Formation and characteristics of nitritation granules cultivated in sequencing batch reactor by stepwise increase of N/C ratio

Ling-yun Li, Yong-zhen Peng, Shu-ying Wang, Lei Wu, Yong Ma, Akio Takigawa and Dong Li

ABSTRACT

The cultivation of nitritation granules in sequencing batch reactor (SBR) by seeding conventional floccular activated sludge was investigated using ethanol-based synthetic wastewater. Reducing settling time offers selection pressure for aerobic granulation, and stepwise increase of influent N/C ratio can help to selectively enrich ammonia oxidizing bacteria (AOB) in aerobic granules. The spherical shaped granules were observed with the mean diameter of 1.25 mm, average settling velocity of 1.9 cm s^{-1} and the sludge volume index (SVI) of 18.5–31.4 ml g^{-1}. After 25 days of operation, the nitrogen loading rate reached 0.0455 kg NH_4^+-N (kg MLSS·d)$^{-1}$, which was 4.55 times higher than that of the start-up period. The mature granules showed high nitrification ability. Ammonia removal efficiency was above 95% and nitrite accumulation ratio was in the range of 80–95%. The nitrifying bacteria were quantified by fluorescence in situ hybridization analysis, which indicated that AOB was 14.9 ± 0.5% of the total bacteria and nitrite oxidizing bacteria (NOB) was 0.89 ± 0.1% of the total bacteria. Therefore, AOB was the dominant nitrifying bacteria. It was concluded that the associated inhibition of free ammonia at the start of each cycle and free nitrous acid during the later phase of aeration may be the key factors to start up and maintain the stable nitritation.

Key words | aerobic granules, ammonia oxidizing bacteria, free ammonia, free nitrous acid, nitritation

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INTRODUCTION

Aerobic granulation is a promising alternative to traditional activated sludge in wastewater treatment plants (WWTP). Compared with conventional flocculent activated sludge, granular sludge has a strong and compact microbial structure, improved settling-ability and higher biomass retention (Morgenroth *et al.* 1997; Yang *et al.* 2003; Zheng *et al.* 2005; Adav *et al.* 2008). The aerobic granulation technology has the potential to respond to the challenges of nitrogen removal from wastewater as well.

Recently, there has been a lot of reports regarding of cultivation of aerobic granules in aerobic upflow fluidized reactor or sequencing batch reactor (SBR) (Shin *et al.* 1992; Hu *et al.* 2005). In their reports, aerobic nitrifying granules have been cultured using short settling time and a relative high shear. Moreover, many researchers have focused on the research in SBR for the formation identification, characteristics description, spatial distribution of bacterial species and application of aerobic granule. Spherical and elliptical granules with the average diameter of 0.35 mm were detected at the bottom of the reactor after 300 days of operation (Tsuneda *et al.* 2003). Liu *et al.* (2004) cultivated nitrifying granules with strong structure and good settleability in terms of specific gravity, SVI and cell hydrophobicity by controlling the substrate N/COD ratio in SBR. Shi suggests that the maximum number of the ammonia-oxidizing bacteria (AOB) and Nitrobacter occupy 79.1% of the total bacteria in nitrifying granules, and AOB are close to the surface of granules, while the nitrite-oxidizing bacteria (NOB) are found in the deeper layer of granules (Shi *et al.* 2010).

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Due to extremely low growth rate and lack of production of extracellular polymeric substances, it takes a long time to develop the granules of nitrifying bacteria. The main purpose of this work is about how to achieve nitrifying granulation rapidly with synthetic wastewater (ethanol as carbon source) in a partial nitrification SBR process. The distribution of AOB and NOB in the nitrifying granules has been investigated for the better understanding of nitrifying granulation process and for the potential application of it in treatment of high ammonium content wastewaters.

MATERIALS AND METHODS

SBR operation

The SBR reactor was made of flexible glass, with a working volume of 14 L. One operation cycle was 4 h with feeding of 8 min, aeration of 3.5 h, settling of 15 min, discharge of 5 min and idle time of 2 min. The SBR was controlled by a programmable logic circuit. There was one vertical mixer to provide mixing of mixed liquor. Oxygen was supplied by an air compressor through a fine bubble diffuser. Air flow rate to the SBR during aeration was set to 0.3 $m^3\ h^{-1}$. Hydraulic retention time (HRT) was maintained at 8 h by discharging half of the wastewater (7 L) during the discharge. Temperature was controlled at $25 \pm 0.5\ ^{\circ}C$ with thermostat. The pH and DO sensors were installed for monitoring the signal of pH and DO in the reactor.

Seed sludge and experimental conditions

The seed sludge was taken from a local municipal wastewater treatment plant. The initial concentration of volatile suspended solid in the SBR reactor was about 4,000 mg L^{-1}. In order to acclimate nitrifying granules, the Influent NH_4^+-N concentration has been increased stepwise from 50 to 200 mg L^{-1}. See more detailed experimental conditions in Table 1. The pH was maintained at the range of 7.0–8.0 by addition of $NaHCO_3$ solution during the whole operation (Table 2).

Table 1 | stepwise increase of substrate NH_4^+-N

Cycles	I (1–12)	II (13–40)	III (41–64)	IV (65–186)
Nitrogen Loading NH_4^+-N/COD (mg L^{-1})	50/400	100/400	150/400	200/400

Analytical methods

Chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N) and Mixed Liquor Suspended Solids (MLSS) and Mixed liquor volatile suspended solids (MLVSS) were measured according to standard methods (APHA 1995). DO and pH were measured with a Multi 340i (WTW, Weilheim, Germany). The morphology of aerobic granule was observed with OLYMPUS BX-51 phase-contrast microscope and OLYMPUS C24040 ZOOM digital camera.

Nitrite accumulation ratio (NAR) and free ammonia (FA), free nitrous acid (FNA) concentration

The nitrite accumulation ratio was calculated as follows:

$$\mathrm{NAR}\,(\%) = \frac{NO_2^-}{NO_2^- + NO_3^-} \qquad (1)$$

The concentrations are calculated as a function of pH, temperature (T) and total ammonium as nitrogen (TAN), for FA; and total nitrite (TNO_2), for TNA from the equilibrium described below:

$$\mathrm{FA}\,(\text{mg-N/L}) = \frac{\mathrm{TAN}}{1 + (10^{-\mathrm{pH}}/K_e^{\mathrm{NH}})}, \quad K_e^{\mathrm{NH}} = e^{-6334/(273+T)} \qquad (2)$$

$$\mathrm{FNA}\,(\text{mg-N/L}) = \frac{[\mathrm{TNO_2}]}{1 + (K_e^{\mathrm{NO}}/10^{-\mathrm{pH}})}, \quad K_e^{\mathrm{NO}} = e^{-2300/(273+T)} \qquad (3)$$

Table 2 | Characteristics of the artificial wastewater

Composition	Concentration	Nutrient solution (mg L^{-1})	
COD	400 (mg L^{-1})	$FeCl_3 \cdot 6H_2O$	1.5
PO_4^{3-}-P	4 (mg L^{-1})	H_3BO_3	0.15
$MgSO_4 \cdot 7H_2O$	0.02 (g L^{-1})	$CuSO_4 \cdot 5H_2O$	0.03
$CaCl_2 \cdot 2H_2O$	0.01 (g L^{-1})	KI	0.18
Nutrient solution	0.575 (ml L^{-1})	$MnCl_2 \cdot 4H_2O$	0.12
pH controlled by addition of $NaHCO_3$ solution	7.5–8.0	$Na_2MoO_4 \cdot 2H_2O$	0.06
		$ZnSO_4 \cdot 7H_2O$	0.12
		$CoCl_2 \cdot 6H_2O$	0.15
		EDTA	10

Parameters including physical (size, settling velocity, density, specific gravity (SG), sludge volume index(SVI), specific surface area, moisture content, integrity coefficient) and chemical (oxygen utilization rate (OUR)) were investigated to study characteristics of aerobic granules with the methods reported by (Beun *et al.* 2000).

Scanning electron microscope (SEM) observation

The morphology of the bacteria was examined with high resolution SEM (FEI QUANTA 200, FEI Company in USA). Samples were pre-treated by fixing with 2.5% glutaraldehyde in a 0.1 M phosphate buffer. Subsequently, the samples were washed and dehydrated in a series of ethanol solution (50, 70, 80, 90, and 100%). The dewatered samples were dried by the critical point method and were further sputter coated with gold for SEM observation.

Fluorescence in situ hybridization (FISH) analysis

FISH was used to analyze the quantitative changes of nitrifying microbial communities in the activated sludge which was described by (Amann 1995). Sludge samples were taken from the SBR at various times and were analyzed for AOB and NOB. The probes applied include EUB_{Mix} (EUB338, EUB338-II and EUB338-III, specific for all bacteria) (Daims *et al.* 1999), NSO1225 (specific for Betaproteobacterial AOB) (Mobarry *et al.* 1996), NIT3 (specific for Nitrobacter sp.) (Wagner *et al.* 1996) and Ntspa662 (specific for Nitrospira genera) (Daims *et al.* 2001). 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.

RESULTS AND DISCUSSION

Granulation of nitrifying sludge

The reactor was operated for over 50 days under three operational phases: start-up period, self-aggregation phase and maturation stage, as characterized below.

The start-up period (cycle 1–cycle 114)

Figure 1 shows clearly the variations of settling time, SVI and MLVSS during the granulation. The biomass concentration of the grey colour floccular sludge was 3,200 mg L^{-1} after seeding with the SVI value of 136.98 ml g^{-1}. The initial settling time was 15 min in the first 12 cycles and reduced to 12, 8, 5, 3 and 2 min gradually, the biomass in term of MLVSS and SVI in the reactor decreased and reached a relatively low value of about 1,200 mg L^{-1} and 40 mL h^{-1} on the cycle of 120. Previous studies showed that the settling time has been as one of a significant impact factors on the aerobic granulation and that a short settling time was favourable for the granule formation (Shi *et al.* 2010). However, a too short settling time is bad for the enrichment of the autotrophic nitrifying bacteria due to their low growth rate. In order to maintain a large amount of AOB and/or NOB for granulation, the settling time of the SBR gradually decreased from 15 to 2 min. The volume exchange ratio was maintained at 50% in this experiment. The high wastewater volume exchange ratio and the short settling time decreased MLVSS concentration with poor settling property microbial cells wash-out.

The self-aggregation period (cycle 114–cycle 150)

The aerobic granulation, from dispersed sludge to mature granules, is a gradual and slow process.

During the first 114 cycles of operation, the suitable aeration volume and hydrodynamic shear force promoted the aggregation of the nitrifying bacteria. Small granules, with an average diameter of 0.3 mm, can be seen by eyes with settling time maintained 2 min on the 150th cycle. As shown in Figure 1, MLVSS concentration are stabilized eventually, the SVI value reduced continuously from an initial value of 139.8 mL g^{-1} and stabilized at 20 mL g^{-1}.

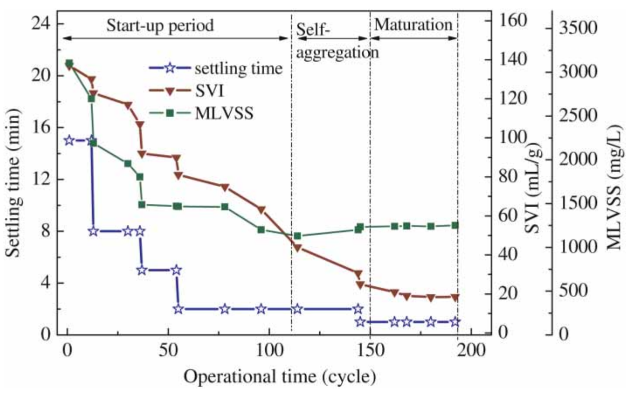

Figure 1 | Variations of settling time, SVI and MLVSS during the granulation.

The maturation period (cycle 151–cycle 180)

When the biomass concentration and SVI reached a pseudo-steady state, the settling time was fixed at 2 min. A stable biomass concentration was observed after 150 cycles of operation. At the end of operation, the SVI decreased to only 18.5 mL g^{-1}, suggesting that the mature granules sludge had a more excellent settling capacity compared with the seeding sludge.

SEM observation and physical characteristics of granules

The optical microscopy and SEM pictures of the sludge in the granulation process were shown in Figure 2. Initially the sludge had a floc structure with a mean floc size of 0.10 mm, irregular and loose-structured morphology which transformed to a spherical granule structure in 180 cycles. The colour of activated sludge changed gradually from brown to yellow. The nitrifying granules had a compact and round-shaped structure with a clear outer shape (Figure 2(e)) and the average diameters reached 0.8 mm in 30 days. An SEM image of a granule, its surface and interior at a high magnification in Figure 2(f) indicated that thick clusters of small rod-shaped cells were the dominant population structure. It was confirmed that the bacteria formed dense layers on the granule surface. No filamentous bacteria were observed on the granule surfaces. It seemed that the nitrifying granules were composed of clusters of small aggregates with internal cavities and a network of cell-free channels. Structurally similar networks formed by channels and cavities between cell aggregates were previously detected in microbial granules of different microbial origins and compositions and could be interpreted as facilitating the exchange of nutrients and gases (Hu *et al.* 2005; Kim & Seo 2006).

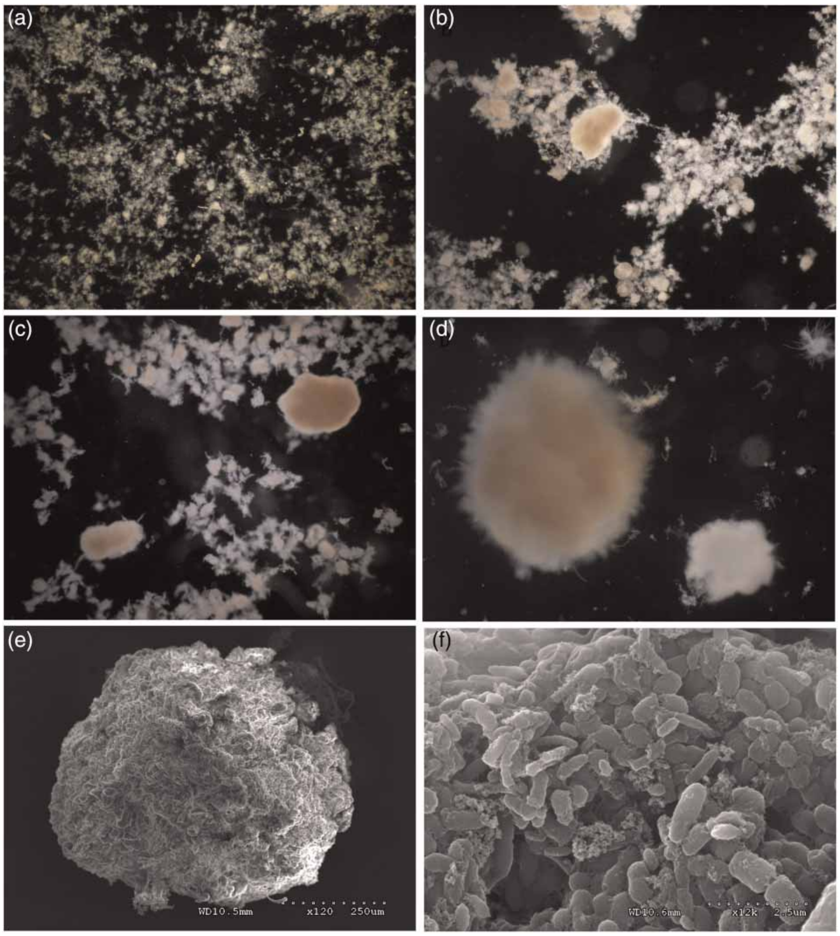

Figure 2 | Phase contrast microscopy and scanning electron microscopic pictures of the granules during the cultivation. (a) Seed sludge; (b) 50 cycle; (c) 80 cycle; (d) 114 cycle; (e–f) 180 cycle.

Efficient aerobic granules with wide diverse microbial species, compact structure and excellent settling capabilities have been formed in SBR. The size of granule determined by SEM was in the range of 0.8–1.3 mm, averaged 1.25 mm. The mature aerobic granules exhibited many excellent attributes compared with floccular sludge, shown in Table 3. The mature nitrifying granules showed excellent settling ability with moisture content of 96.1% and specific gravity of 0.52 g g^{-1}. The average settling velocity of the granules was 1.9 cm s^{-1}. It was observed that after only 2 min of settling, the large-sized nitrifying granules were well settled, leaving a clear supernatant in the granular SBR (GSBR).

The activities of nitrifying bacteria populations in granules was described by oxygen utilization rate (OUR). When the substrate N/COD ratio was maintained at 200/400, the OUR of steady-state aerobic granules was 0.069 mg O_2·(g min)$^{-1}$ which indicated that activities of AOB and NOB in the granules were high. Data of similar activity were reported by Yang *et al.* (2003) and the activities of AOB and NOB significantly increased with the increase of the substrate N/COD ratio.

Organics removal and partial nitrification achievement in SBR

As showed in Figure 3, there was a good and stable organics removal performances in 50 days Continuous operation of GSBR, with the influent COD at about 400 mg L^{-1}. The effluent COD concentration was stabilized at 34.8–62.81 mg L^{-1}, and the total COD removal efficiency was 84.3–91.3%.

Variations of NH_4^+-N, NO_2^--N and NO_3^--N concentrations during the whole operation were illustrated in Figure 4. During the start-up period, low N/COD ratio of 50/400 was maintained from cycle 1 to cycle 12. The influent NH_4^+-N decreased rapidly and converted to nitrite and nitrate. No lag in nitrate production with respect to nitrite formation was observed. Then the influent NH_4^+-N concentration increased to 100 mg L^{-1} to enhance the nitrification ability from cycle 13. In this step (cycle 13–cycle 38), the average value of NH_4^+-N removal efficiency was above 90%. Meanwhile, The NAR increased from a low level of 10.4% to 61.5%, with a high effluent NO_2^--N concentration of 31.25 mg L^{-1}. The NAR increased continuously with the influent N/COD ratio150/400 during the consequent 40 cycles.

After 78 cycles, the N/COD ratio was up to 200/400. The ammonia in the influent was oxidized completely in 3.5 h and effluent NH_4^+-N was below 10 mL g^{-1}. The NH_4^+-N oxidization efficiency was always above 95% with NAR up to 95% and the effluent nitrate was below 4.0 mg L^{-1}. In general, the NH_4^+-N loading was enhanced from 0.01 kg NH_4^+-N·(kg MLSS·d)$^{-1}$ to 0.0455 kg NH_4^+-N·(kg MLSS·d)$^{-1}$, 4.55 times

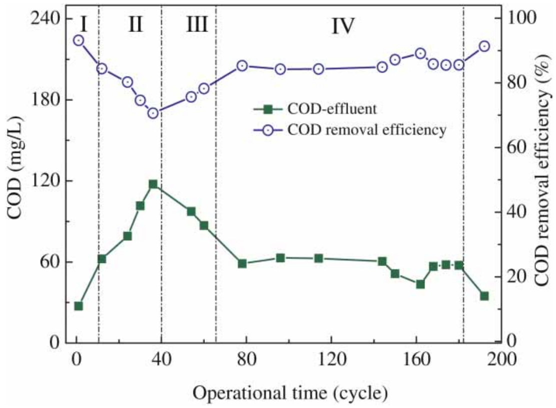

Figure 3 | Organics removal during the whole operation.

Table 3 | Characteristics of sludge granules

Indexes	Value
Appearance	Ellipse or sphericity
Moisture Content/%	96.1
Gravity/g g^{-1}	0.52
Wet denisity/g m^{-1}	1.68
Average diameter/mm	1.25
Quantitative distribution	4.17×10^5
Specific surface area/m^2·(m^3)$^{-1}$	0.5×10^4
Integrity coefficient/%	80.4
Settling velocity/cm s^{-1}	1.9
OUR/mg·(g min)$^{-1}$	0.069

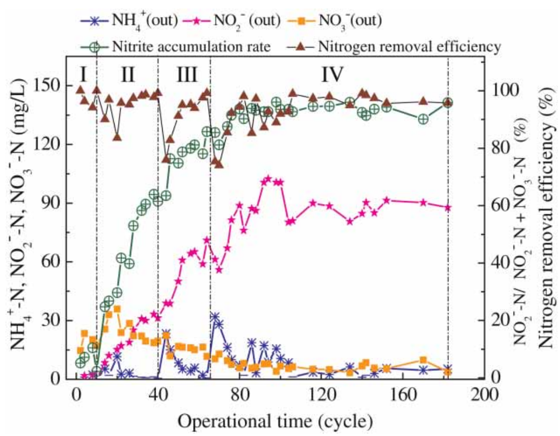

Figure 4 | Nitrification performance during the granulation process.

increase compared with the ammonia load of seed sludge. To maintain a stable partial nitrification, the sludge residence time (SRT) and the MLVSS concentration was kept at 18–20 days and 1.4 g L^{-1} respectively by withdrawing excess sludge from the GSBR. Stable ammonia removal capacity was also obtained in the GSBR. As shown in Figure 4, the effluent ammonia and its removal efficiency was 1.12–5.55 mg L^{-1} and 91.4–96.0% respectively.

Community analyses of AOB and NOB in the nitrite accumulating granules

The samples used for the experiment were the nitrifying granules cultured with a diameter of 1.25 mm. Both groups of nitrifying bacteria, i.e. AOB and NOB, were detected with the NSO190, NIT3 and Ntspa662 probes, respectively based on the total bacteria by quantitative analyses of FISH images. The seeding sludge in this study was typical activated sludge with complete nitrification to nitrate and had average values of 2.8 ± 0.6% AOB and 5.9 ± 0.2% NOB. In start-up phase with low FA concentrations for 12 cycles, the percentage of nitrifying bacteria in biomass has increased; however, the amount of NOB was still much more than that of AOB. By increasing influent N/COD ratio (100/400, 150/400, 200/400), FA inhibition played an important role in the following phase for 168 cycles, AOB became the dominant nitrifying bacteria and very few *Nitrospira* were detected.

Figure 5 illustrated the FISH images of the nitrifying granules collected from the GSBR on cycle 180. The investigation of the distribution of nitrifying bacteria populations revealed that only 0.89 ± 0.1% of the total bacteria belonged to NOB while AOB occupied 14.9 ± 0.5% of the total bacteria. The results showed that AOB were selectively enriched and grown in the GSBR and they became the most dominant bacteria in the granule within the SBR operation time.

Mechanism of partial nitritation achievement in GSBR

Nitrifying bacteria are divided into two distinct groups, AOB and NOB, that have a relationship which is synergistic and competitive. The AOB utilize ammonium as their donor and release nitrite as the oxidized product. The NOB utilize nitrite as their donor, with nitrate being the oxidized product. The accumulation of nitrite results from the imbalance of nitritation and nitratation activities which is due to selective inhibition of NOB by inhibiting parameters such as FA, FNA and dissolved oxygen limitation (DO) (Peng *et al.* 2004; Yang *et al.* 2007). FA and FNA are known to inhibit nitrification, especially nitrite oxidation (Peng *et al.* 2008; Ma *et al.* 2010).

Although many researchers reported the concentrations of FA and FNA that might inhibit the growth of NOB and cause the accumulation of AOB, the critical values recorded in the literatures were different. NOB is known to be inhibited at the free ammonia concentration of 0.1–1.0 mg N L^{-1}

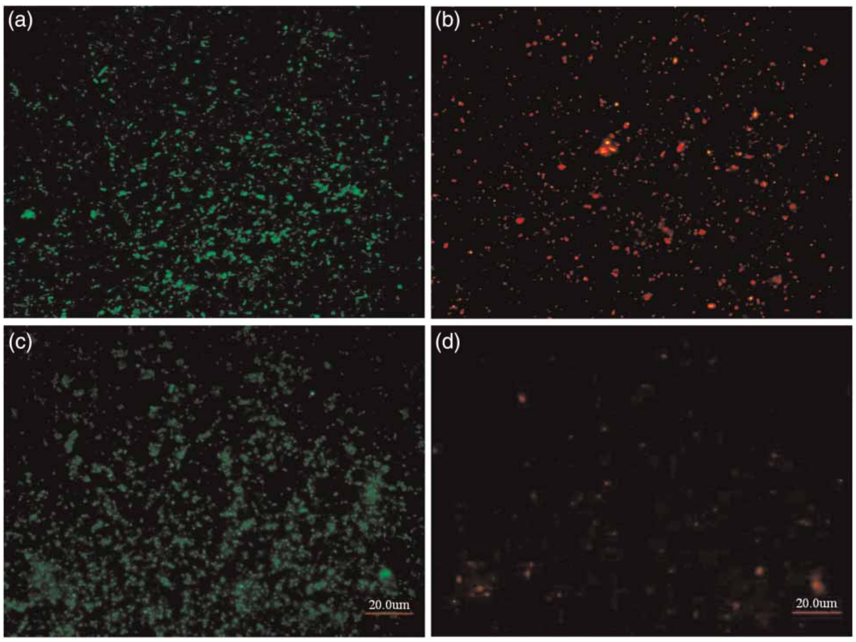

Figure 5 | FISH images of aerobic granules on 180th cycle. (a, c) EUBmix for all bacteria (b) NSO190 for AOB (d) Ntspa 662 for NOB.

(Anthonisen *et al.* 1976). Chang *et al.* (2002) also reported that nitrite could be accumulated at a free ammonia concentration as low as 0.2 mg N L^{-1}, while (Kim & Seo 2006) achieved nitrite accumulation by selective enrichment of AOB and wash-out of NOB under the condition of the initial FA concentration 2.4 mg L^{-1}. Additionally, Anthonisen *et al.* (1976) and Hellinga *et al.* (1998) reported inhibition concentrations were 0.22 and 0.2 mg HNO_2-N·L^{-1} for nitrite oxidation, respectively. Recently, Vadivelu *et al.* (2006) reported that FNA initiated the growth inhibition of *Nitrobacter* at a concentration of 0.01 mg N L^{-1} and the growth of the *Nitrobacter* completely ceased at a nitrous acid concentration of 0.02 mg N L^{-1}. Similar results were obtained by Kim & Seo (2006) and confirmed that FNA was a very important control factor to achieve partial nitrification to nitrite.

Additionally, oxygen limitation is the critical point for stable maintaining of partial nitrification via nitrite due to the stronger DO affinity of AOB than NOB at low DO concentrations, AOB outcompete NOB (Guo *et al.* 2009). However, it is interesting that nitrite still accumulated and nitrate production was ignored although the DO concentration in this study varied from 3.0 to 5.6 mg L^{-1}, which was higher than the normal inhibition level. So oxygen limitation can be eliminated from the candidate.

In this investigation, the initial concentration of NH_4^+-N was 50 mg L^{-1} as the influent was diluted with the solution remaining in the SBR by 1:1 and ammonia was completely oxidized in 100 min. the FA concentration in the first cycle was 0.553 mg N·L^{-1} which was not up to the inhibition level as reported in the literature(1.0 mg N·L^{-1}). During the NH_4^+-N oxidation, NO_3^--N concentration increased to 30 mL g^{-1} until NH_4^+-N was complete oxidized to NO_2^-, and further oxidized to NO_3^-. Nitrous acid concentration, calculated from the equilibrium equation of NO_2-N, was less than 0.003 mg L^{-1} and far below the inhibition level. At the cycle 14 and cycle 50, the initial concentration of NH_4^+-N increased to 100 and 150 mg L^{-1}, respectively and the FA concentration calculated as 3.75 and 6.91 mg N·L^{-1} respectively, which was high enough to inhibit NOB. As the NH_4^+-N was completely oxidized, very few NO_2^--N was further oxidized to NO_3^--N. FNA started inhibiting nitrite oxidation at 0.0069 mg N·L^{-1}, and the nitrate production was found to be affected significantly when FNA concentration exceeded 0.01 mg N·L^{-1} (Figure 6). The FNA concentrations reached up to 0.0175 mg N·L^{-1} (cycle 76) and 0.023 mg N·L^{-1} (cycle 98), which were consistent with the inhibition level reported in the literature as 0.02 mg N·L^{-1}. Nitrite accumulation ratio was up to 60%. When the initial NH_4^+-N concentration increased to 200 mg·L^{-1}, FA was 6.91– 7.26 mg N·L^{-1}. So most NH_4^+ was converted to NO_2^- and very few NO_2^- was further oxidized to NO_3^- after complete oxidation of NH_4^+. However, there was no complete inhibition of nitrite oxidation even if the biomass being exposed to the highest level of FNA (up to 0.023 mg N·L^{-1}) in this study. By the above operational pattern, nitritation was successfully established with NAR above 95%. It seemed that the combined inhibition of free ammonia and free nitrous acid selectively suppressed the growth of NOB and NOB was gradually washed out of the GSBR. Initial FA level in the SBR was high enough to selectively inhibit NOB to accumulate nitrite and it can be used as a very important control factor to achieve partial nitrification. When NH_4^+-N was oxidized, nitrite started to increase. Then FNA could also inhibit nitrite oxidation during the later phase of aeration when nitrite concentration was very high.

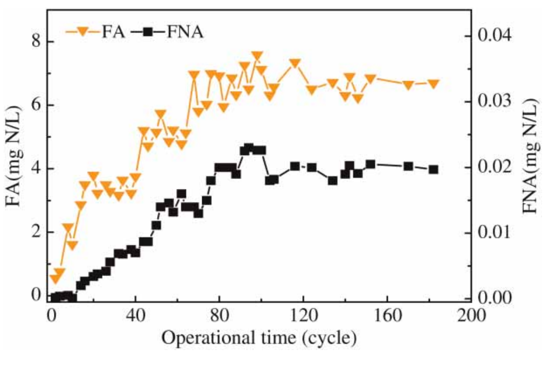

Figure 6 | Concentrations of FA and FNA during nitrite accumulation (FA: start of each cycle, FNA: end of aeration).

CONCLUSION

Aerobic nitrifying granules, which were enriched with AOB, were successful cultivated in a SBR reactor by shortening the settling time during the experiment. With the granulation of the seed sludge, the sludge volume index drastically decreased from 139.8 to 18.5 mg L^{-1} in 50 days. The mature aerobic granules exhibited many advantages: regular, smooth and nearly round in shape, excellent settle ability, dense and strong microbial structure and high biomass retention compared with the floccular sludge.

The influent ratios of N/COD were stepwise increased to improve the removal of ammonia and simulate the growth of AOB. The initial high FA and high FNA during the later phase of aeration promoted nitritation and inhibited nitratation and nitrite accumulation was established rapidly. Nitrifying microbial communities were also optimized and AOB became the dominant nitrifying bacteria with the abundance of 14.9 ± 0.5% to the total bacteria and NOB was only 0.89 ± 0.1% to the total bacteria according to FISH analysis. From the results, it can be concluded that the maintenance of high nitrite accumulation can be conjectured in two ways: the selective inhibition of nitratation and wash-out of NOB. Therefore, the possibility of GSBR application was verified in our study and it is an attractive nitrogen removal technology for wastewater with high ammonia nitrogen.

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