56th day when it was increased to 1000 mg N/L. Since then, influent nitrogen concentration was maintained at this level till the end of the operation. Meanwhile, settling time of the reactor was shortened gradually from 30 min to 7 min within 40 days to stimulate the formation of nitrifying granules.

After 45-day’s acclimation, granules ratio of the mixture of flocs and granules in the reactor increased up to about 70%, indicating the dominance of the nitrifying granules. Liu suggested using the sludge volume percentage with size below 200 μm (SVP-SB200) to indicate the dominance of granules when SVP-SB200 is below 50% [22]. Considering the slow growth rate and relatively smaller size of nitrifying granules, the sludge was assumed as granule dominant when the sludge volume percentage with size below 150 μm is below 50% in this study. From thereon, fast size increment of the nitrifying granules was observed. The mean size doubled within 40 days from 147 μm to 286 μm (Fig. 1 (A)). In the meantime, SVI30 in the both reactors maintained at around 45 ml/g (Fig. 1 (B)), indicating good settling ability of the nitrifying granular sludge. However, biomass concentration did not show the same trend as size in the reactor, which kept decreasing from initial 6 g/L to around 3 g/L as MLSS and 5 g/L to 1.7 g/L as MLVSS (Fig. 1 (C)), respectively. Since shorting settling time was adopted, nitrifying flocs with poor settling ability was washed out of the reactor. Persistent washout of the slow settling nitrifying flocs and the slow growth rate of the nitrifying bacteria resulted in a slow accumulation of biomass, which led to great decrease of the biomass during the initial 50-day’s operation.

![Fig. 1: Characteristics of the nitrifying granules during the whole operation period. (A) Size; (B) SVI30; (C) MLSS and MLVSS](image)

Then, in the following 50 days, dramatic changes occurred in the reactor. Firstly, the value of SVI30 decreased from 45 ml/g to 10-15 ml/g in about 10 days, revealing a big increase of settling ability of the
nitrifying granules. Secondly, size of the nitrifying granules decreased to 138 μm. The big decrease of size of the nitrifying granules was proved to be caused by the disintegration of the nitrifying granules into flocs and aggregates. Fig. 2 (A) shows detailed size distribution statue during disintegration of the nitrifying granules. It’s obvious that the size distribution was in a narrow range with little small-size flocs inside before the disintegration of the nitrifying granules, while it turned to be a much wider range with a lot of flocs inside during the size reduction period. However, the nitrifying granules gradually recovered in the following operational days with the size distribution from a relatively wider range to a narrower one, which probably resulted from the washout of small size flocs and the prevailing of even-sized granules. Fig. 2 (B) gives the volume percentage of flocs (SVP-SB150) in the sludge on the corresponding day in Fig. 2 (A). SVP-SB150 was below 20% in the dominance of nitrifying granules (on the 55th day), while it increased up to 65% during the disintegration period. Since there was no any external perturbation in the reactors during the operation, the disintegration of the nitrifying granule was proposed to be of internal origin. At the same time, MLSS and MLVSS in the reactors stopped decreasing, remaining at around 3 g/L and 2 g/L, respectively.

After the disintegration of the nitrifying granules, a rapid evolution of the nitrifying granules in the reactor was observed. The size of the nitrifying granules gradually increased to 235 μm. MLSS and MLVSS began to rise and sharply increased to around 10 g/L and 7 g/L, respectively, in two months. It seemed that nitrifying granules finished self-adjustment and prospered with large quantities after the unstable and critical state.

The value of SVI5/SVI30 maintained in the range of 1-1.2, indicating the dominance of the nitrifying granules in the whole operational process.

After about 200 day’s operation, nitrifying granules was successfully cultivated with high biomass concentration and settling ability.

3.2. Performances of the Nitrifying Granules

As can be seen in Fig. 4, ammonia removal efficiency maintained higher than 99% throughout the whole operation period, with effluent ammonia concentration lower than 6 mg N/L. NO₂-N and NO₃-N concentration during the whole operating process were also traced, which revealed complete nitrification in the reactor during most of the experiment period. Although nitrifying granules experienced size reduction from day 70 to 120, the ammonia nitrogen removal was not impacted negatively during the period. In addition, the decrease of biomass concentration during the first 50-day operation did not compromise ammonia nitrogen removal either. Fig 5 shows cycle profile of the nitrifying granules on the 160th day of the operation, during which nitrifying granules recovered from the disintegration and retained stable. To reduce the influence of the residue from last cycle, the upper liquid in the leftover was changed with tape water. In the cycle, ammonia was totally oxidized within 3 hours, during which there were a big accumulation of nitrite, indicating that the limiting step of the process was nitrite oxidation by nitrite oxidizing bacteria (NOB). Upon the depletion of ammonia, nitrite concentration increased to the climax and then quickly oxidized to nitrate since there was no oxygen limitation caused by ammonia oxidation. The end product of the oxidation process was totally nitrate, indicating a complete nitrification of the process.
3.3. FA and FNA Conditions in the Reactors

FA and FNA variations in the cycle of reactor operation on the 160th day are shown in Fig. 6. It can be seen that the concentration of FA increased fast to the climax, i.e., 108.8 mg N/L, within half an hour of the cycle. Then it decreased gradually to zero during subsequent 2 hours of the cycle. FNA concentration gradually increased to the climax, i.e., 0.044 mg N/L, on the depletion of FA, and then decreased to zero in the subsequent 2 hours of the cycle.

3.4. Denaturing Gradient Gel Electrophoresis Analysis (DGGE) of the Nitrifying Granules

Fig. 7 shows DGGE band profile of the PCR amplification products obtained from the nitrifying granules. Samples were collected in the developing process of the nitrifying granules, including disintegration of the nitrifying granules. The changing trend of the bands pattern shows that the number of the bands decreased during granulation period and increased as mature bacteria community in the granules formed. The position of the major bands did not shift during the course of granulation (band 1, 2, 4, 5, 6), while the number of the major bands increased after the formation of mature granules. This is coincident
with previous research, which demonstrated that the diversity of the population may increase with the growth of the granules [23].

Fig. 6: DGGE profiles of the communities in the nitrifying granules

4. Discussion

Nitrification is affected by many factors, such as substrate concentration, pH, dissolved oxygen (DO), temperature [24]-[27]. Among them, the effects of substrate are strong and complicated due to its inhibition effects when unionized, i.e., the formation of FA and FNA. They are reported to inhibit the growth and/or energy generation of a wide range of bacteria. Different threshold values were proposed for nitrification inhibition, which are very sensitive to bacteria adaptation. It was stated that the FA inhibition threshold is 10-120 mg/L for ammonia oxidation, and 0.1-1.0 mg/L for nitrite oxidation. While FNA inhibition threshold is 0.1-4.0 mg N/L for ammonia oxidation and 0.01-0.83 mg N/L for nitrite oxidation, [20]-[29]. In this study, both the FA concentration and FNA concentration increased to a climax value during one cycle, i.e., 108.8 and 0.044 mg N/L, respectively, when influent ammonia concentration increased to 1000 mg N/L under well controlled temperature, pH and DO. The high FA and FNA concentration may thereby exert high inhibition to the system. However, the nitrifying granules exhibited good performance (99% nitrogen removal efficiency) and stability (SVI$_{30}$ value of around 15 ml/g) during the whole operation under stepwise loading increasing strategy in the influent. This shows that the nitrifying granules may have strong resistance to high inhibitions with stepwise acclimation, which may facilitate their nitrification of ammonia under high loading rates.

As a kind of aggregated cells, aerobic granules can be expected to have better protection from high substrate loading rates and inhibitory of toxic substrates. The tolerance towards the disadvantage conditions is not only caused by the fast removal of the substrates outside biomass, but also by the granules’ intrinsic structural advantage as aggregated cells, which cause diffusion limitation inside granules. Though diffusion barrier might limit oxygen and nutrient transportation to the core of the aggregates, it can protect the inner layer of microorganisms against exposure to disadvantageous conditions. Therefore, as one of the major producer of diffusion limitation, size of the granules should be optimally developed with enough resistance to the disadvantage conditions but little barrier to the nutrient transportation. In this study, it was found that the size of the nitrifying granules was increased with the fast loading increasing in the feed, which could be a positive responds of the nitrifying granules to the disadvantageous environment, i.e., the stepwise accumulated FA and FNA in the bulk liquid. The high growth rate of bacterial strains was widely reported to encourage proliferation of microbes, which may cause a rapid increase in size of the granules with a loose structure and low density [30], [31]. In the meantime, the size of the micro-colonies was reported to be able to determine whether fragmentation took place under the shear conditions applied and that whether this shear could erode or further fragmentation [32]. It is thus speculated that the structure of the granules could start to be damaged when the size of the granules grew continuously to a larger one that limited mass transfer greatly. Once the interior structure became too weak to withstand the applied shear force, the nitrifying granules were
broken up into small pieces. Therefore, it is possible that the fast increase of size of the nitrifying granules was the main cause of the break-down of size of the nitrifying granules on the 60th operational day.

However, the disintegration of the nitrifying granules did not negatively affect ammonia removal efficiency of the system, which was confirmed by the unchanged ammonia removal efficiency and settling ability of the nitrifying granules after the disintegration. It’s reported that different populations had large variations in their micro-colony strength and in their resistance to micro-colony break-up due to physical-chemical effects from various chemicals [33], [34]. Nitrifiers are known to grow in dense micro-colonies and micro-colonies generally seem to form the strongest fraction of the flocs. Gilda reported that the overall nitrogen removal in their system was not affected by the physical separation of ammonium and nitrite oxidation, in which most NOB was located in the granules and AOB in flocs [35]. Considering that nitrifying granules in this study are dominant of AOB and NOB (data not shown), it is reasonable to speculate that the disintegration of the nitrifying granules could be mainly the separation of the AOB and NOB inside, which have high flexibility in the forms of cooperation. Overall nitrogen removal efficiency was thus not much negatively affected.

Loss of granule stability and deterioration of the whole system was one of the major barriers to practical applications for long-term operation. However, in this study, the disintegration of the nitrifying granules did not lead to the deterioration of the system accordingly. On the contrary, the system maintained a good performance through self-adjustments of size of the nitrifying granules, showing stronger self-healing ability than that of the heterotrophic granules. This discrepancy of the performances of the granules may be attributed to the microbial difference. Not as the loosely aggregating form of the nitrifying bacteria (AOB and NOB) in nitrifying granules mentioned above, most granules with complicated bacteria have closely related microbial communities. The physical break-up of the micro-colonies could lead to big shock to microbial communities who depend closely on each other for balanced life and hence the deterioration of the whole system. Therefore, nitrifying granules may have a higher self-healing ability than that of the heterotrophic granules, which make it a good option for stable and effective nitrification under high ammonia loading conditions.

However, it should be noted that the disintegration of the nitrifying granules in the development is not the doomed process. Without the outgrowth of size, the nitrifying granules in our parallel experiment experienced a stepwise growth in terms of size of the granules [36]. In addition, the DGGE results clearly showed that the main microbial communities in the two experiments were almost same under the same operational conditions, though there was a big difference on the evolution process of the physical characteristics of the nitrifying granules. These results clearly demonstrated that nitrifying granules have a more flexible developing mode than heterotrophic granules due to their stronger self-healing ability, which make the system more stable and promising in practice.

5. Conclusions

The nitrifying granules able to treat ammonia as high as 1000 mg N/L were successfully cultivated in the reactor of SBR. During the operation period, the nitrifying granules disintegrated first and then recovered by themselves. However, the main characteristics of the nitrifying granules, such as treatment capacity, settling ability, biomass concentration did not deteriorate. These results demonstrated that nitrifying granules have good performance and stability under high loading rates. At the same time, they have a more flexible developing mode than mix-cultured granules due to their stronger self-healing ability. These good attributes of the nitrifying granules make their nitrification more stable and promising in practice.

6. References


