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Strategies to improve aerobic granular sludge stability and nitrogen removal based on feeding mode and substrate

Quan Yuan, Hui Gong, Hao Xi, Heng Xu, Zhengyu Jin, Nasir Ali, Kaijun Wang**

State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China.

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ABSTRACT

A systemic strategy was proposed to improve aerobic granular sludge (AGS) stability and nitrogen (N) removal efficiency by optimizing feeding mode and substrate aiming at complicated wastewater characteristics. Key functional groups at the genus level identified by high-throughput sequencing were evaluated as well. The results showed that anaerobic feeding mode and acetate promoted the compact AGS formation with excellent total nitrogen (TN) removal efficiency (averaging 91.7% ± 4.1%) at various dissolved oxygen conditions. While the aerobic feeding mode led to a loose AGS structure with a vulnerable anaerobic core and poor TN removal efficiency (averaging 58.8% ± 7.4%). Simultaneous nitrification and denitrification process played the dominant role in N removal in compact AGS over the alternating nitrification and denitrification process. High-concentration glucose undermined feast–famine condition with filamentous bacteria growth out of granule and decreased TN removal efficiency to 67.3% ± 15.2%. Lower food to microorganism ratio may result in a lower N removal rate attributed to the sharply increased biomass concentration fed by glucose. Ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, denitrifying bacteria, and denitrifying phosphorus accumulation organisms enriched during AGS granulation also contributed to the efficient N removal. The proposed strategy provided insights into the relationship between various factors and stable AGS formation, and systemic operation methods for various complicated wastewater treatment.

Introduction

Aerobic granular sludge (AGS) in a sequencing batch reactor (SBR) is one of the new attractive technologies for biological wastewater treatment, and has been implemented in treatment of both municipal (Swiatczak and Cydzik-Kwiatkowska, 2018; Li et al., 2014) and a wide range of industrial wastewater by some researchers (Zhang et al., 2016; Jiang et al., 2017; Nancharaiah and Venugopalan, 2011). Furthermore, it demonstrated unique features including granules without carrier material, excellent settling properties, great biomass enrichment, simple single-tank concept with simultaneous chemical oxygen demand (COD), nitrogen (N) and phosphorus (P) removal, low operation costs compared with conventional

* *Corresponding authors.* *E-mails:* gongh14@tsinghua.org.cn (Hui Gong), wkj@mail.tsinghua.edu.cn (Kaijun Wang).

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activated sludge processes (Liu and Tay, 2015; Moreira et al., 2015; Liu et al., 2014). However, one of the main problems encountered in the operation of a granular sludge SBR is the instability of AGS. A stable and reliable operation of an aerobic granule-based reactor is desirable, but some of the aerobic granules formed in previous studies appeared to have relatively low stability as compared to anaerobic granules. The poor stability of AGS limits its application in the field of wastewater treatment (Zheng et al., 2006). Besides, operation strategies for industrial wastewater treatment varied greatly due to the complicated wastewater characteristics, and no systemic operation strategy has been proposed for guidance (Kozlovskiy et al., 2017; Tomei et al., 2016; Zhu et al., 2013).

Factors affecting AGS stability were of great varieties, including organic strength (Long et al., 2015; Thanh et al., 2009), substrate characteristics (Kreuk et al., 2010; Pronk et al., 2015), feeding mode (de Kreuk et al., 2005; Beun et al., 1999; Cydzik-Kwiatkowska et al., 2016), selective pressure (Beun et al., 1999), shear force (Tay et al., 2001) and so on. AGS was able to withstand high organic loading rates (OLRs) ranging from 2.5 to 15 kg-COD/(m^3·d) (Long et al., 2015; Thanh et al., 2009) with easily degradable carbon source, while the increased OLR led to the development of fast-growing microorganisms (fungi and filamentous bacteria) and AGS instability, especially when the influent contained particulate substrates (Kreuk et al., 2010; Corsino et al., 2018; Li et al., 2010). Fast feeding mode with various operation regimes and anaerobic feeding modes were widely applied to cultivate AGS aimed at certain pollutant removal (Beun et al., 2002; Wu et al., 2012), while the stability of AGS needs further improvement. Limited settling time created high selective pressure to remain granules with good settling ability and washout flocs, and a relatively high shear force was favorable for granulation (Beun et al., 1999). When looked into the influencing factors connections, we found that factors are interrelated with each other. For example, Beun et al. (2002) cultivated stable AGS at saturated DO condition, but when Mosquera-Corral et al. (2005) decreased DO to moderate level to optimize N removal, the granules began to disintegrate and biomass washout occurred. de Kreuk et al. (2005) adopted anaerobic feeding mode to select slow-growing bacteria as the anaerobic core and found that the anaerobic core made high local shear less critical. The influencing factors showed a strong relationship and interconnection, and single factor consideration would lead to neglect of the integrity, resulting in practical operation failure. In this study, strategies were proposed to improve aerobic granule formation and N removal by optimizing feast–famine condition. Anaerobic granule has caught much attention and achieved great successful application around the world with their mature granule stability through advanced processes development including upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB). It is proposed that the anaerobic core in aerobic granule is of great importance for AGS cultivation as the experience of the successful anaerobic granular sludge cultivation. Although AGS is generally mentioned aerobic, the physical anaerobic core within AGS and operational alternative anaerobic/aerobic control demonstrated "anaerobic-in-aerobic" with feast–famine condition optimization. Due to the high interactive influence between AGS cultivation factors under various wastewater characteristics, this research tried to interpret the various options and accordingly provided strategies to improve AGS stability.

Nitrogen removal performance in AGS was evaluated as well. Anoxic and oxic phases were normally exerted in different compartments for nitrification and denitrification in the traditional N removal process. Due to the mass transfer resistance in compact AGS, anoxic zone existed in the inner part providing conditions for simultaneous nitrification and denitrification (SND). The impact factors for efficient N removal included DO (de Kreuk et al., 2005), particle size (de Kreuk et al., 2005), granule structure (Chen et al., 2011), operation mode (Chen et al., 2011), substrate, and so on. It was vital to control DO to balance the oxic zone for nitrification and anoxic zone for denitrification (Beun et al., 2001) with integrated consideration of particle size. Operation modes such as alternating oxic–anoxic mode (Adav et al., 2009), continuous or on/off aeration with controlled DO for SND (Adav et al., 2009) and so on were developed to enhance denitrification rate and alternating nitrification and denitrification (AND) process in AGS.

The carbon source is of vital importance to the selected slow-growing bacteria in AGS due to the availability of volatile fatty acids (VFA) (He et al., 2018). The application of AGS in treating wastewater containing high proportion particulate organic carbon has been studied in lab-scale or pilot-scale SBRs (Wagner et al., 2015). During the long-term SBR operations, the AGS process gradually becomes less stable with filamentous outgrowth on the surface, leading to biomass washout and reactor failure. The hydrolysis process of the particulate organic matter becomes the rate-limiting step for their conversion to readily biodegradable substrate that can serve as a necessary carbon source for denitrification or biological phosphorus removal (Kreuk et al., 2010; Wagner et al., 2015). Some substrates, such as alcohol, cannot be converted into storage polymers in the anaerobic feeding period, and the remaining alcohol in the liquid phase was converted aerobically (Pronk et al., 2015). The glucose- and acetate-fed granules were cultivated and compared under the fast feeding mode instead of the anaerobic feeding mode (Tay et al., 2013). AGS fed by molasses wastewater under aerobic condition has been cultivated successfully but failed after 130 days operation (Morgenroth et al., 1997). Little is known about the feature of glucose-fed or overload glucose-fed AGS under anaerobic feeding condition.

In this research, the effects of the substrate (acetate and glucose) and feeding mode (fast feeding mode and anaerobic plug-flow feeding mode) on AGS formation and stability, biomass concentration, particle size distribution, and N removal efficiency, including microbial community variation were revealed and analyzed. Based on the comprehensive analysis, strategies to improve AGS formation and stability were discussed and proposed with insights into the relationship between various impact factors and stable AGS cultivation, and systemic operation methods for various complicated wastewater treatment.

1. Materials and methods

1.1. Experimental devices

Three identical SBRs labeled R1, R2, and R3 were used with a total volume of 3.0 L and an internal diameter of 80 mm each. The schematic diagram of the SBR system for AGS cultivation is shown in Fig. 1.

The reactors contained a fine bubble aerator in the bottom and the air upflow velocity was controlled at about 1.0 cm/sec by a gas flowmeter. DO in the bulk varied along the operation due to the biomass concentration change, and was kept between 1.8 and 4.2 mg/L in three reactors. The influent pH was controlled at 7.2 ± 0.8. The reactors were operated at room temperature, of approximately 25°C, The volume exchange ratio (VER) was set as 50%, and hydraulic retention time (HRT) was 6 hr. The OLR varied from 1.6 kg-COD/(m^3·day) (period 1, from day 1 to 41) to 3.6 kg-COD/(m^3·day) (period 2, from day 42 to 102) to accelerate AGS formation during operation. The systems operated cyclically using a programmable logic controller (PLC).

Sodium acetate was fed for R1 and R2 while glucose for R3 (counted as COD): 400 mg/L for period 1 and 900 mg/L for period 2. 26 mg/L NH_4^+-N, 20 mg/L $K_2HPO_4·3H_2O$, and 10 mg/L KH_2PO_4 were added as N and P source for period 1 and the influent N and P concentrations increased with increasing OLR accordingly. The following elements remained the same: $MgSO_4·7H_2O$: 25 mg/L, $CaCl_2·2H_2O$: 20 mg/L, KCl: 30 mg/L. 1 mL/L trace element was added and the composition was (mg/L): $CuSO_4·5H_2O$ 50, H_3BO_3 50, $MnSO_4·H_2O$ 50, $Na_2MoO_4·2H_2O$ 10, $ZnCl_2·7H_2O$ 10, $CoCl_2·6H_2O$ 50 (de Kreuk et al., 2005).

The inoculum activated sludge was taken from the aeration tank in Shahe Wastewater Treatment Plant, Beijing, China. The mixed liquor suspended solids (MLSS) concentration of the inoculum was 6.0 g/L, and the mixed liquor volatile suspended sludge (MLVSS) concentration was 3.9 g/L.

1.2. SBR procedures

To elucidate the influence of the anaerobic plug-flow feeding duration on granule formation, 5 min of anaerobic plug-flow feeding followed by the aeration mode (referred to as aerobic feeding mode hereafter) and 60 min of anaerobic plug-flow feeding mode (referred to as anaerobic feeding mode hereafter) were compared in R1 and R2 with sodium acetate as the sole carbon source. Sodium acetate and glucose was compared in R2 and R3 under anaerobic feeding mode as shown in Table 1.

1.3. Batch experiments

To evaluate the effect of DO on N removal of aerobic granule with a comparison to traditional activated sludge (AS), batch experiments were conducted for 2 hr in a total volume of a 1.0-L bottle with various air flow rates of 0.5, 1, 2, and 3 L/min. The corresponding DO were about 1.5 ± 0.3, 2.5 ± 0.7, 4.6 ± 0.7, and 6.3 ± 0.8 mg/L, respectively. The granules were taken from R2 (day 90), and AS were from the same WWTP where the AGS inoculum was from. The sludge had been washed by deionized water three times to remove residual organic matter or nutrient. Stirring speed of 50 r/min was applied to augment mass transfer without destructing the granules. The biomass concentration in all bottles was controlled at about 5 g/L. Sodium acetate and NH_4Cl were dosed to create the initial COD and NH_4^+-N concentration 300 and 50 mg/L, respectively. Samples were taken at an interval of 15 min for COD and N concentration analysis throughout the reaction.

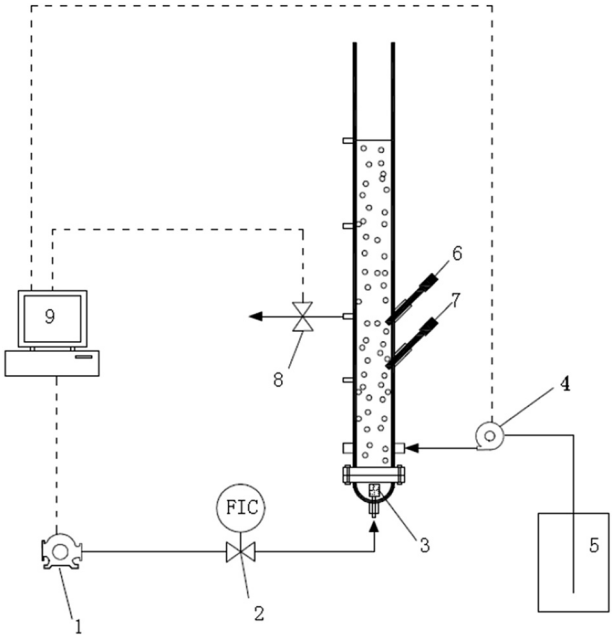

Fig. 1 – Schematic diagram of the SBR system for AGS cultivation (1) Aeration machine, (2) Airflow controller, (3) Bubble stone, (4) Influent pump, (5) Influent tank, (6) DO probe, (7) pH probe, (8) Effluent valve, (9) programmable logic controller (PLC).

To study the effect of a layered structure on N removal efficiency, the granules taken from R2 at day 90 were crushed manually with an average particle size of 60 μm. Three bottles contained the granules from R2, the crushed granules, and the AS with biomass concentration of 5 g/L. The air flow rate was controlled at 1 L/min. The initial COD and N concentration were 300 and 50 mg/L, and the samples for COD and N analysis were taken at an interval of 15 min throughout the reaction.

1.4. Measurements

MLSS, MLVSS, COD, TN, NH_4^+-N, NO_2^--N, and NO_3^--N were measured according to the standard methods (APHA 1998), while particle size distributions were measured by sieving the granules using 0.5, 1.0 1.5 and 2 mm sieves. Only 50 mL of mixed sludge was taken from reactors to avoid turbulence. The sludge retention time (SRT) for R2 and R3 was calculated using the biomass concentration in the reactor and the effluent. The morphology of aerobic granules was observed by using scanning electron microscope (SEM) with pretreatment procedures described by Tay et al. (2001), including fixing, washing, dehydration, and drying with the final step of sputter-coated with aurum.

Table 1 – Operating parameters of the reactors.

Reactor	Procedures					Substrates
	Influent (min)	Aeration (min)	Settling (min)	Withdrawal (min)	Cycle (hr)	
R1	5	165	5	5	3	Sodium acetate
R2	60	110	5	5	3	Sodium acetate
R3	60	110	5	5	3	Glucose

To evaluate microbial community variation during aerobic granule formation, the inoculum sludge and granules in R2 at day 100 were compared. Granule samples in R2 were taken during the aeration phase to obtain a representative population present in the reactor.

The total genomic DNA of sludge samples was extracted using a Powersoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA), and amplified using primer set 341F (5′-CCTACGGGNGGCWGCAG-3′)/805R (5′-GACTACHVGGGTATCTAAT-3′) under the following conditions: 94°C for 3 min; 5 cycles of 94°C for 30 sec, and 45°C for 40 sec, followed by 65°C for 30 sec; 20 cycles of 94°C for 20 sec, and 55°C for 20 sec, followed by 72°C for 30 sec with a final extension 72°C for 5 min. The polymerase chain reaction products were saved for Illumina MiSeq sequencing (Shanghai Sangon Biotech Co., Ltd).

1.5. Biomass yield rate calculation

A linear fitting analysis was conducted to calculate the biomass growth rate using the software Origin 8.0. Since no external sludge and little effluent suspended sludge (SS) were discharged during operation, the slopes of fitting lines were used to characterize the sludge growth rate, and the unit was gVSS/(L·day). The proportion of COD used for sludge growth was calculated according to

$$Y = \frac{S \times C_{COD/biomass} \times V}{(C_{in} - C_{out}) \times Q} \times 100\% \quad (1)$$

In which S (g-VSS/(L·day)) is the slope of the fitting line, $C_{COD/biomass}$ (1.42 g-COD/g-VSS) is the stoichiometric coefficient of COD-biomass (Chuang and Ouyang, 2000), V (L) is the reactor volume, C_{in} (g/L) and C_{out} (g/L) are the influent and effluent COD concentration, and Q (L/day) is the flow quantity of the reactor.

2. Results

2.1. Granule morphology

The morphological differences between granules in the three reactors are shown in Fig. 2. The granules in R1 displayed a fluffy, irregular and loose-structured morphology (Fig. 2a and d), while the matured aerobic granules in R2 had a regular and spherical outer shape (Fig. 2b and e). The feeding mode had a substantial impact on granule morphology. The aerobic granules in R3 had a regular shape surrounded by filamentous structure (Fig. 2c and f). The presence of glucose caused a sharp proliferation of such structures. AGS in R2 and R3 with extended anaerobic duration had more compact structures than that of R1.

After one week cultivation, granules firstly appeared in R1. Another week later, granules formed in R2 and R3 as well. The biomass growth and size distribution in R1 were considerably different from R2 under different feeding modes (Fig. 3). The initial MLVSS in R1 and R2 were higher than 5 g/L and dropped rapidly to less than 3 g/L due to the limited settling time. The biomass concentration remained stable in the period 1. When the influent COD increased at day 42, MLVSS in R1 and R2 also started to increase. The biomass concentration in R2 gradually increased, showing a linear-like trend despite the twice drops during period 2 which were accidentally caused by improper reactor cleaning, and the MLVSS reached 12.2 g/L at day 102. The size distribution was tested at day 50, and more than 50% of granule ranged between 1.0 and 1.5 mm. While R1 showed the opposite behavior. The granules in R1 were bigger than those of R2, and the distribution was predominantly bigger than 2 mm or smaller than 0.5 mm. The bigger granules began to break into pieces, and the small flocs were washed out at day 51, and the biomass concentration began to decrease to 5 g/L or lower due to the granules breakage. The feeding mode had a strong influence on the biomass concentration and granule size distribution. The carbon source also affected granule formation. MLVSS in R3 fed by glucose had a similar tendency as R2 fed by sodium acetate, but when OLR increased to 3.6 kg/(m^3·day) at period 2, the biomass concentration in R3 grew much more rapid than that of R2. MLVSS in R3 reached 22.4 g/L at day 100. A linear fitting was conducted for R2 and R3 during day 42 to day 102, and the slope indicated the biomass growth rate of 0.097 gVSS/(L·day) for R2 and 0.288 gVSS/(L·day) for R3. The particle size distribution in R3 was similar to that of R2, and the granules in the range between 1.0 and 1.5 mm accounted for more than 60%. Both the feeding mode and carbon source strongly affected the biomass concentration, and feeding mode also had a strong influence on the particle size distribution.

2.2. COD and N removal efficiency

COD and N removal efficiencies were evaluated during the operation of all three reactors (Fig. 4). The effluent COD in all reactors were lower than 60 mg/L, regardless of influent COD fluctuation (365–550 mg/L for period 1 and 801–979 mg/L for period 2). COD removal rate exceeding 90% could be easily achieved despite granules shape or cultivation mode, and all the granules formed under different conditions demonstrated excellent COD removal efficiency. The feeding modes and carbon sources investigated had no remarkable effects on COD removal.

NH_4^+-N and TN removal efficiency in three reactors were evaluated. All the effluent NH_4^+-N in three reactors were nearly 0 mg/L, and the removal rates were nearly 100% (Fig. 4b). However, the granules showed distinct differences in TN

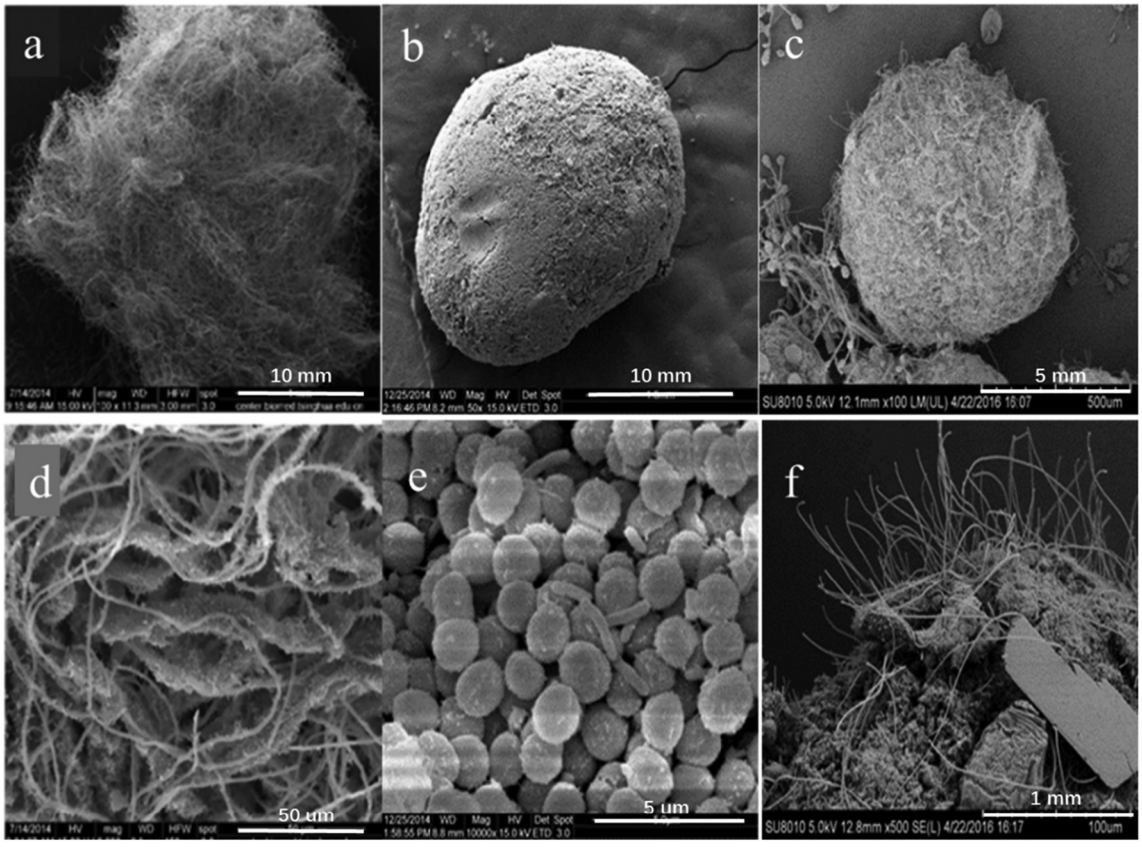

Fig. 2 – Granule morphology in three reactors (a) and (d) for granules in R1, (b) and (e) for granules in R2, and (c) and (f) for granules in R3.

removal efficiency (Fig. 4c). The effluent TN concentration in R2 was always less than 7 mg/L except for the sudden influent TN concentration increase at day 42, reaching an excellent TN removal rate of 91.7% ± 4.1% in period 2. NO_3^--N was the main N form in the effluent of three reactors. Compared to R2 with the anaerobic feeding mode, the effluent TN concentration in R1 averaging 20.8 mg/L was much higher, and the removal rate was 58.8% ± 7.4%. The TN removal in R3 fed by glucose showed a similar tendency as that of R2 at the beginning, but appeared higher effluent TN concentration afterward, resulting in a lower removal efficiency (67.3% ± 15.2%) than that of R2 fed by sodium acetate. Evidently, the long anaerobic duration (R2) achieved much better TN removal efficiency than the short one (R1). It seems that the alternating aerobic and anoxic condition by the extended anaerobic duration enhances the denitrification process in R2. However, SND process was also reported as an important factor for the granules efficient N removal. To figure out the dominant process in granules in R2 between AND and SND, N removal efficiency were tested at various DO without anaerobic period in Section 2.3.

2.3. N removal at variable DO in AGS relative to AS

In order to elucidate the importance of SND process of AGS in R2, batch experiments were conducted for 2 hr with initial COD and NH_4^+-N concentration of 300 and 50 mg/L respectively with four different air flows. In order to eliminate AND process, no anaerobic period was introduced and the DO was recorded in 2 hr. Table 2 shows the NH_4^+-N and TN removal rate.

The AGS showed stable NH_4^+-N and TN removal efficiency exceeding 92% with different air flows, while AS showed increased NH_4^+-N removal rate but a tendency of rising up at the beginning and declining in late for TN. The higher DO concentration (<4.6 mg/L) in the bulk promoted conversion of NH_4^+-N to NO_3^--N, providing the substrate for denitrification process. However, DO exceeding 4.6 mg/L created a smaller anoxic area for denitrification in the suspended sludge system, and TN removal rate dropped. Whereas the various DO did not have much effect on the granule system. AGS still showed excellent N removal efficiency without the anaerobic period for denitrification, indicating that SND was more crucial than AND in AGS in R2, and the compact layered structure of the AGS in R2 was supposed to ensure SND process (He et al., 2016). In order to testify this hypothesis, N removal efficiency of the crushed granules was compared with the complete AGS and AS by batch experiments.

As illustrated above, the COD and N removal efficiencies of complete AGS from R2 (Fig. 5a), crushed AGS from R2 (Fig. 5b), and AS (Fig. 5c) were compared under the same condition. DO in the reactors varied between 1.5 and 3.0 mg/L. Both the granules and AS showed similar and favorable COD and NH_4^+-N removal efficiencies. However, the details of TN removal

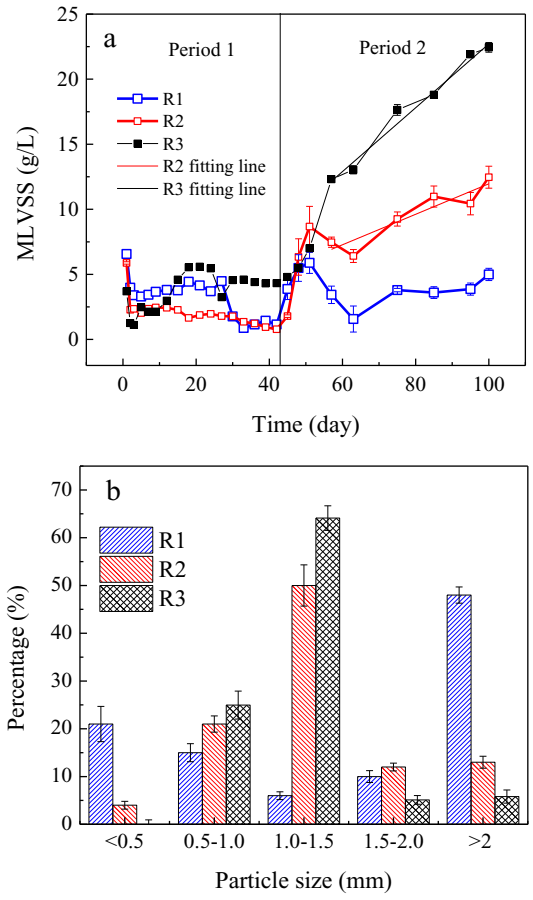

Fig. 3 – MLVSS (a) and particle size distribution at day 50 (b) of various reactors.

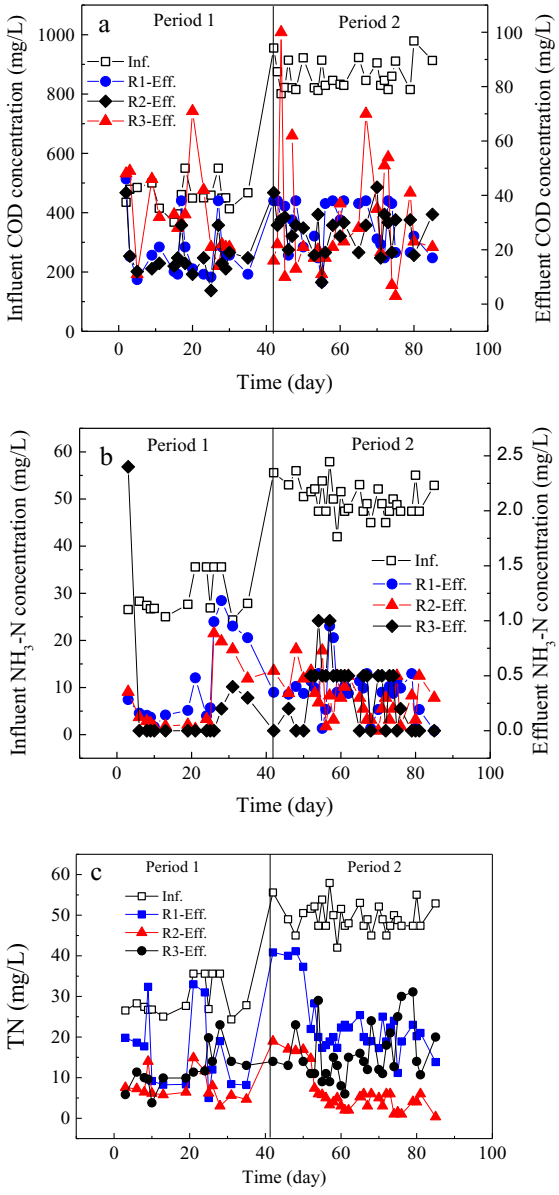

Fig. 4 – COD (a), NH_4^+-N (b) and TN (c) removal efficiency in the reactors.

were substantially different. The NO_3^--N in the complete AGS was always lower than 1 mg/L during the entire reaction process, and the effluent TN concentration was less than 1.6 mg/L and removal rate higher than 96%. The NO_3^--N concentration in the effluent increased to 9.3 mg/L when the granules were crushed and the inner anoxic-environment structure damaged, and the TN removal rate decreased to 80%. The effluent TN concentration in the AS system was maximum (15 mg/L) with less than 70% removal rate, and NO_3^--N was the main N form in the effluent, indicating a lower capacity of denitrification in relation to AGS. Compared with the AS (as shown in Fig. 5b), the crushed granules with similar particle size had a higher TN removal rate. Section 2.4 illustrates microbial communities of sludge as there might be some microbiota differences in both types of sludge.

2.4. Microbial community

High-throughput sequencing was employed to explore the microbial population dynamics over granulation. Table 3 shows the basic diversity analysis for the AS and the granules in R2.

A total of 10,158 and 17,487 sequences were retrieved from the AS and AGS in R2, respectively. 942 operational taxonomic units (OTUs) were clustered in the AS and 1975 OTUs in the R2, indicating a higher community richness and diversity in R2 after granulation. The richness indices ACE and Chao1 showed a similar tendency. Higher Shannon index in R2 indicated higher community diversity. The increased community richness and diversity may be due to the abundant biomass concentration obtained by the granules.

The phylogenetic classifications of functional groups at the genus level were analyzed. As shown in Table 4, we found 2, 2, and 24 kinds of ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and denitrifying bacteria (DNB) respectively. As shown in Table 6, the relative abundance and sequence number of house-keeping genes of AOB and NOB in R2 increased sharply during granulation. The DNB sequence number increased from 2360 to 3237, and the diversity of DNB increased as well. *Paracoccus*, *Azoarcus*, and *Thauera* were the dominant DNB in AS, accounting for 72.4% of

Table 2 – COD and N removal by complete aerobic granule and traditional activated sludge at variable air flow.

Air flow (L/min)		0.5	1	2	3
DO (mg/L)		1.5 (0.3) *	2.5 (0.7)	4.6 (0.7)	6.3 (0.8)
AGS in R2	NH_4^+-N (%)	93.4 (0.4)	98.1 (0. 5)	99.7 (0.4)	99.8 (0.1)
	TN (%)	92.2 (0.8)	96.6 (0.4)	95.8 (0.7)	92.9 (0.9)
AS	NH_4^+-N (%)	85.2 (0.7)	97.9 (0.3)	99.2 (0.2)	99.6 (0.1)
	TN (%)	58.4 (2.9)	61.6 (2.5)	63.8 (1.6)	54.9 (4.6)

* Standard deviations shown in brackets.

Table 3 – Alpha indices for samples from AS and R2.

Samples	Sequences	OTU	Shannon	ACE	Chao1
AS	10,158	942	4.6	3774.3	2222.4
AGS in R2	17,487	1975	6.2	4661.5	3726.3

the total DNB in the AS (Table 5). The AGS in R2 showed more uniform distribution of DNB, and the dominant genus shift from *Paracoccus*, *Azoarcus* and *Thauera* to *Denitratisoma*, *Novosphingobium*, *Planctomyces*, *Ferruginibacter* (Liu et al., 2018;

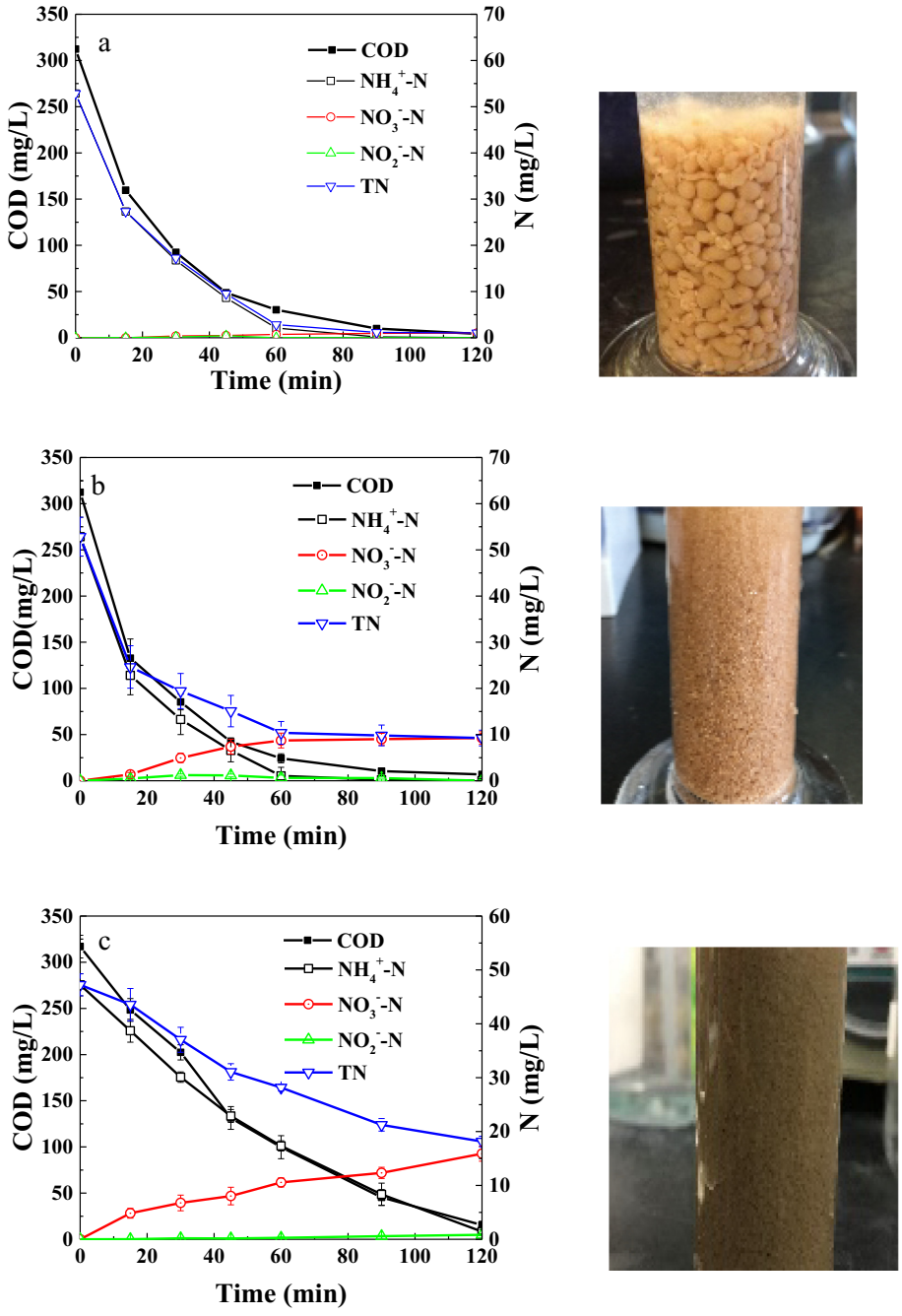

Fig. 5 – COD and N removal comparison of batch experiments between complete AGS (a), crushed AGS (b) and AS system (c). The glass cylinder showed in the pictures is 100-mL graduated cylinder with 28 mm internal diameter.

Table 4 – The key functional groups involved in nitrogen removal at the genus level.

Key functional groups at genus level		Relative abundances (%)	
		AS	R2
AOB	*Nitrosomonas*	0.02	0.12
	Nitrosospira	0.10	0.95
NOB	*Nitrobacter*	0.00	0.01
	Nitrospira	0.02	4.37
DNB	*Comamonas*	0.02	0.47
	Acidovorax	0.12	0.25
	Aeromonas	0.00	0.06
	Arcobacter	0.00	0.14
	Azoarcus	4.76	0.09
	Azospira	0.03	0.45
	Bacillus	0.01	0.01
	Denitratisoma	0.03	2.28
	Flavobacterium	2.85	0.01
	Novosphingobium	0.00	1.66
	Rhodobacter	2.96	1.85
	Thauera	7.06	1.10
	Zoogloea	0.00	0.11
	Paracoccus	5.00	1.33
	Planctomyces	0.02	1.34
	Ferruginibacter	0.01	2.85
	Hyphomicrobium	0.04	1.70
	Steroidobacter	0.02	2.62
	Klebsiella	0.01	0.03
	Eubacterium	0.00	0.07
	Rhizobium	0.23	0.01
	Delftia	0.03	0.01
	Stenotrophomonas	0.02	0.00
	Thiobacter	0.01	0.07
DPAO	*Dechloromonas*	0.14	0.61
	Pseudomonas	0.06	0.06
	Bacillus	0.01	0.01
	Stenotrophomonas	0.02	0.00
	Serratia	0.07	0.10
	Hyphomicrobium	0.04	1.70
	Aminobacter	0.01	0.12

Hu et al., 2012), *Hyphomicrobium, Steroidobacter*, and *Zoogloea* (Reino et al., 2016; Fra-Vazquez et al., 2016).

An obvious increase of denitrifying phosphorus accumulation organisms (DPAO) was observed in R2 relative to the AS, from 0.35% to 2.6% of relative abundance, and from 36 to 455 of the sequence number (Table 5). DPAO was important for AGS formation and N removal, and the dominant DPAO were *Hyphomicrobium* and *Dechloromonas* in R2.

The major shift in bacteria assemblage observed during granulation indicated that the microbial population in the AS was distinctly different from that in mature granules. The increased AOB, NOB, DNB, and DPAO may also result in higher N removal efficiency in the crushed granules than the AS.

3. Discussion

3.1. Extending anaerobic period for anoxic zone formation in AGS

Nitrification is an easy step to achieve in case of sufficient aeration during N-removal process, while denitrification is normally a rate-limiting step due to the deficient anoxic condition and/or carbon source supply. Denitrification occurs either in the anoxic zone inside single granules (SND) or anoxic condition (phase) provided in some parts of an entire cycle (AND). The loose structure may be in favor of DO penetration compared with the compact structure, therefore leading to a less anoxic zone for denitrification. Compared with the AGS in R1, the extended anaerobic period in R2 may have two effects on N removal: enhancing the AND process (Chen et al., 2011) and favoring the compact structure formation which facilitates the SND process. The batch experiment results revealed that exceeding 90% TN removal rate can be achieved easily by the AGS in R2 at various DO conditions. Without the anoxic period in the batch experiment, the effect of AND process can be eliminated. When the AGS in R2 were crushed, the TN removal rate decreased from 96% to 80%. Evidently, SND process had a more dominant influence on N removal in the AGS. The compact granule structure is an important factor to influence the nitrification rate and the denitrification rate due to the mass transfer resistance apart from granule size (Kishida et al., 2006) and facilitates SND process in the AGS. The extended anaerobic duration favored anaerobic zone in aerobic granule, leading to AGS formation with compact structure, and then enhanced the SND process. The granule cultivated in anaerobic feeding mode fed by acetate in R2 was mainly composed of DPAO, AOB, and NOB, which are the key functional group in AGS (Kreuk et al., 2007). The anaerobic feeding mode prolonged anaerobic duration and the acetate would be taken in by DPAO, therefore promoted DPAO proliferation, and created a compact anaerobic core in aerobic granular sludge. The enriched AOB, NOB, DNB, and DPAO during granulation also contributed to the efficient N removal.

3.2. Stimulating filamentous and heterotrophic bacteria growth by glucose

As shown in Section 2.1, the slope of the fitting line in R2 and R3 were 0.097 and 0.288 gVSS/(L·day), the average influent

Table 5 – Relative abundance and OUT numbers of the key functional groups involved in nitrogen removal.

Key functional groups at the genus level	AS		AGS in R2	
	Relative abundance (%)	Sequences	Relative abundance (%)	Sequences
AOB	0.12	12	1.07	187
NOB	0.02	2	4.38	766
DNB	23.23	2360	18.51	3237
DPAO	0.35	36	2.60	455

Table 6 – COD distribution for R2 and R3.

Rectors	Items	Influent	Effluent	Removal (in CO_2 form)	Biomass yield
R2 (sodium acetate)	Mass flow (gCOD/day)	10.35	0.32	9.62	0.41
	Mass distribution (%)	100.00	3.10	92.90	4.00
R3 (glucose)	Mass flow (gCOD/day)	10.35	0.36	8.76	1.23
	Mass distribution (%)	100.00	3.50	84.60	11.90

COD concentration during day 42 to day 102 was 863 mg/L, and effluent COD for R2 and R3 were 26.9 and 29.7 mg/L with 6 hr HRT, respectively. According to Eq. (1), the COD used for biomass yield and metabolism (considered as removal in CO_2 form) can be calculated, and the result is shown in Table 6.

Due to the long SRT applied in R2 and R3 (both were about 60–120 days), the biomass yield (0.04 gCOD/gCOD for R2 and 0.12 gCOD/gCOD for R3) was quite lower than the reported yield of 0.33 gCOD/gCOD on starch with the biomass concentration of 1.8 gVSS/L and SRT of 10 days (Kreuk et al., 2010). Compared with the starch, substrate such as acetate or glucose could remain a higher biomass concentration and a longer SRT in the reactor. According to Kang and Yuan's (2017) study, the proper food to microorganism (F/M) was necessary to maintain AGS property. Due to the high biomass concentration, the F/M in R3 was much lower than that of R2. The lower F/M may be one of the reasons for the lower N removal rate in R3 due to the insufficient carbon source for denitrification.

The COD used for biomass yield in R3 was three times of that of R2, indicating that the biomass concentration fed by glucose grew much faster than that by acetate. Compared with glucose, acetate is more widely used for microbial metabolism. Sometimes, glucose or other non-volatile fatty acids (nVFAs) carbon source may have to be hydrolyzed to VFA before utilized by the microorganism. As illustrated by Kreuk et al. (2007) in the AGS model, the granule cultivated in anaerobic feeding mode fed by acetate was mainly composed of DPAO, AOB, and NOB, and VFA was the only carbon source for DPAO. When the glucose concentration was high enough to exceed the hydrolysis rate, the hydrolysis process would limit the substrate conversion. The glucose would accrete fast-growing heterotrophic bacteria growth, resulting in the sharp increase of biomass concentration and filamentous bacteria growth, and finally leading to the AGS instability.

3.3. Strategies for stable AGS formation and operation

To obtain stable AGS, a systemic strategy tree was proposed based on the present study and the references (Fig. 6). The wastewater characteristics and operational options were analyzed aiming at the complicated and various wastewater, including organic strength, substrate characteristics, feeding mode, shear force, and selective pressure, which were widely studied by other researchers.

According to the organic strength of wastewater, the strategies varied to the longer cycles for high organic strength wastewater and the shorter cycles for low organic strength wastewater to achieve the balance of feast period and famine period (Corsino et al., 2018). Longer cycles would provide a longer famine period and a bigger famine to feast period ratio, which was favorable for the development of bacteria with low growth rates (0.5/day) and inhibited the fast-growing microorganisms. According to the substrate characteristics, the substrate can be divided into refractory and easily

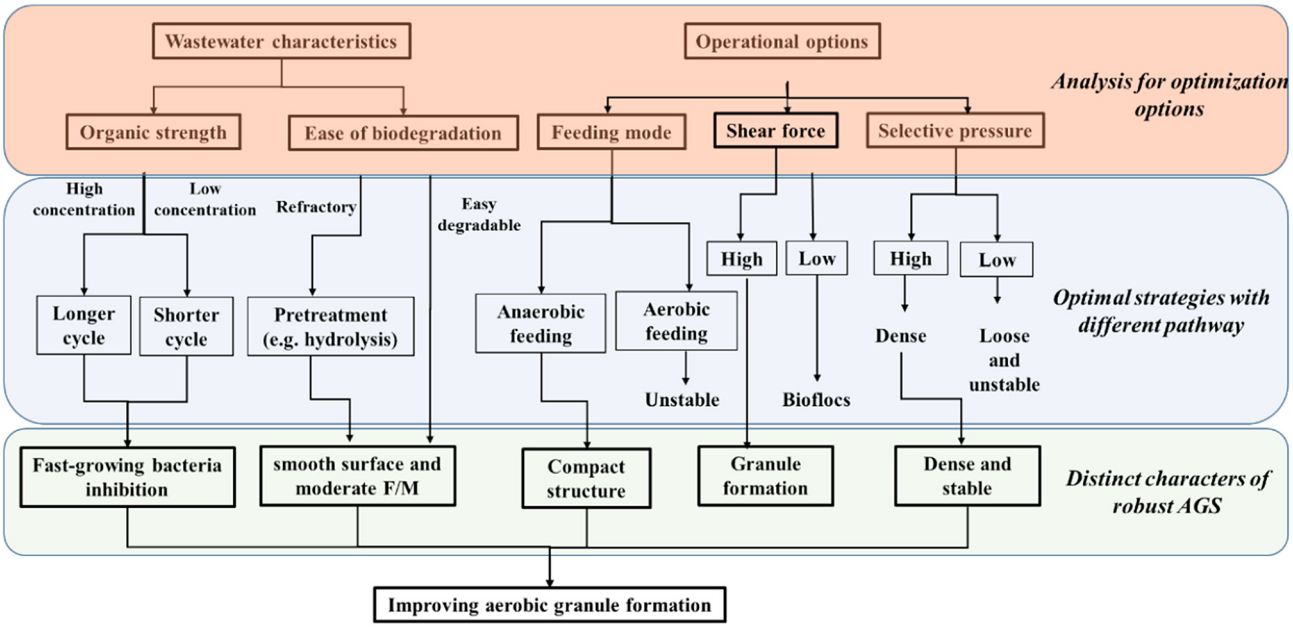

Fig. 6 – A strategy tree for stable AGS cultivation and operation.

degradation parts. Due to the limited hydrolysis process in the AGS, the refractory substrate always leads to the filamentous structure and finally resulted in AGS instability. Accordingly, a pre-hydrolysis process seems helpful to enhance the limited step, converting the refractory substrates into VFAs to inhibit filamentous bacteria growth and obtain a smooth surface and a moderate F/M for efficient N removal. As shown in the above results, feeding modes had a great effect on AGS structure. The anaerobic feeding mode cultivated the AGS with compact structure, while the aerobic feeding cultivated the AGS with a loose structure and finally broke up. The high shear force was a key factor for AGS formation, and that was the reason for traditional sludge remained in flocs for more than 100 years. The higher selective pressure controlled by the shorter settling time would select granules with a dense structure and washout the granules with bad setting ability.

4. Conclusions

In this study, a systemic strategy was proposed to improve aerobic granule stability and N removal. The granule stability, N removal efficiency and microbial dynamics of AGS during formation under different anaerobic durations and substrates were evaluated. The results showed that the anaerobic feeding mode favored AGS formation with a compact structure, while the aerobic feeding cultivated AGS with a loose structure at moderate DO. SND was the dominant process for efficient N removal in the compact AGS over AND. High-concentration glucose would accelerate fast-growing bacteria growth, leading to a sharp increase of biomass concentration in the system due to the limited hydrolysis rate, and the lower F/M may be the reason for decreased N removal efficiency. The relative abundance and sequence number of the key functional bacteria groups involved in N removal including AOB, NOB, DNB, and DPAO increased greatly during granulation and favored the stability and efficient N removal in the AGS. The systemic strategy based on wastewater characteristic and operation options was proposed for insights into the relationship between various influencing factors and complicated wastewater.

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