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Stability of aerobic granular sludge under condition of low influent C/N ratio: Correlation of sludge property and functional microorganism

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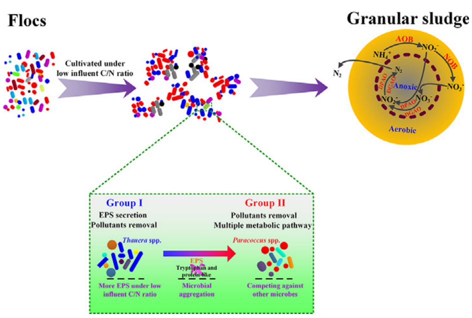
GRAPHICAL ABSTRACT



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ABSTRACT

Aerobic granular sludge process treating domestic wastewater with low C/N ratio is necessary to be studied for rapid urbanization in China and other countries. In this study, two parallel reactors with different influent C/N ratio (15 in R1, 5 in R2) were established. Compared to the disintegrated granule in R1 with high influent C/N ratio, granules with large size (650 μm) and compact structure (integrity coefficient < 0.1) were stable in R2 along with influent C/N ratio decreased to 5. High-through sequencing illustrated the functional microbes like *Thauera* and *Paracoccus* enriched under low influent C/N ratio, and principal component analysis further showed these microbes were positive correlation with tryptophan and protein-like substances in extracellular polymeric substances (EPS) and granular strength. It was indicated that under low influent C/N ratio, several resistant microbes like *Thauera* (19.5%) enriched and then secreted tryptophan and protein-like substances, and stable granules with multi-functional microbes could be formed finally.

1. Introduction

In recent years, the influent of most municipal wastewater treatment plants in China and other countries shows the characteristics of low ratio of carbon to nitrogen (C/N). As described by previous study (Sun et al., 2010; Pronk et al., 2015; Derlon et al., 2016), the C/N ratio of most typical influent in domestic wastewater plants is about 10.5–12.5 in the world. In recent decades, lots of domestic wastewater

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treatment plants are accepting part of industrial wastewater, so their influent has the C/N ratio lower than 5 (Su and Yu, 2005; Sun et al., 2010; Ye et al., 2011; Wang et al., 2014; Liu et al., 2015; Meng et al., 2015; Hao et al., 2016). On the other hand, the gradually increment of effluent quantity from wastewater treatment plants makes it necessary to denitrify with low organic carbon demand or external carbon sources added. Thus, lots of emerging technologie such as partial nitrification and denitrification and anammox appear in recent decades (Oshiki et al., 2013; Zhang et al., 2017; Zhao et al., 2018b).

As we know, the biological denitrification efficiency is highly related to the DO, temperature, type of carbon source and other factors. Among them, C/N ratio could be a crucial complex factor in the operation of biological wastewater treatment system as described by previous study (Sun et al., 2010; Ye et al., 2011; Hu et al., 2014; Luo et al., 2014; Wang et al., 2014; Wang et al., 2018) The microorganism species in biological denitrification are essential for total nitrogen (TN) removal. The low ratio of carbon to nitrogen (C/N) could enrich the nitrifying bacteria and increase the tolerance of microbes to high-level free ammonia (Huang et al., 2014; Meng et al., 2015; Ferhan and Başak, 2016; Zhao et al., 2018a). Moreover, lots of microbes with multiple nitrogen metabolic pathways utilizing NO_3^--N or NO_2^--N as substrate for nitrogen removal are enriched under low C/N ratio (Ma et al., 2009; Wan et al., 2013; Hu et al., 2014; Wang et al., 2018). The TN removal efficiency and rate increased with decreased C/N ratio in stable biological wastewater treatment system as described by Meng et al. (2015). On the other hand, the varied influent C/N changes the component of extracellular polymeric substances (EPS) significantly, and the extracellular protein (PN) content as well as bound force of EPS increase with decreased C/N ratio in activated sludge for stability of the system (Gül and Ferhan, 2016; Sun et al., 2016; Niu et al., 2017). However, hazardous of excessive free ammonia limits the aggregation of functional microbes in wastewater treatment system could worsen the denitrification property (Park and Bae, 2009; Sun et al., 2012).

Aerobic granular sludge is a promising technology for wastewater treatment as its abundant microorganism, excellent settling ability and compact structure as well as the tolerance for fluctuant influent loading. However, the unstability of aerobic granular sludge is the emerging issue to be solved. Up to now, most of aerobic granular sludge is cultured under condition of high C/N over 8 in all scales as reported by previous study to ensure the fast formation and stability of aerobic granules (Chiu et al., 2007; Lemaire et al., 2008; Zhou et al., 2015; Zhou et al., 2016). According to the possible secretion of hydrophobic PN by the functional microbes under lower C/N ratio with nitrogen metabolic pathways (Ye et al., 2011; Wang et al., 2014; Geyik and Cecen, 2016; Hao et al., 2016) and lower influent C/N ratio of most domestic wastewater treatment plants nowadays (Sun et al., 2010; Pronk et al., 2015; Derlon et al., 2016), two parallel reactors with high and low influent C/N ratio of domestic wastewater (15 in R1, 5 in R2) were established. The objectives of this study were to investigate the effect of low influent C/N ratio on the property of granular sludge and enrichment of functional microbes. Based on the function of PN content in sludge EPS for the cellular hydrophobicity and microbial aggregation, the EPS components were analyzed qualitatively by 3D-EEM with PARAFAC, and the microbial community structure of granular sludge was analyzed by 16S rRNA high-throughput Illumina sequencing. Finally, the possible mechanism for the stability of aerobic granular sludge under low influent C/N ratio was illustrated using principal component analysis (PCA).

2. Materials and methods

2.1. Operation mode of reactors

Two identical sequencing batch reactors (SBR) namely R1 and R2, were seeded with activated sludge of 4.0 $g{\cdot}L^{-1}$ taken from a wastewater treatment plant, Hangzhou, China. Each reactor had the volume of 4 L with 10 cm in diameter and 50 cm in height, and the superficial upflow air velocity of each reactor was 1.5 $cm{\cdot}s^{-1}$. The SBR was operated with a 4-h cycle comprising 5 min feeding, 40 min idle, 185 min aeration, 5 min settling and 5 min discharge from the middle of the reactor. The dissolved oxygen (DO) was kept at 7.5–8.5 mg/L in both reactors. Moreover, the sludge retention time (SRT) of aerobic sludge in both reactors was determined by the sludge discharged with effluent, and the SRT was maintained at 6–15 days during the reactor operation.

The influent C/N ratio was set at 15 in R1 with influent COD and TN of 1000 and 65 mg/L during the whole operation. The influent C/N ratio of R2 decreased to 5 with detailed operation mode as follows: (1) During Phase I (1–37 day), the influent COD and TN was set at 1000 and 65 mg/L respectively, and influent C/N ratio was about 15; (2) During Phase II (38–57 day), the influent COD decreased gradually from 1000 to 700 mg/L and the influent TN was still at 65 mg/L, so the influent C/N ratio decreased from 15 to 10; (3) During Phase III (58–73 day), the influent COD was still at 700 mg/L while the influent TN increased from 65 to 140 mg/L, so the influent C/N ratio decreased from 10 to 5; (4) During Phase IV (74–95 day), the influent C/N ratio was constant with 5 with influent COD and TN of 700 and 140 mg/L respectively (shown in Appendix A).

Synthetic wastewater used as feeding water with C/N of 15, comprised the following (mg/L): sodium acetate, 1008.3; NH_4Cl, 185.35; KH_2PO_4, 27.17; $K_2HPO_4{\cdot}5H_2O$, 34.72; yeast, 16.15; peptone, 24.2; $CaCl_2$, 80; $MgSO_4$, 30; and a trace element solution composed of the following components: H_3BO_3, 0.05; $CuSO_4{\cdot}5H_2O$, 0.05; $ZuSO_4{\cdot}7H_2O$, 0.05; $AlCl_3$, 0.09; $CoCl_2$, 0.05; $MnSO_4{\cdot}H_2O$, 0.05; $(NH_4)_2Mo_7O_{24}$, 0.05; $NiCl_2{\cdot}6\ H_2O$, 0.09; and $FeSO_4{\cdot}7H_2O$, 0.05. The ambient temperature in the laboratory was 25 ± 2 °C. Influent C/N ratio of R2 changed via regulating the content of sodium acetate and NH_4Cl .The influent pH was 7.1 ± 0.1 during the whole operation as the pH was regulated by KH_2PO_4 and $K_2HPO_4{\cdot}5H_2O$. Accordingly, the free ammonia (FA) concentration was 0.65 and 0.65–1.52 mg NH_3/L in R1 and R2, respectively (Park and Bae, 2009).

2.2. Analysis methods

2.2.1. Water quality

The effluent of each reactor was sampled three times a week. The water was centrifuged at 4000 rpm for 10 min to test chemical oxygen demand (COD) and total nitrogen (TN) according to the standard methods (APHA, 2005). The samples for detection of NH_4^+-N, NO_2^--N and NO_3^--N were filtered by 0.45 μm glass fiber filter and tested by standard methods (APHA, 2005).

2.2.2. Analysis of sludge property

2.2.2.1. Sludge physical properties. The sludge physical properties included the mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI), and granular size. MLSS and SVI_{30} were measured according to the standard methods (APHA, 2005) three times every week. The mean size and size density distribution of aerobic granules were measured by analyzers for granular size and shape (QICPIC, Sympatec, Germany) according to the international standards (ISO, 2001), and the granulation degree was calculated as the proportion of granule with size greater than 200 μm. The granular span value was introduced to quantize the uniformity of granular size as described by Zhou et al. (2016) and calculated as follows:

$$\text{Span value} = \frac{d_{90}-d_{10}}{d_{50}}$$

where, d_{10} is the diameter of granules larger than 10% of total particle volume (μm), d_{50} is the mean diameter of granules (μm), and d_{90} is the diameter of granules larger than 90% of total particle volume (μm).

2.2.2.2. Granular strength. The granular strength was described as the integrity coefficient and measured according to Ghangrekar et al. (2005). Briefly, granules taken from reactors were added into flasks and diluted using distilled water to a final volume of 100 mL. The flasks were agitated using an orbital shaker at 200 rpm for 5 min, and then the mixed solution was put into a 150-mL measuring cylinder to separate ruptured granules. The dry weights of settled granules and residual granules in the supernatant were measured. The ratio of solid in the supernatant to total weight of granular sludge was used for the granular strength measurement, which is expressed in percentages as an integrity coefficient.

2.2.2.3. Relative hydrophobicity of granules. The relative hydrophobicity (RH) of sludge was measured as follows: 30 mL sludge mixture was sampled from reactor and was washed three times, and then was resuspended using Tris-HCl (0.05 mM, pH = 7.1). The suspension liquid was dispersed in ice bath by ultrasonic with power of 50 w for 2 min. The dispersed liquid ($MLSS_{before}$) was mixed with hexadecane and shaked for 5 min. When the two phases had separated completely (about 30 min), the aqueous phase ($MLSS_{after}$) was transferred into another beaker.

$$RH=1-\frac{MLSS_{after}}{MLSS_{before}}$$

2.2.3. Analysis of EPS components

EPS was extracted from the sludge sample using a heating method (Li and Yang, 2007). The polysaccharide (PS) content in the EPS was quantified using the phenol–sulfuric acid method with glucose as the standard (Dubois et al., 1956), and the protein (PN) content in sludge EPS was determined by a modified Lowry colorimetric method with bovine albumin serum as the standard (Lowry et al., 1951).

The excitation-emission matrix of the EPS extracted above was analyzed using a fluorospectrophotometer (Shimadzu F-4500) according to Luo et al. (2014): Scanning emission spectra was obtained from 250 to 550 nm in 5-nm increments, and the excitation wavelength varied from 200 to 400 nm in 5-nm increments. Excitation and emission slits were kept at 5 nm and 10 nm, respectively, while the scanning speed was set at 1200 nm·min^{-1}. A parallel factor (PARAFAC) analysis was employed to process the EEM data for the variation of EPS components. Raman scattering and Rayleigh scattering were eliminated according to Sheng et al. (2013). The processed EEM data was handled by MatLab R2016a (MathWorks Inc., USA) software including the non-negative constraint, estimation of loading leverage for all samples, removal of outliers, and split-half analysis and validation for a suitable number of components. The highest peak fluorescence intensity of each component was defined as F_{max} value, which are used to represent the relative concentrations of individual fluorescent dissolved organic matter components (Stedmon and Bro, 2008).

2.2.4. Analysis of sludge microbial community

The genomic DNA of granular sludge samples was extracted following the protocol of the Power Soil DNA extraction kit (MO BIO Laboratories Inc.). The total DNA extracted from sludge samples was used as a template, and the V3-V4 region of the bacterial 16S rRNA was amplified with the primers (338F 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R 5′-GGACTACHVGGGTWTCTAAT-3′). All reactions were carried out in 25 μL (total volume) mixtures containing approximately 25 ng of genomic DNA extract, 12.5 μL of PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. PCR reactions were performed in a Master Cycler Gradient thermocycler (Eppendorf, Hamburg, Germany) set to the following conditions: initial denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 10 min. The PCR products of the samples were sequenced with the Illumina MiSeq platform (PE300, CA, USA).

2.2.5. Statistical analysis

Statistical analysis between all the variables was conducted by the *t*-test using SPSS (SPSS 17.0). *p* value less than 0.05 was considered to be statistically different, and *p* value less than 0.01 was considered to be different significantly. The Principal Component Analysis (PCA) analysis was carried out using Matlab 2016a based on the results of sludge property and microbial community during aerobic sludge granulation.

3. Results and discussion

3.1. Performance of aerobic granular sludge reactor under different influent C/N ratios

The sludge concentration and settling property under different influent C/N ratio were investigated during the reactor operation. The two reactors processed in parallel during initial 37 day (Phase I), and sludge started granulation after the operation time of 12 day with granulation degree of more than 10% (Zhou et al., 2016). Moreover, the influent C/N ratio in R2 decreased. Compared with R1 operated under condition of high influent C/N ratio, the influent C/N ratio decreased via decreasing influent COD firstly for weakening the effect of high nitrogen loading on aerobic granules. Under this operation, the size distribution of granules (Fig. 1b) in R2 was gradually concentrated with the span value decreasing during Day 38–57 (Phase II), and the span value was constant at 1.5 during Day 48–57 in Phase II. At the same time, the intensity (integrity coefficient = 0.11) and hydrophobic property (RH = 0.61) of granules in R2 were significantly higher than those of R1 (integrity coefficient and RH of 0.15 and 0.56, respectively) (Fig. 1c-d).

Granules were stable gradually under decreased influent C/N ratio via decreasing influent COD during Phase II, and the influent NH_4^+-N was further increased in order to decrease influent C/N ratio from Day 58. Following this operation, the Span value of granules in R2 was decreasing continuously (Fig. 1b), and the granulation degree of sludge in R2 was constant over 80% (Fig. 1 a). By contrast, the granulation degree of sludge in R1 decreased significantly, and was less than that in R2 on Day 73 (Fig. 1a). The operation mode of decreasing influent C/N ratio via increasing influent NH_4^+-N could maintain higher granular size and uniform size distribution (Phase III; Day 58–73). Moreover, the intensity and hydrophobic property of granules in R2 were further enhanced with integrity coefficient and RH of 0.058 and 0.70 in Phase III (Fig. 1c-d). The influent C/N ratio in R2 was constant with 5 from Day 74 (Phase IV), and granules in R2 were stable with granulation degree of 87.24% while the granulation degree of sludge in R1 was less than 25% (Fig. 1a). At the same time, the MLSS in R2 (8050 mg·L^{-1}) was greater than that in R1 (6754 mg·L^{-1}) with higher MLVSS/MLSS of 0.75 at the end of Phase IV although decreased after Day 65, because of the limitation of influent NH_4^+-N at high level as shown in Fig. 1e. Granules in R2 also had excellent settling ability with lower value of SVI_5 and SVI_5/SVI_{30} (less than 30 mL·g^{-1} and 1.1, respectively) during Phase IV (Fig. 1f). Results showed that granules cultured under low influent C/N ratio (5) could achieve excellent hydrophobicity and favor the formation of granular sludge with larger size and compact structure.

As shown in Fig. 2, the COD removal efficiency was similar in two reactors before Phase IV. After then, the removal efficiencies of COD and TN in R1 decreased sharply from 99.6% and 81.5% to 94.2% and 65.3%, along with the disintegration of granular sludge. However, the removal efficiencies of COD and TN were still maintained at 99.6% and 79.5% in R2. According to the disintegration of granules in R1 during Phase IV, it was not necessary to compare the stable granules in R2 with disintegrable granules or flocs in R1 for long time.

The nitrification activity of sludge in both reactors was excellent as the NH_4^+-N removal efficiency was over 95% and effluent NO_2^--N was lower than 0.1 mg/L during the whole operation (shown in Appendix A). Interestingly, the intact granules and stable TN removal were achieved under condition of low influent C/N ratio. Fig. 2 showed the

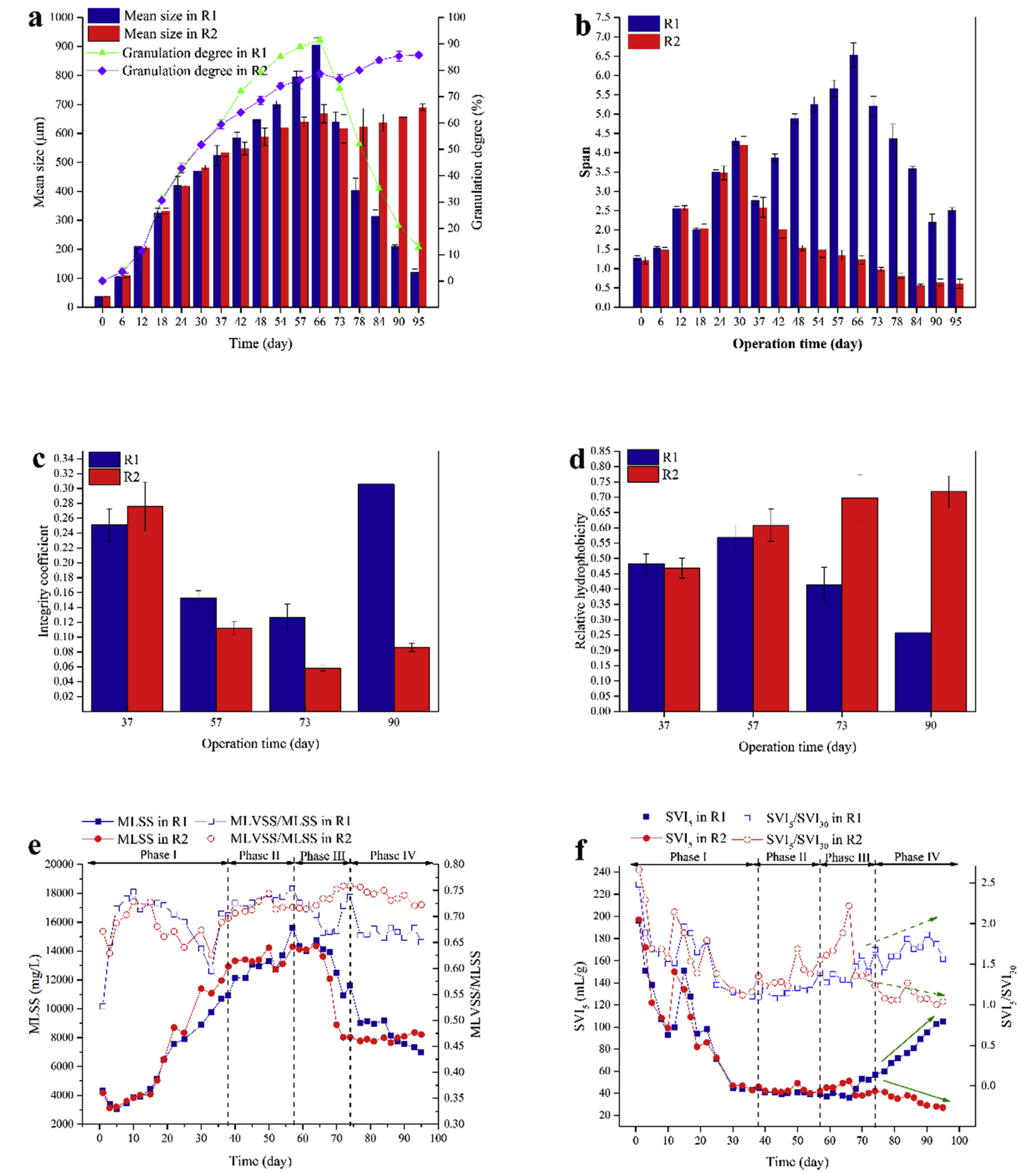

Fig. 1. Physical characteristic of aerobic granular sludge in different reactors: a. Granular size; b. Span value of granular size; c. Integrity coefficient of granular sludge; d. Relative hydrophobicity of granules; e. MLSS and MLVSS/MLSS; f. SVI_5 and SVI_5/SVI_{30}.

TN removal efficiency was about 80% at different influent C/N ratio in R2. For better indicating the possible nitrogen removal process under low influent C/N ratio, the stoichiometric formula of nitrification (Kishida et al., 2006) was used as follows:

$$55\ NH_4^+ + 76\ O_2 + 109\ HCO_3^- \rightarrow C_5H_7NO_2 + 54\ NO_2^- + 57\ H_2O + 104\ H_2CO_3,$$

$$400\ NO_2^- + NH_4^+ + 4\ H_2CO_3 + HCO_3^- + 19O_2 \rightarrow C_5H_7NO_2 + 3\ H_2O + 400\ NO_3^-$$

With the data analysis (shown in Appendix A), 59.8 mg/L of NH_4^+-N was removed on Day 30, so the NO_3^--N concentration in the effluent

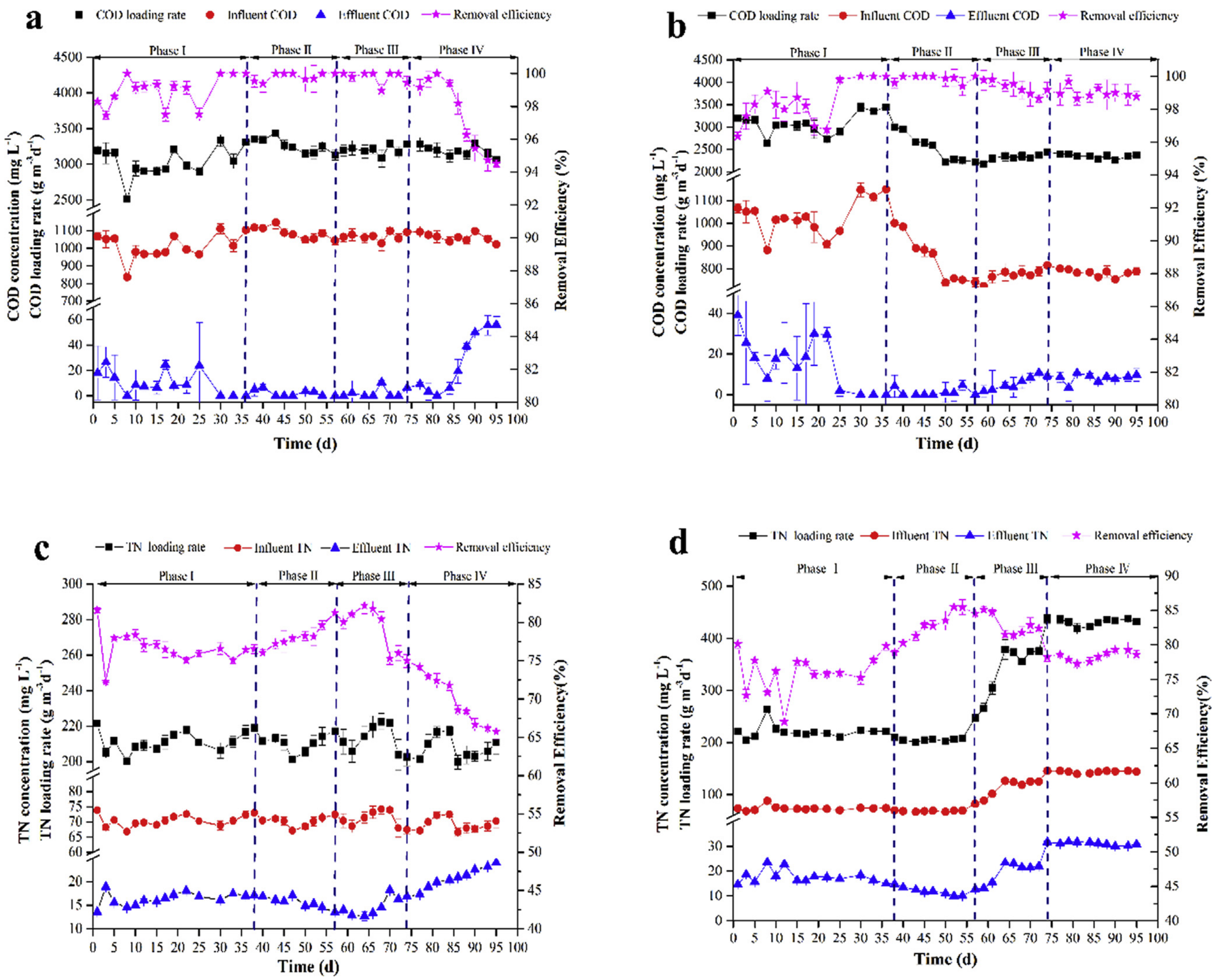

Fig. 2. Pollutant removal property of aerobic granular sludge: COD removal in R1 (a) and R2 (b); TN removal property in R1 (c) and R2 (d).

should be 58.7 mg/L according to the stoichiometric formula. However, the effluent NO_3^--N was 19.4 mg/L and TN removal amount was 45.7 mg/L in R2, it meant that the additional NO_3^--N (39.3 mg/L) was removed by denitrification (86.0% of TN removal amount). Similarly, the removal amounts of NH_4^+-N and TN were 109.4 and 105.3 mg/L on Day 85 respectively, and the effluent NO_3^--N was 29.8 mg/L. So, the additional NO_3^--N was 77.6 mg/L to be removed by denitrification (73.7% of decreased TN), which was lower than that on Day 30. The results indicated that other nitrogen oxide such as NO_2^--N was used as substrate for denitrification (Kishida et al., 2006; Sun et al., 2010; Meng et al., 2015; Wang et al., 2018). Moreover, the pollutant removal process of R2 during the SBR cycle changed obviously with decrease of influent C/N ratio. On Day 30, the COD concentration decreased sharply from the start to 90 min of the cycle. By contrast, the COD concentration maintained at high level after 120 min of SBR cycle in the operation time of 85 day, because the high NH_4^+-N content in the reactor might be the main reason for the restraint of COD removal (Ma et al., 2009; Park and Bae, 2009; Wan et al., 2013; Wang et al., 2018). The accumulated COD could provide carbon source for denitrification. On the other hand, lots of microbes with multiple nitrogen metabolic pathways utilizing NO_3^--N or NO_2^--N as substrate for nitrogen removal might be enriched under low C/N ratio (Wang et al., 2018).

3.2. Granular characteristics and EPS components

The EPS property was further studied in view of its matrix for aerobic granular sludge and important role for the stability of aerobic granular sludge as described previously (Al-Halbouni et al., 2008; Sheng et al., 2013; Hao et al., 2016). As shown in Fig. 3, the EPS content of the two reactors increased significantly during Phase I, the PN content increased from 278.7 and 213.6 mg g^{-1} VSS to 436.5 and 373.2 mg g^{-1} VSS in R1 and R2, respectively. The PS content increased from 74.4 and 66.9 mg g^{-1} VSS to 118.4 and 106.5 mg g^{-1} VSS in R1 and R2, respectively. The corresponding PN/PS ratio increased to 5.01 and 4.86 at the end of Phase I. However, the PN/PS ratio was constant at about 5.0 in R2 with decreasing influent C/N ratio after Phase I, and the PN/PS ratio decreased significantly in R1 during Phase III and was less than 2.0 during Phase IV. Moreover, the hydrophobicity of granules in R2 was gradually better than that of R1 especially during Phase III and IV. The higher PN/PS ratio of EPS led to excellent hydrophobicity to enhance microbes accumulation and granular stability, the results were consistence with previous study (Ye et al., 2011; Wang et al., 2014; Geyik and Cecen, 2016; Niu et al., 2017).

For further investigating the key PN to illustrate the enhancement of influent C/N on granular stability in the aspect of EPS secretion, the EPS components were analyzed by 3D-EEM with PARAFAC. Three main component, i.e. soluble microbial byproduct-like, tryptophan and protein-like and humic acid-like were confirmed in this study (Fig. 4).

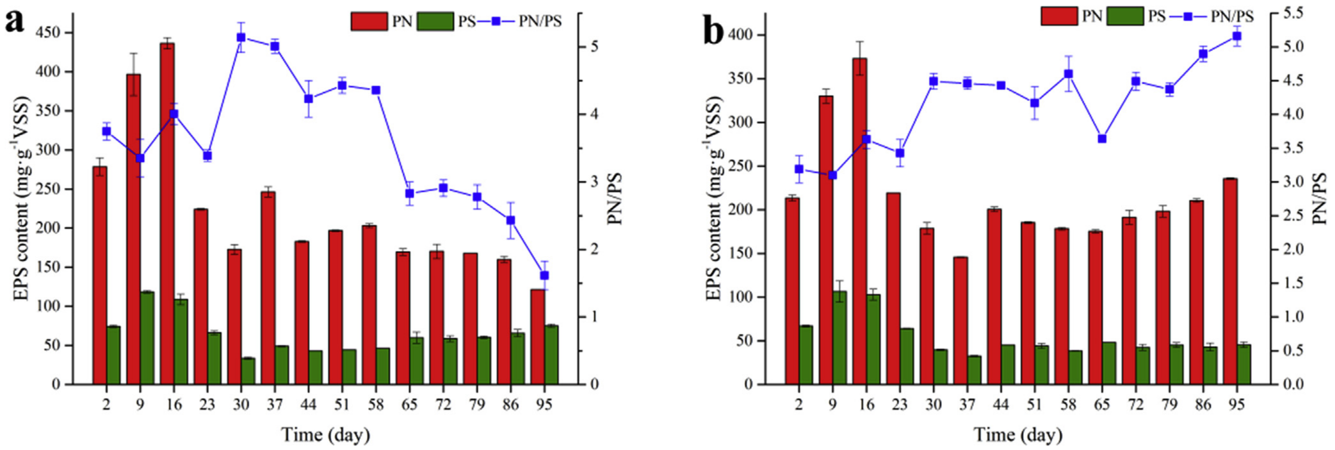

Fig. 3. Variation of EPS component of sludge in R1 (a) and R2 (b).

Tryptophan and protein-like substances were accumulated in granules of R2, especially during Phase III and IV when influent C/N ratio decreased gradually to 5. Nevertheless, the content of soluble microbial byproduct-like substances and humic acid-like substances in granules of R2 decreased after during Phase III and IV. Recent studies have suggested that tryptophan and protein-like substances and hydrophilic acids were abundant in the EPS of mature granules (Luo et al., 2014). Humic acid, mainly derived from adsorption of wastewater components and hydrolysis of other biopolymers such as protein (Zhang et al., 2015), was the main component of membrane foulants in MBRs with high hydrophilicity (Al-Halbouni et al., 2008). It seemed that tryptophan and protein-like substances were the main PN for granular stability via increasing the hydrophobicity of granules while amounts of soluble microbial byproduct-like substances and humic acid-like substances in granules against granular stability.

3.3. Succession of sludge microbiol community

Considering the differences of sludge characteristics under different influent C/N ratio, the microbial community of granular sludge was investigated. As shown in Fig. 5, the main functional microbes *Rhodobacter* spp., *Thauera* spp. and *Pseudoxanthomonas* spp. enriched in both reactors with stable granules on Day 50. Nevertheless, along with the decrease of influent C/N ration in R2, *Thauera* spp. and *Paracoccus* spp. enriched gradually. Among them, the relative abundance of *Thauera* spp. increased from 11.25% on Day 50 to 15.36% on Day 70, and then enriched with relative abundance of 19.5% on Day 90. As described by Cydzik-Kwiatkowska (2015), *Thauera* spp. was the main microorganism of aerobic granular sludge with influent C/N ratio lower than 4, and this kind of microbes might be crucial for aerobic sludge granulation. Luo et al. (2014) also observed that *Thauera* spp. gradually accumulated in granules with decreased C/N ratio. The relative abundance of *Paracoccus* spp. reached at 16.32% and 17.37% on Day 50 and 70, and then increased to 36.9% on Day 90. *Paracoccus* spp. was reported to have high activity in annomonia-riched wastewater (Sun et al., 2012; Remmas et al., 2016). Additionally, previous studies showed that these bacteria (*Thauera* spp. and *Paracoccus* spp.) had the function of EPS secretion and nitrogen removal (Liu et al., 2013). While the relative abundance of the other denitrification microbes, *Rhodobacter* spp. decreased from 22.56% on Day 50 to 7.50% on Day 90, and the content of *Pseudoxanthomonas* spp. with the function of EPS (mainly PS) secretion was stable with relative abundance of 4.56%–5.40%.

By contrast, *Paracoccus* spp., *Thauera* spp. and *Pseudoxanthomonas* spp. were washed out the reactor with granular disintegration in R1 with relative abundance of 8.95%, 10.36% and 6.75% on Day 50 to 4.56%, 7.86% and 2.11% on Day 90. *Rhodabacter* spp. also decreased slightly when granules disintegrated with relative abundance of 25.64% on Day 50 to 21.43% on Day 90. Results showed that the formation of aerobic granular sludge could enrich more microbes with the function of PN secretion and nitrogen removal with decrease of influent C/N ratio.

3.4. Correlation of sludge property and functional microorganism

Principal component analysis (PCA) was carried out to illustrate the relationship among microbial structure, sludge characters and pollutants removal. As shown in Fig. 6, the major microbes, *Paracoccus* spp. and *Thauera* spp. as well as PN, tryptophan and protein-like substances, relative hydrophobicity and TN removal efficiency were circled in a confidence ellipse of 95% in R2 with low influent C/N ratio. These microbes, EPS component and pollutant removal property presented principal component 1 (PC1) and the principal score of sludge on Day 90 obtained highest value of 0.933 in PC1. Among the major microbes in PC1, *Thauera* spp. could tolerate high-level free ammonia than other microbes in activated sludge (Foss and Harder, 1998), and was also the main microbes in lots of wastewater treatment systems with low influent C/N ratio in this and previous studies (Sun et al., 2012; Liu et al., 2013; Remmas et al., 2016) .The large proportion of *Thauera* spp. led to more PN content (mainly tryptophan and protein-like substances) in EPS of granules and protected other microbes from the stress of high influent ammonia (Song et al., 1998; Mao et al., 2014; Gül and Ferhan, 2016; Hao et al., 2016; Prombutara and Allen, 2016). Based on the characteristic of *Thauera* spp. mentioned above, the microbes was thought as the main resistant microbes under high-level free ammonia in this study. Stable granules with excellent hydrophobicity maintained when influent C/N ratio decreased. More microbes related to nitrogen removal, i.e. *Paracoccus* spp. then were enriched in stable aerobic granular sludge under low influent C/N ratio as more PN content led to the less stress of high influent ammonia. On the other hand, most species of *Paracoccus* had higher specific growth rate (0.41–0.77 h^{-1}) than *Thauera* spp. (0.21–0.38 h^{-1}) with the same substrates, and had multiple nitrogen metabolic pathway using NO_3^--N, NO_2^--N, NO and N_2O as substrates (Liu et al., 2013). These characteristic led to *Paracoccus* spp. enrich with decreased C/N ratio for NO_3^--N and NO_2^--N degradation during SBR cycle in Phase IV (shown in Appendix A).

The suitable environment was made by PN secreted by *Thauera* spp. under low influent C/N ratio, *Paracoccus* spp. was gradually enriched with highest relative abundance and further decreased the stress of high influent ammonia via enhancing nitrogen removal. These microbes provided the suitable niche for other microorganisms enriched in aerobic granular sludge under low influent C/N ratio. The stable granules with excellent hydrophobicity and enhanced pollutant removal efficiency finally formed. By contrast, although *Paracoccus* spp., *Rhodobacter* spp. *Pseudoxanthomonas* spp., PN content, relative

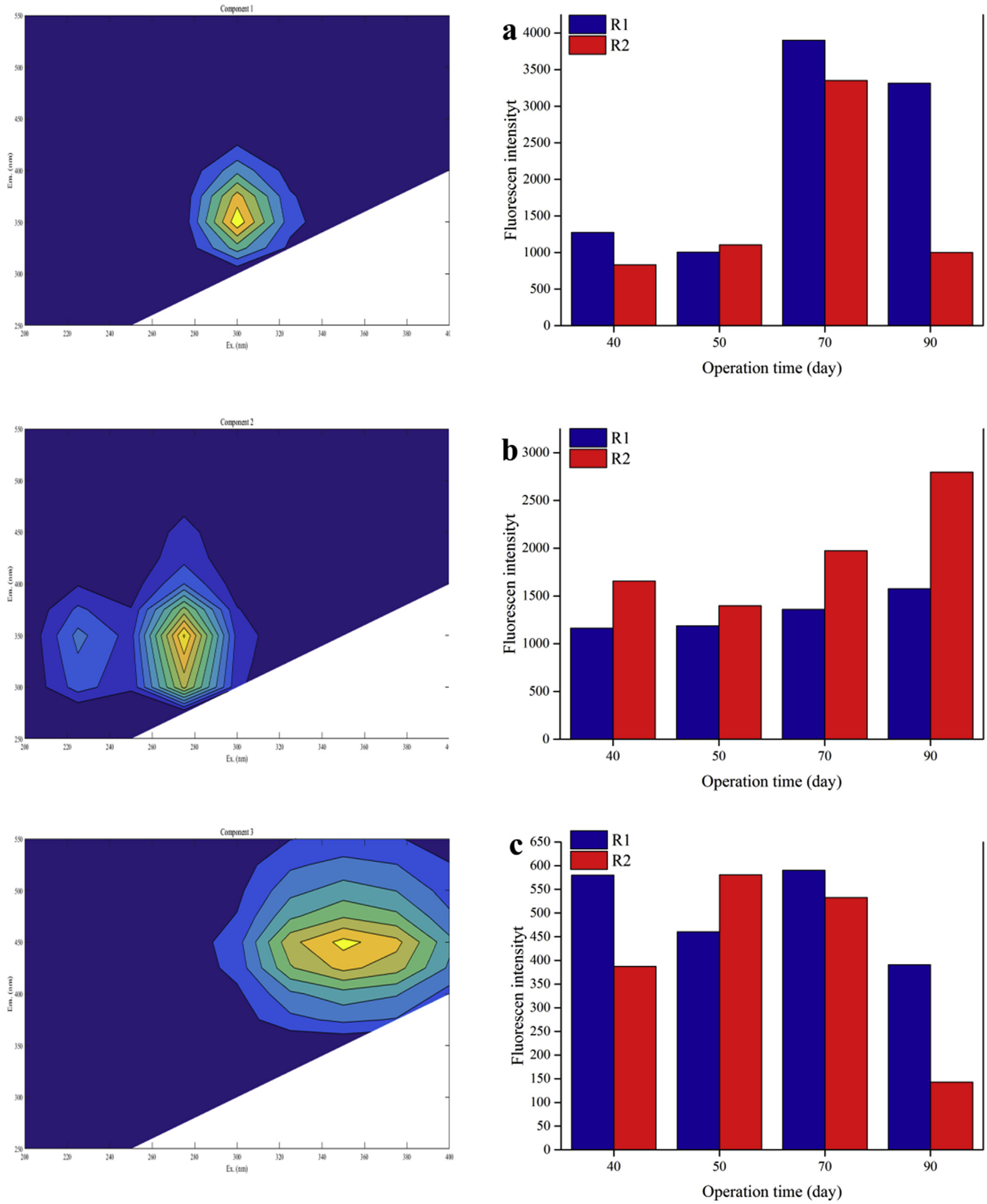

Fig. 4. Peak intensity variation of PARAFAC-derived components in sludge EPS during aerobic sludge granulation: a. soluble microbial byproduct-like substances; b. tryptophan and protein-like substances; c. humic acid-like substances.

hydrophobicity and TN removal efficiency of R1 were circled in a confidence ellipse of 95% and represented PC1, the principal score of sludge on Day 90 was -1.01. The functional microbes related EPS secretion and pollutant removal efficiency were washed out of the reactor with the disintegration of granular sludge under high influent C/N ratio.

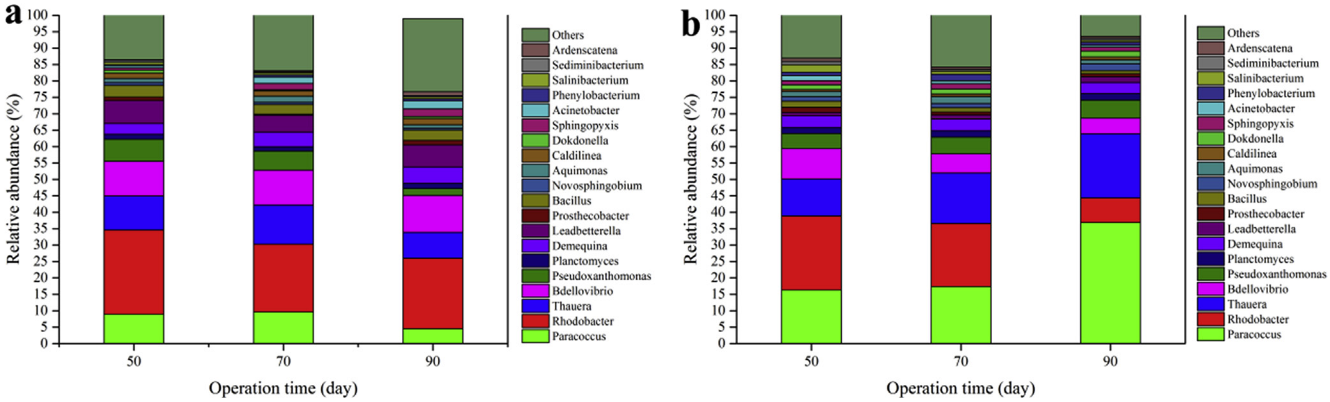

Fig. 5. Succession of microbial community in R1 (a) and R2 (b) during reactor operation.

4. Conclusion

Compact granular sludge with excellent pollutants removal property was formed under condition of low influent C/N ratio, and several resistant microbes such as *Thauera* spp. were enriched and more EPS especially tryptophan and protein-like substances were secreted simultaneously. Under this suitable niche, the fast-growth-rate microbes like *Paracoccus* spp. with function of nitrogen removal could be better colonised with less stress of high-level ammonia, and favor the formation of stable granular sludge and efficient removal of pollutants.

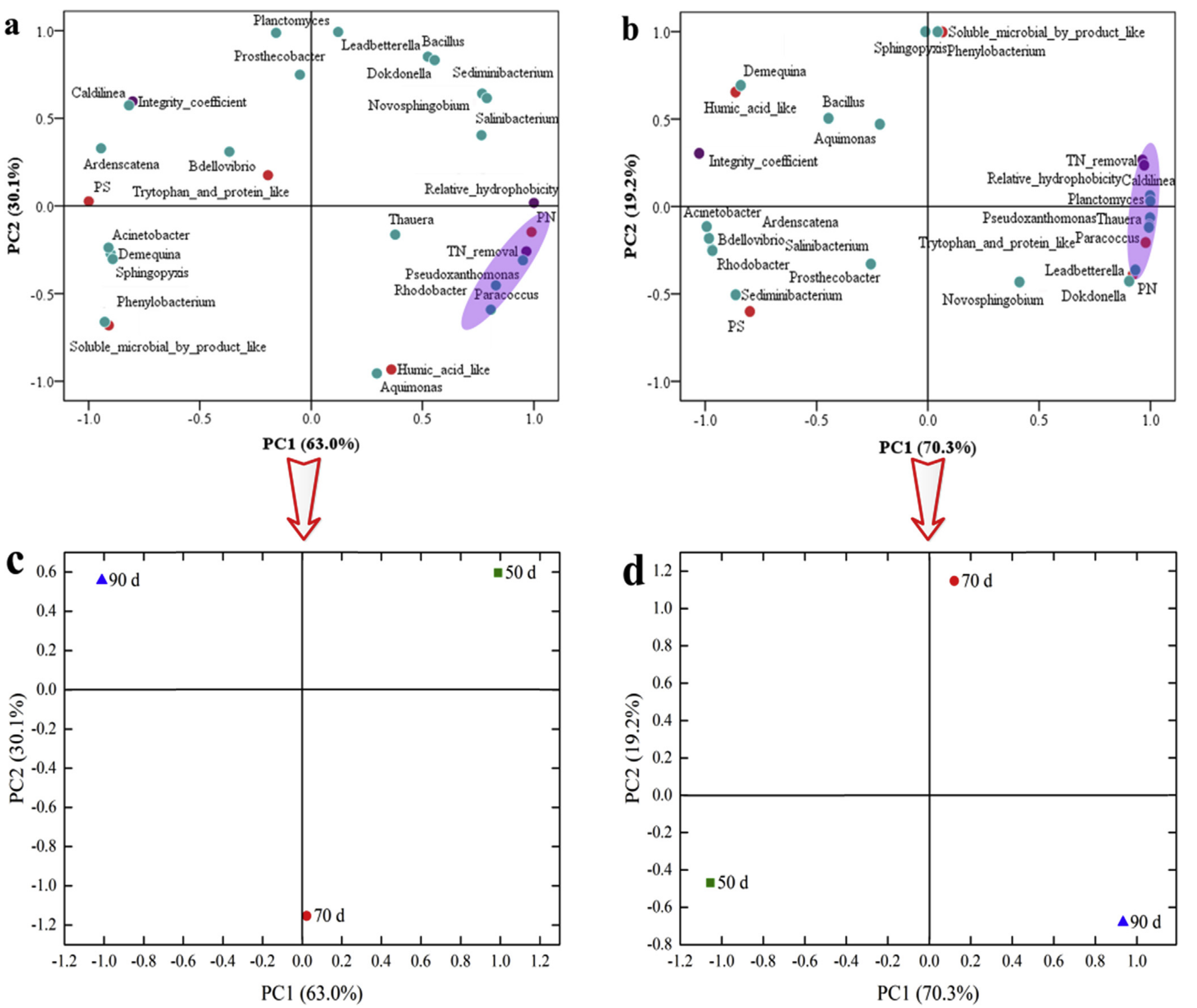

Fig. 6. Principal component analysis (PCA) of microbial community, EPS component and granular sludge characteristic: Loading plot of component in R1 (a) and R2 (b); score plot of samples in R1 (c) and R2 (d).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.09.045.

References

Al-Halbouni, D., Traber, J., Lyko, S., Wintgens, T., Melin, T., Tacke, D., Janot, A., Dott, W., Hollender, J., 2008. Correlation of EPS content in activated sludge at different sludge retention times with membrane fouling phenomena. Water Res. 42, 1475–1488.

APHA, 2005. Standard methods for the examination of water and wastewater, Washington, DC, USA.

Chiu, Z.C., Chen, M.Y., Lee, D.J., Wang, C.H., Lai, J.Y., 2007. Oxygen diffusion in active layer of aerobic granule with step change in surrounding oxygen levels. Water Res. 41, 884–892.

Cydzik-Kwiatkowska, A., 2015. Bacterial structure of aerobic granules is determined by aeration mode and nitrogen load in the reactor cycle. Bioresour. Technol. 181, 312–320.

Derlon, N., Wagner, J., da Costa, R.H.R., Morgenroth, E., 2016. Formation of aerobic granules for the treatment of real and low-strength municipal wastewater using a sequencing batch reactor operated at constant volume. Water Res. 105, 341–350.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Ferhan, Ç., Başak, K., 2016. Inhibitory effect of silver on activated sludge: effect of organic substrate and the carbon to nitrogen ratio. J. Chem. Technol. Biot. 91, 1190–1198.

Foss, S., Harder, J., 1998. *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, menthol, and eucalyptol) and nitrate. Syst. Appl. Microbiol. 21, 365–373.

Gül, G.A., Ferhan, Ç., 2016. Production of protein-and carbohydrate-EPS in activated sludge reactors operated at different carbon to nitrogen ratios. J. Chem. Technol. Biot. 91, 522–531.

Geyik, A.G., Cecen, F., 2016. Production of protein- and carbohydrate-EPS in activated sludge reactors operated at different carbon to nitrogen ratios. J. Chem. Technol. Biot. 91, 522–531.

Ghangrekar, M.M., Asolekar, S.R., Joshi, S.G., 2005. Characteristics of sludge developed under different loading conditions during UASB reactor start-up and granulation. Water Res. 39, 1123–1133.

Hao, L., Liss, S.N., Liao, B.Q., 2016. Influence of COD: N ratio on sludge properties and their role in membrane fouling of a submerged membrane bioreactor. Water Res. 89, 132–141.

Hu, X., Xie, L., Shim, H., Zhang, S., Yang, D., 2014. Biological Nutrient Removal in a Full Scale Anoxic/Anaerobic/Aerobic/Pre-anoxic-MBR Plant for Low C/N Ratio Municipal Wastewater Treatment. Chin. J. Chem. Eng. 22, 447–454.

Huang, W.L., Wang, W.L., Shi, W.S., Lei, Z.F., Zhang, Z.Y., Chen, R.Z., Zhou, B.B., 2014. Use low direct current electric field to augment nitrification and structural stability of aerobic granular sludge when treating low COD/NH4-N wastewater. Bioresour. Technol. 171, 139–144.

ISO, 2001. Representation of results of particle size analysis.

Kishida, N., Kim, J., Tsuneda, S., Sudo, R., 2006. Anaerobic/oxic/anoxic granular sludge process as an effective nutrient removal process utilizing denitrifying polyphosphate-accumulating organisms. Water Res. 40, 2303–2310.

Lemaire, R., Yuan, Z., Blackall, L.L., Crocetti, G.R., 2008. Microbial distribution of *Accumulibacter* spp. and *Competibacter* spp. in aerobic granules from a lab-scale biological nutrient removal system. Environ. Microbiol. 10, 354–363.

Li, X.Y., Yang, S.F., 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Res. 41, 1022–1030.

Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegard, A., 2013. Strains in the genus *Thauera* exhibit remarkably different denitrification regulatory phenotypes. Environ. Microbiol. 15, 2816–2828.

Liu, Y.L., Kang, X.R., Li, X., Yuan, Y.X., 2015. Performance of aerobic granular sludge in a sequencing batch bioreactor for slaughterhouse wastewater treatment. Bioresour. Technol. 190, 487–491.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luo, J.H., Hao, T.W., Wei, L., Mackey, H.R., Lin, Z.Q., Chen, G.H., 2014. Impact of influent COD/N ratio on disintegration of aerobic granular sludge. Water Res. 62, 127–135.

Ma, Y., Peng, Y.Z., Wang, S.Y., Yuan, Z.G., Wang, X.L., 2009. Achieving nitrogen removal via nitrite in a pilot-scale continuous pre-denitrification plant. Water Res. 43, 563–572.

Mao, Y., Xia, Y., Wang, Z., Zhang, T., 2014. Reconstructing a *Thauera* genome from a hydrogenotrophic-denitrifying consortium using metagenomic sequence data. Appl. Microbiol. Biotechnol. 98, 6885–6895.

Meng, J., Li, J., Li, J., Antwi, P., Deng, K., Wang, C., Buelna, G., 2015. Nitrogen removal from low COD/TN ratio manure-free piggery wastewater within an upflow microaerobic sludge reactor. Bioresour. Technol. 198, 884–890.

Niu, W., Guo, J., Lian, J., Song, Y., Lu, C., Li, H., Han, Y., Yin, P., 2017. Rapid start-up of denitrifying granular sludge by dosing with semi-starvation fluctuation C/N ratio strategy. Bioresour. Technol. 241, 945–950.

Oshiki, M., Ishii, S., Yoshida, K., Fujii, N., Ishiguro, M., Satoh, H., Okabe, S., 2013. Nitrate-dependent ferrous iron oxidation by anaerobic ammonium oxidation (anammox) bacteria. Appl. Environ. Microbiol. 79, 4087.

Park, S., Bae, W., 2009. Modeling kinetics of ammonium oxidation and nitrite oxidation under simultaneous inhibition by free ammonia and free nitrous acid. Process Biochem. 44, 631–640.

Prombutara, P., Allen, M.S., 2016. Flocculation-related gene identification by whole-genome sequencing of *Thauera aminoaromatica* MZ1T floc-defective mutants. Appl. Environ. Microbiol. 82, 1646–1652.

Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217.

Remmas, N., Melidis, P., Katsioupi, E., Ntougias, S., 2016. Effects of high organic load on *amoA* and *nirS* gene diversity of an intermittently aerated and fed membrane bioreactor treating landfill leachate. Bioresour. Technol. 220, 557–565.

Sheng, G.P., Xu, J., Luo, H.W., Li, W.W., Li, W.H., Yu, H.Q., Xie, Z., Wei, S.Q., Hu, F.C., 2013. Thermodynamic analysis on the binding of heavy metals onto extracellular polymeric substances (EPS) of activated sludge. Water Res. 47, 607–614.

Song, B., Young, L.Y., Palleroni, N.J., 1998. Identification of denitrifier strain T1 as *Thauera aromatica* and proposal for emendation of the genus *Thauera* definition. Int. J. Syst. Bacteriol. 48, 889–894.

Stedmon, C.A., Bro, R., 2008. Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. Limnology and Oceanography-Methods 6, 572–579.

Su, K.Z., Yu, H.Q., 2005. Formation and characterization of aerobic granules in a sequencing batch reactor treating soybean-processing wastewater. Environ. Sci. Technol. 39, 2818–2827.

Sun, G., Zhu, Y., Saeed, T., Zhang, G., Lu, X., 2012. Nitrogen removal and microbial community profiles in six wetland columns receiving high ammonia load. Chem. Eng. J. 203, 326–332.

Sun, J., Guo, L., Li, Q., Zhao, Y., Gao, M., She, Z., Wang, G., 2016. Structural and functional properties of organic matters in extracellular polymeric substances (EPS) and dissolved organic matters (DOM) after heat pretreatment with waste sludge. Bioresour. Technol. 219, 614–623.

Sun, S.P., Nacher, C.P.I., Merkey, B., Zhou, Q., Xia, S.Q., Yang, D.H., Sun, J.H., Smets, B.F., 2010. Effective biological nitrogen removal treatment processes for domestic wastewaters with low c/n ratios: a review. Environ. Eng. Sci. 27, 111–126.

Wan, C., Sun, S., Lee, D.-J., Liu, X., Wang, L., Yang, X., Pan, X., 2013. Partial nitrification using aerobic granules in continuous-flow reactor: rapid startup. Bioresour. Technol. 142, 517–522.

Wang, B., Zhao, M.Y., Guo, Y.Y., Peng, Y.Z., Yuan, Y., 2018. Long-term partial nitritation and microbial characteristics in treating low C/N ratio domestic wastewater. Environ. Sci.-Wat. Res. 4, 820–827.

Wang, Z., Gao, M., Xin, Y., Ma, D., She, Z., Wang, Z., Sun, C., Ren, Y., 2014. Effect of C/N ratio on extracellular polymeric substances of activated sludge from an anoxic-aerobic sequencing batch reactor treating saline wastewater. Environ. Technol. 35, 2821–2828.

Ye, F., Ye, Y., Li, Y., 2011. Effect of C/N ratio on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge flocs. J. Hazard. Mater. 188, 37–43.

Zhang, L., Narita, Y., Gao, L., Ali, M., Oshiki, M., Okabe, S., 2017. Maximum specific growth rate of anammox bacteria revisited. Water Res. 116, 296–303.

Zhang, W., Peng, S., Xiao, P., He, J., Yang, P., Xu, S., Wang, D., 2015. Understanding the evolution of stratified extracellular polymeric substances in full-scale activated sludges in relation to dewaterability. RSC Adv. 5, 1282–1294.

Zhao, W.H., Huang, Y., Wang, M.X., Pan, C., Li, X.Y., Peng, Y.Z., Li, B.K., 2018a. Post-endogenous denitrification and phosphorus removal in an alternating anaerobic/oxic/anoxic (AOA) system treating low carbon/nitrogen (C/N) domestic wastewater. Chem. Eng. J. 339, 450–458.

Zhao, Y., Feng, Y., Li, J., Guo, Y., Chen, L., Liu, S., 2018b. Insight into the aggregation capacity of anammox consortia during reactor start-up. Environ. Sci, Technol.

Zhou, J.H., Zhang, Z.M., Zhao, H., Yu, H.T., Alvarez, P.J.J., Xu, X.Y., Zhu, L., 2016. Optimizing granules size distribution for aerobic granular sludge stability: effect of a novel funnel-shaped internals on hydraulic shear stress. Bioresour. Technol. 216, 562–570.

Zhou, J.H., Zhao, H., Hu, M., Yu, H.T., Xu, X.Y., Vidonish, J., Alvarez, P.J.J., Zhu, L., 2015. Granular activated carbon as nucleating agent for aerobic sludge granulation: effect of GAC size on velocity field differences (GAC versus flocs) and aggregation behavior. Bioresour. Technol. 198, 358–363.