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AHL-mediated quorum sensing regulates the variations of microbial community and sludge properties of aerobic granular sludge under low organic loading

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ABSTRACT

Aerobic granular sludge (AGS) is promising in wastewater treatment. However, the formation and existence of AGS under low organic loading rate (OLR) is still not fully understood due to a knowledge gap in the variations and correlations of *N*-acyl-homoserine lactones (AHLs), the microbial community, extracellular polymeric substances (EPS) and other physiochemical granule properties. This study comprehensively investigated the AHL-mediated quorum sensing (QS) and microbial community characters in the AGS fed with ammonium-rich wastewater under a low OLR of 0.15 kg COD $(m^3\ d)^{-1}$. The results showed that the AGS appeared within 90 days, and the size of mature granules was over 700 μm with strong settleability and ammonium removal performance. More tightly-bound extracellular polysaccharide and tightly-bound extracelluar protein were produced in the larger AGS. C10-HSL and C12-HSL gradually became dominant in sludge, and short-chain AHLs dominated in water. EPS producers and autotrophic nitrifiers were successfully retained in the AGS under low OLR. AHL-mediated QS utilized C10-HSL, C12-HSL and 3OC6-HSL as the critical AHLs to regulate the TB-EPS in aerobic granulation, and autotrophic nitrifiers may perform interspecific communication with C10-HSL. The correlations of bacterial genera with AGS properties and AHLs were complex due to the dynamic fluctuations of microbial composition and other variable factors in the mixed-culture system. These findings confirmed the participation of AHL-mediated QS in the regulation of microbial community characters and AGS properties under low OLR, which may provide guidance for the operation of AGS systems under low OLR from a microbiological viewpoint.

1. Introduction

Aerobic granular sludge (AGS) is a promising technology for wastewater treatment due to its excellent settling property, small footprint demand and low operation costs (Nancharaiah and Reddy, 2018; van Loosdrecht and Brdjanovic, 2014). AGS is considered a special form of biofilm that results from self-immobilization during the aerobic granulation process without external carriers (Adav et al., 2008a; Su et al., 2016). Though the first successful case of AGS in a lab-scale sequencing batch reactor (SBR) was reported in 1997 through the selection of fast-settling biomass (Morgenroth et al., 1997), full-scale AGS applications were reported very recently (Li et al., 2014a; Pronk et al., 2015). Researchers worldwide have made extensive efforts to interpret the granulation mechanism, verify the potential influencing factors, and

Abbreviations: AHL, *N*-acyl-homoserine lactones; S-AHL, AHL in the sludge phase; W-AHL, AHL in the water phase; C4-HSL, *N*-butyryl homoserine lactone; C6-HSL, *N*-hexanoyl homoserine lactone; C8-HSL, *N*-octanoyl homoserine lactone; C10-HSL, *N*-decanoyl homoserine lactone; C12-HSL, *N*-dodecanoyl homoserine lactone; C14-HSL, *N*-tetradecanoyl homoserine lactone; 3OC6-HSL, *N*-(β-ketocaproyl) homoserine lactone; 3OC8-HSL, *N*-(3-oxooctanoyl) homoserine lactone; 3OC10-HSL, *N*-(3-oxodecanoyl) homoserine lactone; 3OC12-HSL, *N*-(3-oxododecanoyl) homoserine lactone; 3OC14-HSL, *N*-(3-oxotetradecanoyl) homoserine lactone

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apply the AGS into diverse wastewater treatments (de Sousa Rollemberg et al., 2018; Maszenan et al., 2011; Wilen et al., 2018).

As a key influencing factor, the organic loading rate (OLR) has been proven to affect the formation and characteristics of AGS (Ni et al., 2009; Wan et al., 2016; Zhang et al., 2018a). According to the current progress of AGS, the reported OLR value ranges from 0.3 to 15 kg COD $(m^3\,d)^{-1}$ (Chen et al., 2008; He et al., 2019; Liu et al., 2003; Moy et al., 2002; Peyong et al., 2012; Thwaites et al., 2017), while the OLR values of over 1 kg COD $(m^3\,d)^{-1}$ are usually adopted. Higher OLRs usually promote faster formation of larger and looser granules, while lower OLRs lead to either slower formation of smaller and compact granules (Li et al., 2008; Wilen et al., 2018), or even failed formation (Liu et al., 2007; Tay et al., 2004). Several types of practical industrial wastewater, such as fertilizer production wastewater and synthetic ammonia industry wastewater, usually contain low COD and high ammonium concentrations (Keluskar et al., 2013; Yu et al., 2012; Zhang et al., 2012), which result in low OLRs and high ammonium loading rates (ALRs). Nevertheless, the feasibility and granulation process of AGS under low OLR, such as 0.15 kg COD $(m^3\,d)^{-1}$, are rarely reported in ammonium-rich wastewater.

Extracellular polymeric substances (EPS), including extracellular polysaccharide (PS) and extracellular protein (PN), play critical roles in bacteria aggregation and aerobic granulation (Kang and Yuan, 2017; Zhu et al., 2015). PN can alter cell surface hydrophobicity to improve cell attachment and sludge aggregation (Wan et al., 2014; Zhang et al., 2007), while PS can form a network-structure layer as the backbone to wrap bacteria inside (Adav and Lee, 2008; Adav et al., 2008b). Therefore, the research focusing on the regulation and characteristics of EPS is essential in aerobic granulation. Quorum sensing (QS), as a bacterial communication mechanism, has been recognized for its ability to regulate EPS synthesis during aerobic granulation (Tan et al., 2014; Xiong and Liu, 2013). Among various QS signal molecules, *N*-acyl-homoserine lactones (AHLs) secreted and sensed by Gram-negative bacteria have specifically attracted extensive attention (Mukherjee and Bassler, 2019; Shrout and Nerenberg, 2012). AHL-mediated QS favors the formation and stability of AGS through the regulation of EPS, which promotes the microbial attachment growth and sludge hydrophobicity property (Chen et al., 2018a; Huang et al., 2016; Shi et al., 2017). The inhibition of EPS secretion caused by the inactivation of AHLs has been observed in many cases, such as AGS formation (Li et al., 2014b; Li and Zhu, 2014) and membrane biofouling control (Huang et al., 2019; Huang et al., 2018). Meanwhile, the addition of exogenous AHLs enhances the EPS production and granulation process (Li et al., 2015; Wu et al., 2017). However, the spatial and temporal distribution of diverse AHLs in AGS cultivated under low OLR conditions is still rarely reported. In addition, microbial community information, including microbial diversity and composition, is critical for a deeper comprehension of AGS (He et al., 2016; Li et al., 2008; Reino et al., 2016); however, previous reports usually focused on the microbial community under an OLR higher than 1.5 kg COD $(m^3\,d)^{-1}$(Li et al., 2008). As AHL-mediated QS is proven to affect the composition and diversity of microbial community (Li et al., 2015; Valle et al., 2004), investigating the correlation of AHL-mediated QS with microbial community in AGS under low OLR is therefore essential.

This study comprehensively investigates the formation of AGS fed with ammonium-rich wastewater under an OLR of 0.15 kg COD $(m^3\,d)^{-1}$ and especially focuses on the correlations among AHL-mediated QS, microbial community and sludge properties. Several issues were studied. First, the feasibility and properties of AGS under a low OLR were monitored. Second, the existence and distribution patterns of AHLs in AGS were determined. Third, microbial communities were analyzed to reveal the changes of different functional bacteria in AGS. Lastly, correlations among AHL-mediated QS, microbial community and sludge properties were analyzed to discuss the potential role of AHL-mediated QS in AGS under a low OLR.

2. Materials and methods

2.1. Seed sludge and wastewater

The seed sludge was comprised of brown flocs acquired from an aeration tank of a wastewater treatment plant in Harbin, China. Futile sundries were removed from the activated sludge before use. The influent wastewater was artificially prepared with ammonium chloride as the nitrogen source. The influent ammonium was gradually increased from 100 $mg\,L^{-1}$ to 500 $mg\,L^{-1}$. The addition of glucose and sodium acetate in the influent provided the same COD content resulting in the influent COD concentration of 100 $mg\,L^{-1}$ throughout this study. Other ingredients, including 10 $mg\,L^{-1}$ of $PO_4{}^{3-}$-P (KH_2PO_4), 10 $mg\,L^{-1}$ of Mg^{2+} ($MgSO_4.7H_2O$), 5 $mg\,L^{-1}$ of Fe^{2+} ($FeSO_4.7H_2O$), 10 $mg\,L^{-1}$ of Ca^{2+} ($CaCl_2$) and 1 $mL\,L^{-1}$ of a trace element solution, were added in the synthetic wastewater. The trace element solution was prepared according to the procedure of Ma et al. (2015).

2.2. Reactor configuration and operation

A column SBR with an effective volume of 6 L and a height to diameter ratio of 8 was adopted to cultivate the AGS (Fig. S1). The SBR was operated in successive cycle of 8 h. A typical cycle had an anoxic feeding of 5 min, an aeration period, a settling period, a discharging period of 2 min and an idling period of 13 min. The settling period changed according to the practical operation. Compressed air was transmitted through a microporous diffusor in the reactor bottom by an air pump during the aeration period. The flow rate was 0.3 $m^3\,h^{-1}$, and the superficial air velocity was approximately 1.66 $cm\,s^{-1}$. The effluent was discharged through a solenoid valve with an exchange ratio of 50%, which induced a hydraulic retention time of 16 h. The pH of the mixed liquid was maintained at 6–7 by the automatic addition of a sodium bicarbonate solution. The initial biomass in the SBR consisted of 6135 $mg\,L^{-1}$ of mixed liquor suspended solids (MLSS) and 4489 $mg\,L^{-1}$ of mixed liquor volatile suspended solids (MLVSS). The reactor was operated at 21 ± 2 °C. The sludge retention time ranged from 20 to 30 days during the whole operation period due to the washout of flocs in the effluent. The whole operation period lasted for 380 days, and the operation schedule is displayed in Fig. S2. The settling time was gradually shortened from 40 min to 1 min, while the OLR stayed at 0.15 kg COD $(m^3\,d)^{-1}$. The ALR was gradually increased from 0.15 kg N $(m^3\,d)^{-1}$ to 0.75 kg N $(m^3\,d)^{-1}$ during the operation to promote the growth of autotrophic nitrifiers.

2.3. Extraction and analysis of AHLs

All the samples for AHL extraction were obtained after the first hour in the aeration period. The pH of the sludge mixture was adjusted to 2 with 1 $mol\,L^{-1}$ HCl (Gao et al., 2014). The sludge mixture was centrifuged at 8000 r min^{-1} for 10 min, then divided into the water phase (supernatant) and sludge phase (pellet). For the extraction of AHLs in the water phase, the supernatant of 1000 mL was filtered through 0.45-μm PTFE filters, measured to obtain the accurate volume, and extracted thrice with an equivalent volume of ethyl acetate vigorously (Ding et al., 2015; Feng et al., 2014). For the extraction of AHLs in the sludge phase, the pellet after the centrifugation of about 200–250 mL sludge mixture was mixed with ethyl acetate, crushed and homogenized with vortex vibration for 1 min. The mixture was treated with ultrasonic in an ice bath at an intensity of 200 W for 30 min to further promote the release of AHLs into the organic solvent (Tang et al., 2015). Then, the organic solvent was filtered through 0.45-μm PTFE filters. The pellet after the extraction procedure was measured to obtain the accurate dry weight. The remaining sludge pellet and the influent wastewater of suitable volume were dosed into the SBR to recover the system liquid volume. Both the AHL extracts in the water and sludge phases were treated with a rotary evaporator and nitrogen blow equipment (Wu

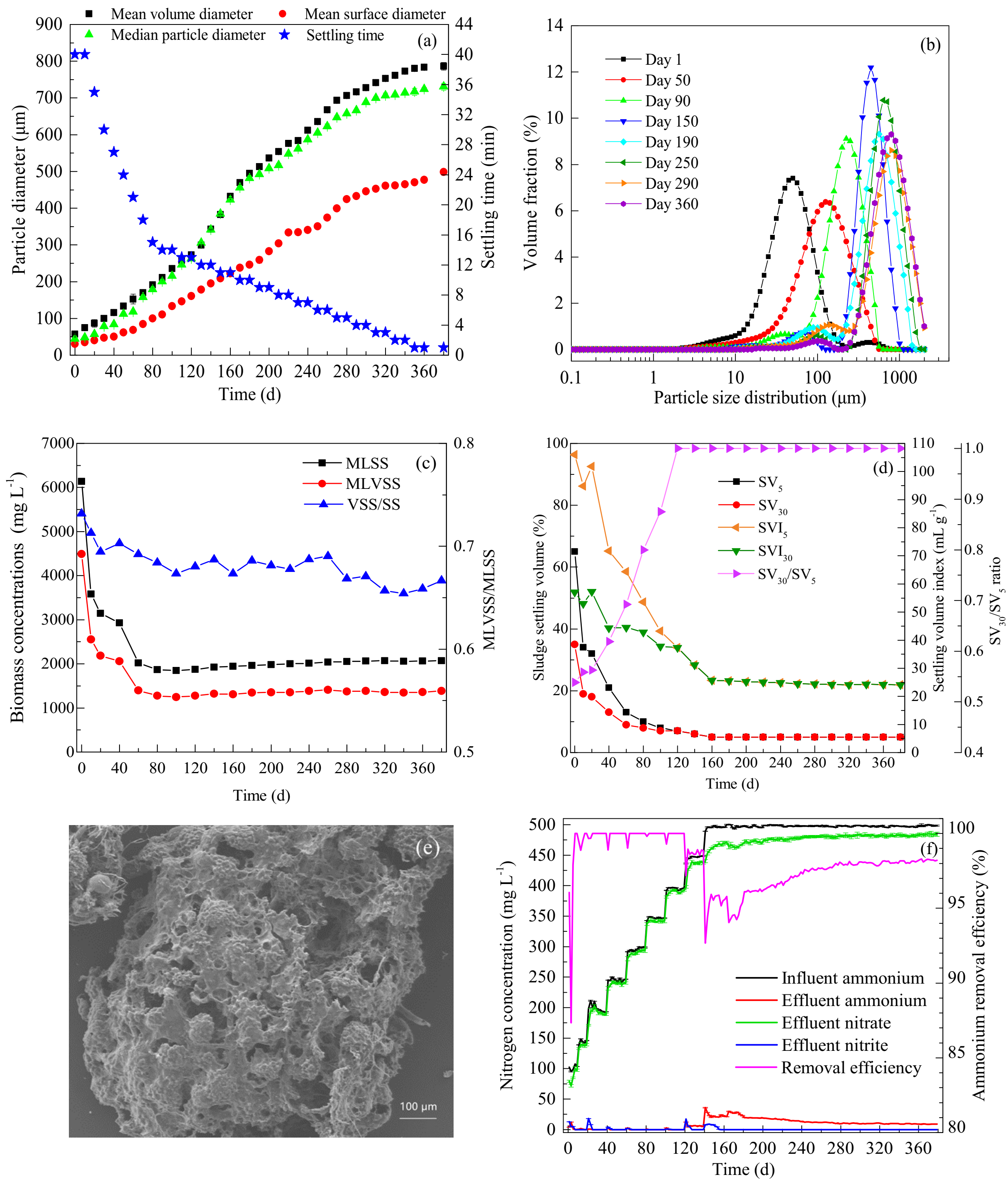

Fig. 1. Properties of AGS during the whole operation process: (a) Sludge size; (b) Particle size distribution; (c) Sludge concentrations; (d) Sludge settleability; (e) Mature granular morphology observation by SEM; (f) Nitrogen transformation.

et al., 2015). The residue was re-dissolved with pure acetonitrile and filtered through 0.22-μm PTFE filters before further analysis by an ultra-performance liquid chromatograph with a tandem mass spectrometer (UPLC-MS/MS).

The UPLC-MS/MS (ACQUITY UPLC-Xevo TQ MS, Waters, USA) was equipped with a BEH-C18 column (2.1 mm × 5 mm with 1.7-μm particle, Waters, USA) and utilized to determine the AHLs. The mobile phase consisted of 0.1% formic acid in acetonitrile with (A) and 0.1% formic acid in pure water (B) with the flow rate as 0.1 mL min^{-1} (Yu et al., 2016). An electrospray positive ionization mode was adopted, and a multiple reaction monitoring (MRM) mode was adopted to analyze the AHLs (Yu et al., 2016). Eleven kinds of standard AHL reagents, including C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3OC6-HSL, 3OC8-HSL, 3OC10-HSL, 3OC12-HSL and 3OC14-HSL, were purchased from Sigma Aldrich (USA) and dissolved in pure acetonitrile. Standard curves of different AHLs were calculated based on the known concentrations and the corresponding peak areas of the quantitative daughter ion (m/z 102). The detailed detection parameters of the different AHLs are listed in Table S1.

2.4. High-throughput sequencing analysis of microbial communities

Sludge samples on Days 1, 60, 100, 140, 180, 240, 300 and 360 were collected and named as S1 to S8, respectively. All the sludge samples were obtained after the first hour in the aeration period to ensure the uniform distribution in the SBR. The genomic DNA extraction, PCR amplification and product quality testing were implemented according to a previous study (Chen et al., 2018b). An Illumina Miseq PE300 platform was chosen to carry out the high-throughput sequencing at Shanghai Majorbio Bio-pharm Technology Co., Ltd. The raw data were analyzed using the free online I-Sanger cloud platform (www.i-sanger.com). The operational taxonomic units (OTUs) were generated by the cluster of normalized sequences at an identity of 97%. An RDP Classifier with a confidence threshold of 70% was used to classify the effective sequences into different taxonomy units, and the Silva 128 ribosomal RNA sequence database was adopted as the taxonomy database. The raw sequencing data were deposited into the NCBI database with an SRA accession of PRJNA513006.

2.5. Analytical methods

Water samples were collected every two days and analyzed after filtration through 0.45-μm filters. Ammonium, nitrite and nitrate were determined by Nessler's reagent spectrophotometry, N-(1-naphthyl)-ethylene diamine photometry and phenol disulfonic acid photometry, respectively. Dissolved oxygen (DO) in the bulk liquor was determined by a dissolved oxygen meter (Oxi730, WTW, Germany). On-line pH and temperature were measured through a real-time monitoring detector (SIN-pH -4, Sinomeasure, China). The loosely-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) were extracted based on a previous study (Li and Yang, 2007), and the sludge samples for EPS extraction were obtained after the first hour in the aeration period to avoid the uneven spatial distribution of the biomass in the SBR. PS and PN in both LB-EPS and TB-EPS were measured by the phenol-sulfuric acid method and a Bicinchoninic Acid Kit (Sigma, USA). The TB-EPS concentrations were calculated as the sum of the tightly-bound PN (TB-PN) and tightly-bound PS (TB-PS) concentrations, and the contents of LB-EPS were the sum of the loosely-bound PN (LB-PN) and loosely-bound PS (LB-PS). The sludge size was measured via a laser particle size analyzer (Mastersizer 2000, Malvern Panalytical, UK).

2.6. Data analysis

The raw data of water quality, sludge size and EPS contents were calculated to obtain the average and standard deviation based on the triplicate tests. Pearson's correlation analysis was performed with Origin 8.5 software to analyze the correlations between different indices, and a p-value < 0.05 was considered significant.

3. Results and discussion

3.1. Formation and properties of AGS under a low OLR

As shown in Fig. 1a, aerobic granules were formed in 90 days with a mean volume diameter of over 200 μm, and the particle diameter was negatively correlated with the settling time, while no obvious breakage of AGS was observed during the whole operation. The aerobic granulation process gradually slowed down after Day 190, when the curves of Day 250, Day 290 and Day 360 were very close (Fig. 1b). The concentrations of MLSS and MLVSS decreased sharply in the beginning, and then remained stable. The ratio of MLVSS to MLSS presented a slightly decreasing trend (Fig. 1c). The sludge settleability was obviously improved during the operation along with a decrease in the sludge setting volume (SV) and settling volume index (SVI) (Fig. 1d). Through the SEM observation, glue-like extracellular substances were found on the surface of a mature granule, and a network structure in the granule was observed shown in (Fig. 1e). The influent ammonium was mainly oxidized to nitrate with little nitrite accumulation (Fig. 1f). When the influent ammonium concentration rose to 500 mg L^{-1}, the ammonium removal efficiency fluctuated and was then maintained at approximately 98% with an ammonium removal rate of approximately 0.74 kg N (m^3 d)$^{-1}$. The decreasing settling time as a hydraulic selection pressure removes the slow-settling flocs and alleviates the fierce substrate competition, which promotes the formation of AGS (Li and Li, 2009; Ni et al., 2009). Aerobic granules with strong settleability and thorough ammonium removal grow slowly in this study, while the granulation period is obviously longer than in previous studies under higher OLR conditions (Li et al., 2008; Ni et al., 2009; Zhang et al., 2018a). The long granulation process may be interpreted by the low EPS production and biomass growth due to the limited influent organics (Liu and Tay, 2015; Rusanowska et al., 2018). Therefore, the formation of AGS with satisfactory settleability, good stability and ammonium removal ability under low OLR and high ALR is achievable, when a prolonged granulation process is induced due to the lack of organic substrates.

3.2. Variations of EPS concentrations of AGS under a low OLR

As shown in Fig. 2a, the contents of TB-PN and TB-PS, and TB-EPS continuously increased during the whole operation process. The

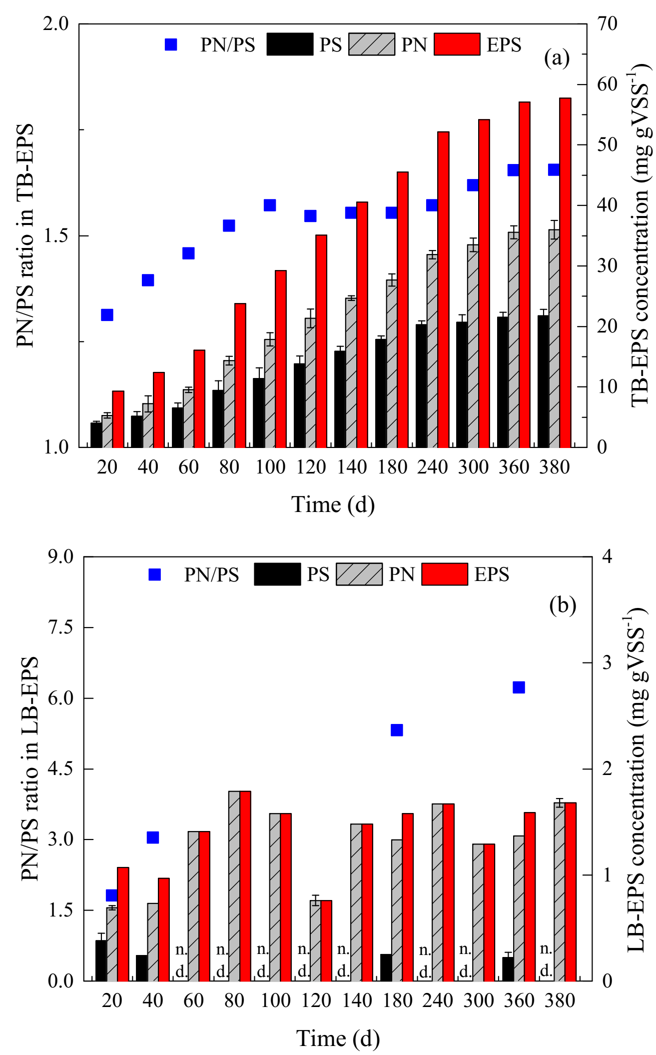

Fig. 2. EPS concentrations of AGS during the whole operation process: (a) TB-EPS; (b) LB-EPS. (n.d. means not detected).

uptrend of TB-EPS concentrations decelerated after Day 240, which was consistent with the variation trend of the granulation process. The TB-PN concentration was always higher than that of TB-PS during the whole process, when the ratio of TB-PN/TB-PS obviously rose. Fig. 2b exhibits that the contents of LB-PN, LB-PS and LB-EPS were very low during the whole operation process under low OLR, while LB-PN was the main component of LB-EPS. The Pearson correlation coefficients of the mean volume diameter with the TB-EPS, TB-PN, TB-PS and the TB-PN/TB-PS ratio are 0.9595 ($p < 0.001$), 0.9626 ($p < 0.001$), 0.9534 ($p < 0.001$), and 0.8388 ($p < 0.001$), respectively, which demonstrates the strong positive correlation between TB-EPS and the granule size.

The content of LB-EPS keeps at a very low level, while excessive LB-EPS is previously reported to play a negative role in cell attachment and aggregation stability (Shi et al., 2017). TB-EPS acts as the main existence form of EPS in AGS under low OLR in this study, however, the EPS contents of AGS in this study are obviously lower than those of AGS obtained under higher OLRs in previous reports (Luo et al., 2014; Yang et al., 2014; Zhu et al., 2015), which is due to the lack of organics supply under low OLR conditions. The components of EPS influence the properties of AGS (Sheng et al., 2010). PN promotes the aerobic granulation via changing the cell surface charge (Zhu et al., 2015) and increasing the sludge hydrophobicity (Zhang et al., 2019), while the loss of PN reportedly induces a significant disintegration of AGS (Xiong and Liu, 2013). Moreover, the increase of the PN/PS ratio is also found, and the ratio is usually reported to favor the formation and stability of AGS due to the high PN content (Kang and Yuan, 2017; Lv et al., 2014a; Zhu et al., 2012). In addition, the slow increase of PS in this work may be caused by the consumption of PS as a growth substrate (Wan et al., 2014). In summary, more TB-PN and TB-PS were produced in the larger AGS than those in the smaller ones, and the obvious increase of TB-PN/TB-PS confirms the critical role of TB-PN in aerobic granulation under low OLR.

3.3. Spatial and temporal distribution of AHLs under a low OLR

As shown in Fig. 3a, the long-chain AHLs were more abundant than the short-chain AHLs (i.e., C4-HSL, C6-HSL and 3O-C6-HSL) in the sludge phase. The contents of C4-HSL, C6-HSL and 3O-C6-HSL increased initially and then decreased gradually. C8-HSL stayed abundant during the operation process, while the concentration of 3OC8-HSL obviously decreased. C10-HSL and C12-HSL gradually accumulated to become dominant, while 3OC10-HSL appeared after Day 180 in the sludge phase. The total AHL concentration in the sludge phase (S-AHL) reached the peak value on Day 240, and slowly decrease from Day 240 to Day 360. As shown in Fig. 3b, C4-HSL, C6-HSL and 3OC6-HSL were more abundant in the water phase than the other AHL types. C8-HSL stayed at a low level during the operation process, while 3O−C8 occurred within the first 100 days. C10-HSL and C12-HSL appeared following Day 60 and Day 100, respectively. The total AHL concentration in the water phase (W-AHL) increased to its peak on Day 140 and then decreased.

Diverse AHLs promote bacterial aggregations such as biofilm formation and aerobic/anaerobic granulation through the regulation of EPS (Ding et al., 2015, Hu et al., 2016, Li et al., 2015, Ma et al., 2018, Tan et al., 2014, Xia et al., 2012). C10-HSL and C12-HSL are the key AHLs in the formation of biofilm in wastewater treatment in a recent report (Wang et al., 2019), while C6-HSL and C12-HSL are possibly involved in the ammonium oxidation metabolism of ammonia-oxidizing bacteria (AOB) (Liu et al., 2019). The long-chain AHLs accumulate more in the sludge phase than in the water phase, which is possibly due to the low aqueous solubility of long-chain AHLs (Feng et al., 2014). More S-AHLs exist in larger AGS than in flocculent seeding sludge, which accords with a previous report (Lv et al., 2014b). In the later period, the slight decrease of W-AHL and S-AHL is possibly induced by the role of bacteria with an AHL quenching ability (Fetzner, 2015),

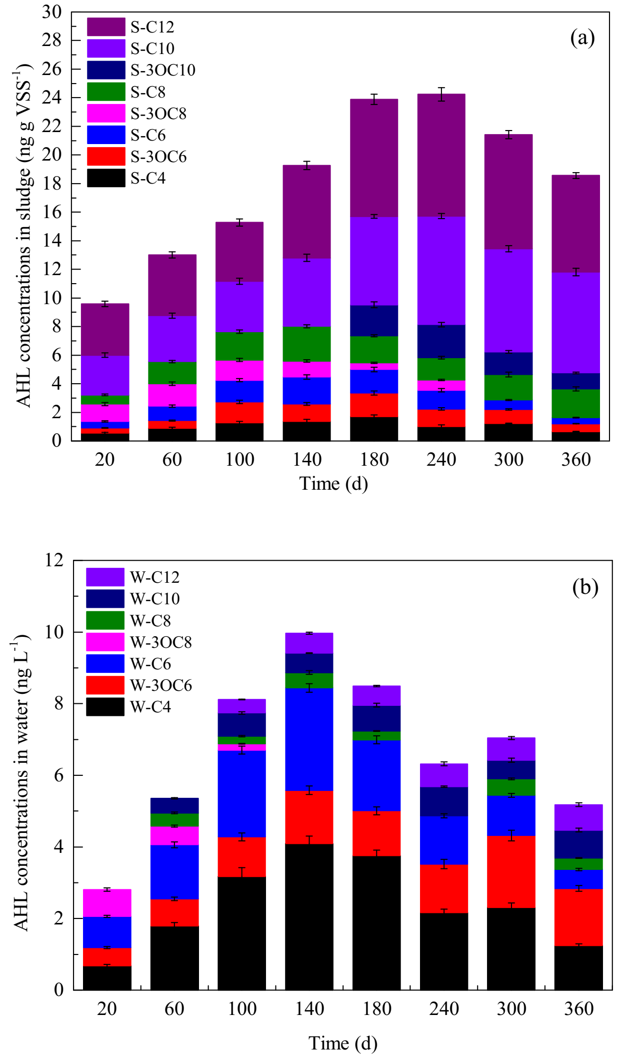

Fig. 3. Distribution of AHLs during the whole operation process: (a) AHL concentrations in the sludge phase; (b) AHL concentrations in the water phase.

which may control the balance of EPS production and substrate transfer, then avoid the overgrowth and instability of AGS. In summary, C10-HSL and C12-HSL gradually became dominant in the sludge phase, and short-chain AHLs dominated in the water phase under low OLR.

3.4. Microbial community analysis of AGS under a low OLR

As shown in Fig. S3a, the sequencing data was extensive enough to reflect microbial community information. The rank-abundance curves displayed that the OTU numbers decreased during the operation process (Fig. S3b), and the distinction between the initial community and later communities became gradually larger (Fig. S3c). The microbial diversity and richness shown in Table 1 overall reduce under a low OLR during the operation, although some fluctuations happened. As shown in Fig. 4, *Proteobacteria* (the relative abundance ranges from 34.48% to 61.29%) and *Bacterioidetes* (18.16%–42.20%) were dominant phyla during the whole operation process. *Actinobacteria* retained in the system with a fluctuant relative abundance of 1.90%–5.88%, while *Nitrospirae* maintained a high abundance at first and then dropped sharply. The microbial composition at the genus level in Fig. 5 reveals that *Nitrosomonas* was enriched during the operation process, while *norank_f_Nitrosomonadaceae* gradually disappeared. For nitrite-oxidizers, *Nitrospira* was relatively abundant in the early and middle stages, and then gradually faded away, while *Nitrobacter* became the dominant

Table 1
Estimators of microbial community diversity and community richness.

Sample	Time[a]	Sobs[b]	Shannon[c]	Simpson[d]	Ace[e]	Chao[f]	Coverage[g]
S1	Day 1	335	4.371833	0.026976	351.42775	359.473684	0.998798
S2	Day 60	340	4.294569	0.029991	347.456418	347.916667	0.999225
S3	Day 100	339	3.659663	0.061808	367.049146	357.8125	0.998333
S4	Day 140	352	3.734982	0.087642	387.474963	397.37037	0.998061
S5	Day 180	346	3.854058	0.059659	379.127399	379.6	0.9981
S6	Day 240	272	3.545489	0.092927	300.503652	302.875	0.998488
S7	Day 300	196	3.1723	0.077055	228.34254	242.866667	0.998527
S8	Day 360	200	3.521735	0.069881	220.316593	217.105263	0.998992

[a] The sampling time of sludge.
[b] The observed OTU numbers reflecting the community richness.
[c] The estimator of community diversity proposed by Shannon.
[d] The estimator of community diversity proposed by Simpson.
[e] The estimated OTU numbers reflecting the community richness.
[f] The estimated OTU numbers calculated by the Chao1 algorithm reflecting the community richness.
[g] The estimator reflecting the sequencing coverage of the sequencing library.

nitrite-oxidizer. EPS-producing genera, such as *Brevundimonas*, *Diaphprobacter*, *Comamonas*, *Ferruginibacter*, *Leadbetterella* and *Pseudoxanthomonas*, and *Thauera* successfully remained or accumulated in the system. Several genera closely related to denitrification, such as *Aeromonas*, *Denitromonas*, *Flavobacterium*, and *Gemmatimonas*, were nearly eliminated during the operation process (Fig. 5).

In this study, EPS producers successfully survived in the system, while some other heterotrophs were inhibited or even eliminated possibly due to the lack of organic substrate under low OLRs. AGS is an effective retention form for bacteria to avoid the washout under harsh situations, and the EPS secretion is essential for the bacterial attachment and the following aerobic granulation (Adav et al., 2008b; Sheng et al., 2010; Zhang et al., 2018b). As a result, EPS producers are urgently critical in the formation of AGS, and the survival of EPS producers may act a precondition of aerobic granulation (Huang et al., 2016; Shi et al., 2017). Besides the EPS producers, two autotrophic nitrifiers, *Nitrosomonas* and *Nitrospira*, are also enriched in AGS. *Nitrosomona* is enriched as a typical AOB to remove the high influent

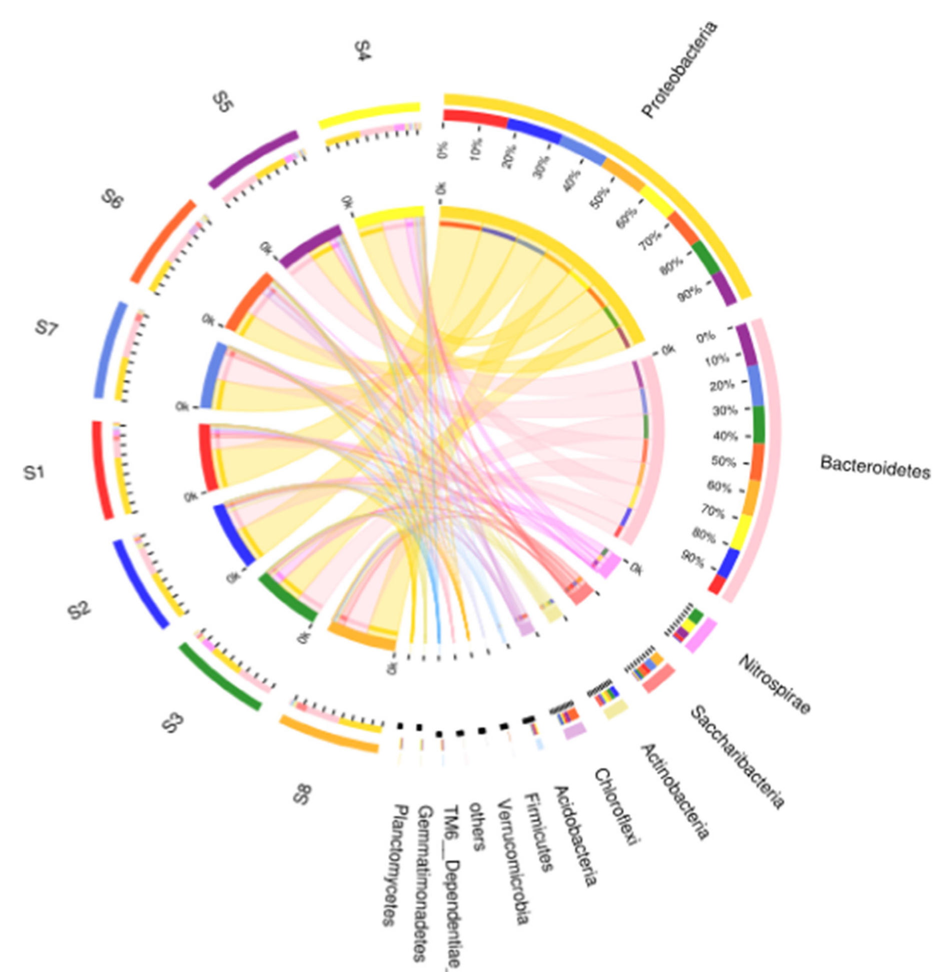

Fig. 4. Circos diagram of microbial composition at the phylum level. (Left and right semicircles represent different samples and different phyla.)

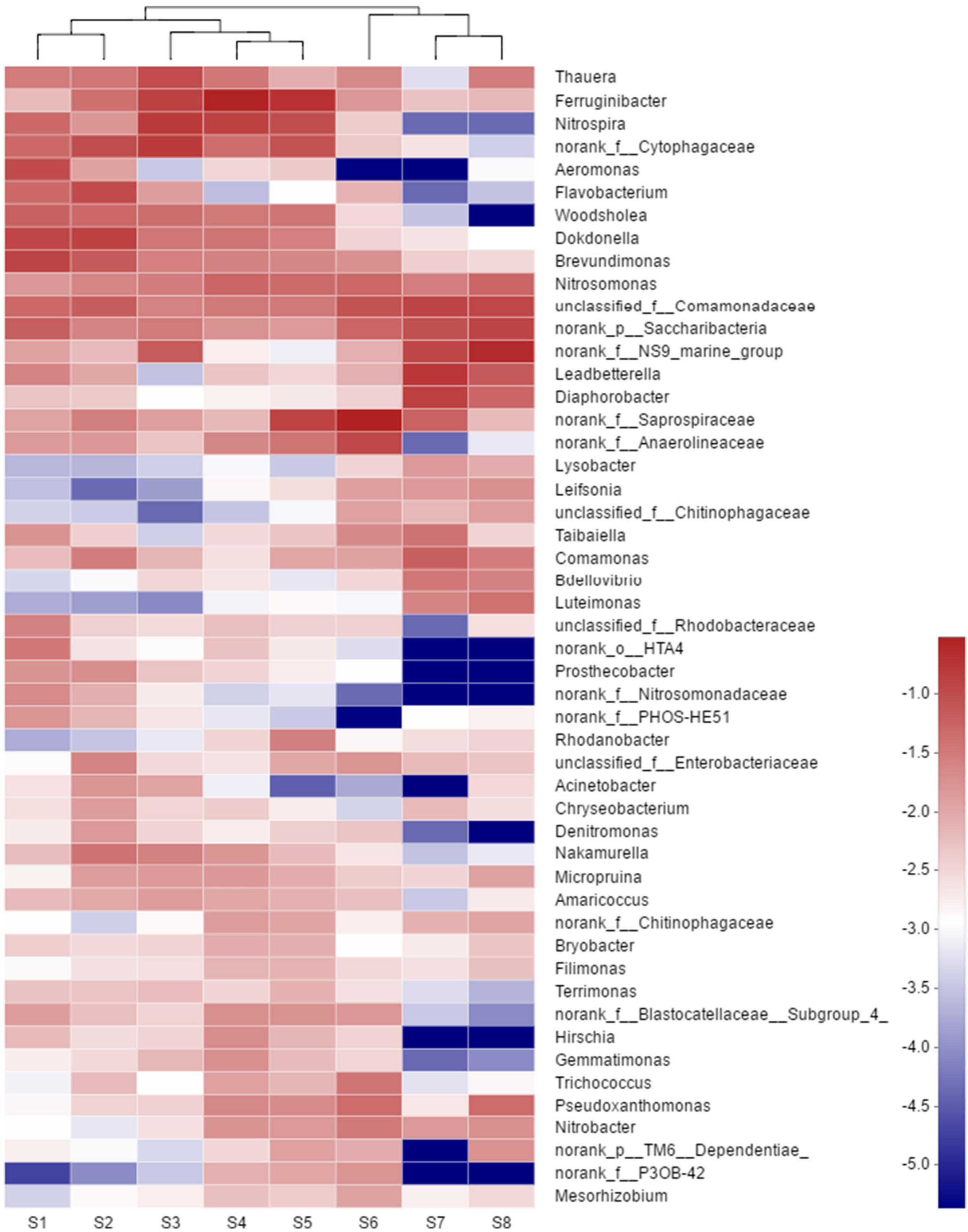

Fig. 5. Heatmap analysis of microbial composition at the genus level with the most abundant 50 genera. (The relative abundances were calculated as the logarithm values.)

ammonium (Zhang et al., 2011). *Nitrobacter* outcompetes *Nitrospira* during the dynamic shift of nitrite-oxidizing bacteria, which is probably due to *Nitrobacter* being more adapted to a sufficient nitrite and oxygen supply than *Nitrospira* (Belmonte et al., 2009; Isanta et al., 2015). According to Ni et al. (2008), the high N/COD ratio benefits the growth of autotrophic nitrifiers. The high ALR and low OLR in this study provide enough growth substrate and potential for autotrophic nitrifiers without the drastic competition of DO against heterotrophic bacteria. Moreover, the secretion of EPS in AGS may also protect the slow-growing nitrifiers from being eliminated (Zhang et al., 2011). In addition, the growth of slow-growing nitrifiers is reported to be beneficial to the AGS formation (Wan et al., 2009). Therefore, the low OLR alters the diversity and composition of microbial communities through the limited organics supply. EPS producers and autotrophic nitrifiers

Table 2
Pearson correlations of different AGS properties with AHL types.

Type	TB-PS	TB-PN	TB-EPS	TB-PN/TB-PS	Volume diameter	SVI_{30}/SVI_5
S-C4	0.35123	0.32074	0.33222	0.39276	0.12296	0.58443
S-3OC6	0.43106	0.40169	0.41277	0.47126	0.18635	0.64547
S-C6	0.10903	0.07287	0.08639	0.19462	−0.18832	0.40939
S-3OC8	−0.83725**	−0.84881**	−0.84475**	−0.64537	−0.93299***	−0.64567
S-C8	0.6048	0.60347	0.60415	0.80024*	0.42133	0.80751*
S-3OC10	0.75255*	0.73472*	0.7416*	0.49333	0.75245*	0.62597
S-C10	0.95646***	0.95271***	0.9544***	0.76185*	0.96252***	0.82467*
S-C12	0.90177**	0.88233**	0.88985**	0.66723	0.83686**	0.84003**
S-AHL	0.89144**	0.86879**	0.87751**	0.73558*	0.76822*	0.90514**
W-C4	0.30907	0.28041	0.29119	0.40312	0.03402	0.59348
W-3OC6	0.90173**	0.90756**	0.90566**	0.87344**	0.86751**	0.87481**
W-C6	−0.0822	−0.10924	−0.09918	0.08896	−0.36526	0.24692
W-3OC8	−0.93792***	−0.93026***	−0.9334***	−0.92385**	−0.80143*	−0.99927***
W-C8	0.28305	0.29637	0.29149	0.48636	0.26179	0.39471
W-C10	0.81235*	0.80812*	0.80994*	0.88685**	0.69814	0.8767**
W-C12	0.97513***	0.97379***	0.97459***	0.89124**	0.88943**	0.9548***
W-AHL	0.44251	0.42037	0.42876	0.56289	0.18149	0.70967*

The significant differences are indicated as follows:
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

successfully survive in the system, which favor the formation and stability of AGS under the low OLR conditions.

3.5. Correlation of AHL-mediated QS with AGS properties

As shown in Table 2, the W-3OC6-HSL, W-C10-HSL, W-C12-HSL, S-C10-HSL and S-C12-HSL had strong positive correlations with AGS properties including TB-PS, TB-PN, TB-EPS, granular size (volume diameter) and settleability (SVI_{30}/SVI_5). C4-HSL and C6-HSL in both sludge and water had no significant correlation with these sludge properties, while 3OC8-HSL in both sludge and water exhibited strong negative correlations with them. The role of AHL-mediated QS in the regulation of EPS production has been confirmed by the strong positive correlations between several AHLs and TB-EPS contents in this study, in accordance with previous results (Tan et al., 2014; Yuan et al., 2017). In the meantime, the S-AHL exerts significantly more influence on the regulation of EPS secretion than W-AHL based on the correlation coefficients of both S-AHL and W-AHL with AGS properties. And the AHL-mediated QS may indirectly influence the settleability and size of AGS through the regulation of EPS because the composition and content of EPS actually affect the properties of bacteria aggregation (Shi et al., 2017). The easier inactivation of AHLs in the water phase may induce the weak correlations of C4-HSL, C6-HSL and W-AHL with the AGS properties in this study (Yates et al., 2002). Since the ratio of PN/PS can act as an indicator to monitor the granulation degree (Zhu et al., 2015), the strong positive correlation of S-AHL with PN/PS confirms the significance of AHL-mediated QS in the regulation of TB-PN production and in the following formation of AGS under low OLR. In summary, AHL-mediated QS utilizes C10-HSL, C12-HSL and 3OC6-HSL as the critical AHLs to regulate the production and composition of TB-EPS in aerobic granulation.

3.6. Correlation of AHL-mediated QS with bacterial genera

As shown in Fig. 6, AHLs including S-C4-HSL, S-3OC6-HSL, S-C8-HSL and S-3O-C10-HSL significantly correlated with few genera, while four AHLs, S-C6-HSL, S-3OC8-HSL, S-C10-HSL and S-C12-HSL, had significant correlations with widespread genera. *Nitrobacter* positively correlated with S-3OC10-HSL ($p > 0.05$), S-C10-HSL ($p < 0.05$), S-C12-HSL ($p < 0.05$) and S-AHL ($p < 0.05$), while *Nitrosomonas* had a positive correlation with S-C10-HSL ($p > 0.05$) (Fig. 6). For AHLs in the water phase, W-C4-HSL, W-C8-HSL and W-C10-HSL have significant correlation with few genera, while W-3OC6-HSL, W-C6-HSL, W-3OC8-HSL and W-C12-HSL showed significant correlations with many genera (Fig. 7). According to previous studies, AHL synthases exist in *Nitrobacter winogradskyi* (Mellbye et al., 2016; Shen et al., 2016). *Nitrobacter winogradskyi* may produce C10-HSL according to Mellbye et al. (2015), and *Nitrosomonas europaea* produces C10-HSL as well (Burton et al., 2005). These two autotrophic nitrifiers are assumed to have an interspecific communication during aerobic granulation under a low OLR for that they may produce and sense the identical AHL (Mellbye et al., 2017). Moreover, the S-AHL has a wider relationship with bacterial genera than W-AHL, which indicates that the content and types of AHLs in sludge are critical for the bacterial communication in AGS. However, bacterial genera with high correlations are not always the direct AHL producer or quencher (Tan et al., 2014), since some AHL-irrelevant genera without the producing or quenching abilities of AHL may benefit or suffer from the AHL-mediated QS induced by other genera, which induces the strong correlations. In addition, AHL-mediated QS in complex environments is easily influenced by many abiotic and biotic factors that disrupt the correlation analysis between QS and microbial communities (Boyer and Wisniewski-Dye, 2009; Huang et al., 2016; Mukherjee and Bassler, 2019).

3.7. Correlation of AGS properties with bacterial genera

As shown in Fig. 8, *Leifsonia*, *Nitrobacter*, and *unclassified_f_chitinophagaceae* were positively correlated with the production of TB-PN and TB-EPS. *Luteimonas*, *Leifsonia*, *norank_p_Saccharibacteria* and *norank_f_NS9_marine_group* were positively correlated with the PN/PS ratio. Seven genera, including *Leifsonia*, *Bdellovibrio*, and *unclassified_f_Comamonadaceae,* were significantly positively correlated with the granule diameter representing the granulation degree. Two main nitrifiers, *Nitrosomonas* and *Nitrobacter*, were positively correlated with the SVI_{30}/SVI_5 representing the granule settleability ($p < 0.05$), while *Prosthecobacter*, *Dokdonella*, *Acinetobacter* and ten other genera showed significantly negative correlations. Some EPS producers, such as *Thauera* and *Comamonas*, show no significant correlations with TB-PN, TB-PS or TB-EPS in this study. The relative abundances of different EPS producers fluctuate during the whole operation, and diverse EPS producers may dominate in different periods, which induces weak correlation of EPS producers with the EPS indices. The proliferation of slow-growing nitrifiers is beneficial to the formation of AGS (Wan et al., 2009), and AGS with strong settleability may promote the retention of

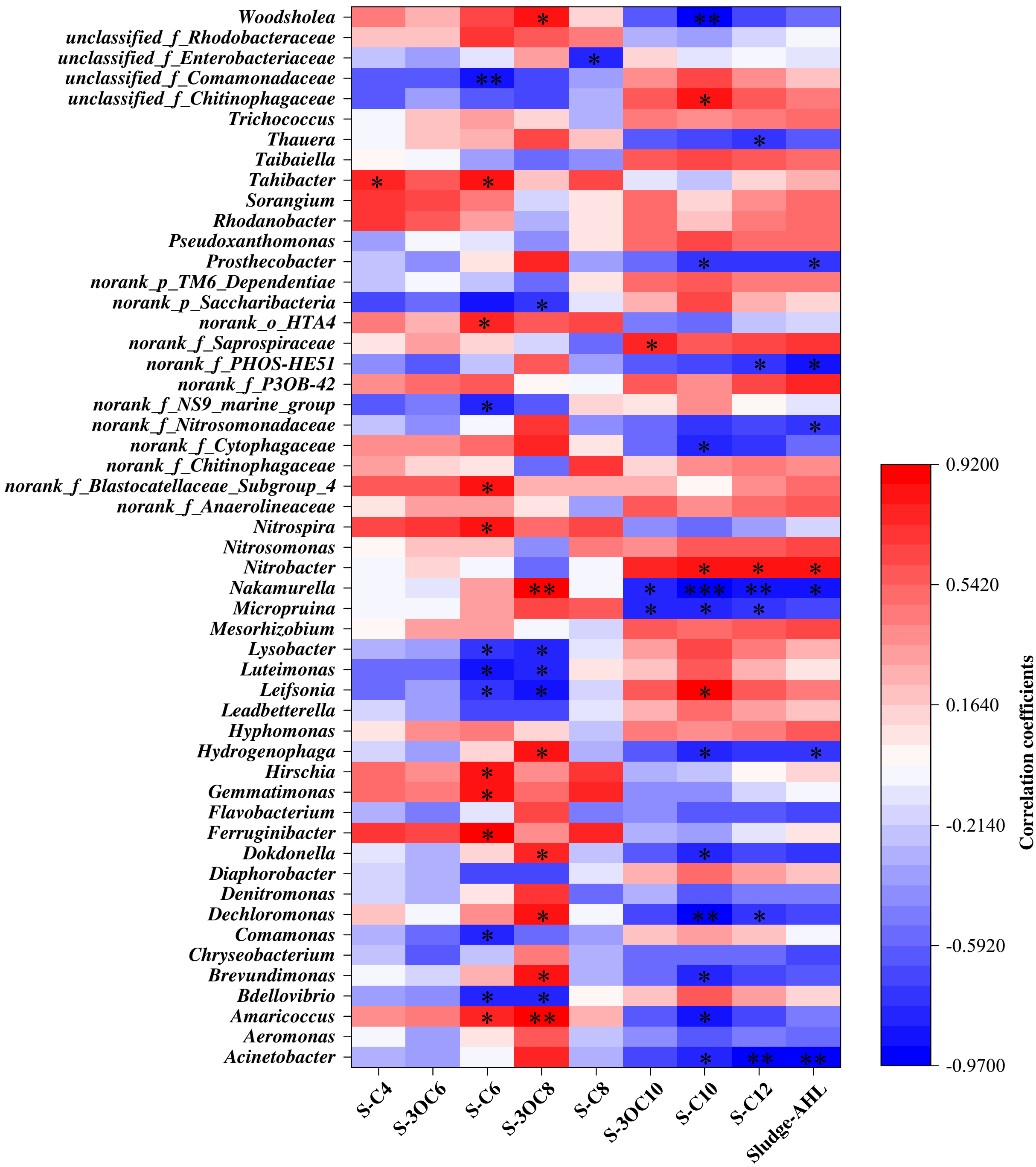

Fig. 6. Pearson correlations of AHLs in sludge with bacterial genera. (*p < 0.05, **p < 0.01 and ***p < 0.001).

nitrifiers (Zhang et al., 2011), which may accord with the positive correlations of nitrifiers and SVI_{30}/SVI_5. The genus *norank_p_Saccharibacteria* may contribute to the increasing hydrophobicity (Chen et al., 2019), which shows a significant correlation with the TB-PN/TB-PS. In summary, the slow-growing autotrophic nitrifiers is closely related to the AGS settleability, while the correlations between EPS producers and AGS properties are not obvious in this study due to the fluctuation of microbial community composition in this mixed-culture system.

4. Conclusions

This study comprehensively investigated the AHL-mediated QS and microbial community characters in the AGS fed with ammonium-rich wastewater under a low OLR of 0.15 kg COD $(m^3\ d)^{-1}$. Obtaining a stable AGS with satisfactory settleability and ammonium removal ability at a low OLR of 0.15 kg COD $(m^3\ d)^{-1}$ was feasible when the low OLR induced a prolonged granulation process due to the lack of organic substrates. More TB-PN and TB-PS were produced in the larger AGS, and the obvious increase of TB-PN/TB-PS confirmed the critical role of TB-PN in aerobic granulation. C10-HSL and C12-HSL gradually became dominant in the sludge phase, and short-chain AHLs dominated in the water phase under low OLR. EPS producers and autotrophic nitrifiers successfully survived in the system, which favored the formation and stability of AGS under low OLR. AHL-mediated QS utilized C10-HSL, C12-HSL and 3OC6-HSL as the critical AHLs to regulate the production and composition of TB-EPS in aerobic granulation. Autotrophic nitrifiers may perform interspecific communication with C10-HSL under low OLR. The correlations of bacterial genera with AGS properties and

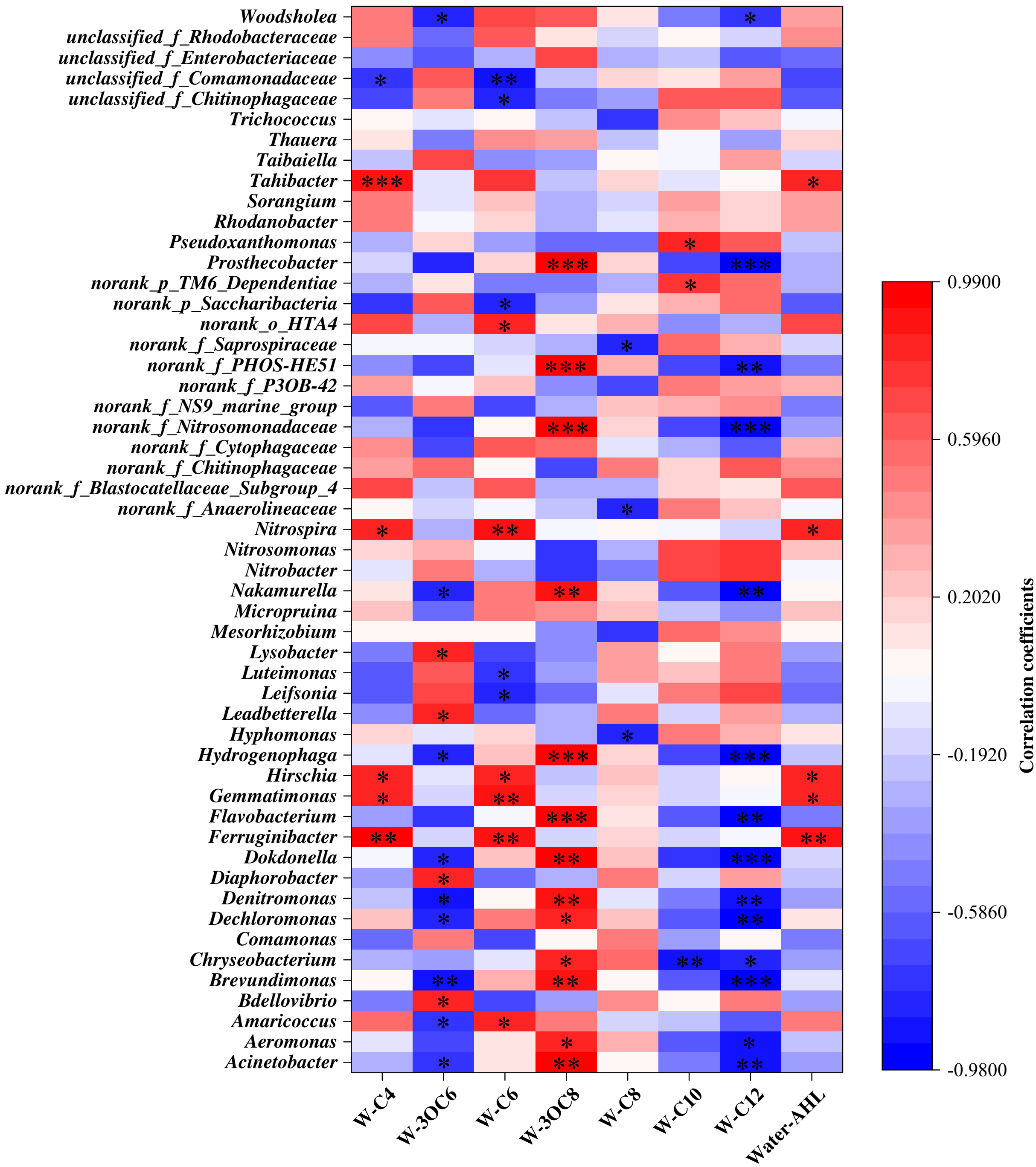

Fig. 7. Pearson correlations of AHLs in water with bacterial genera. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

AHL-mediated QS were complex under low OLR for the dynamic fluctuations of microbial composition and other variable factors in the mixed-culture system. The findings of this work may supplement the comprehension of QS regulation in the formation of AGS under low OLR from a microbial viewpoint.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.104946.

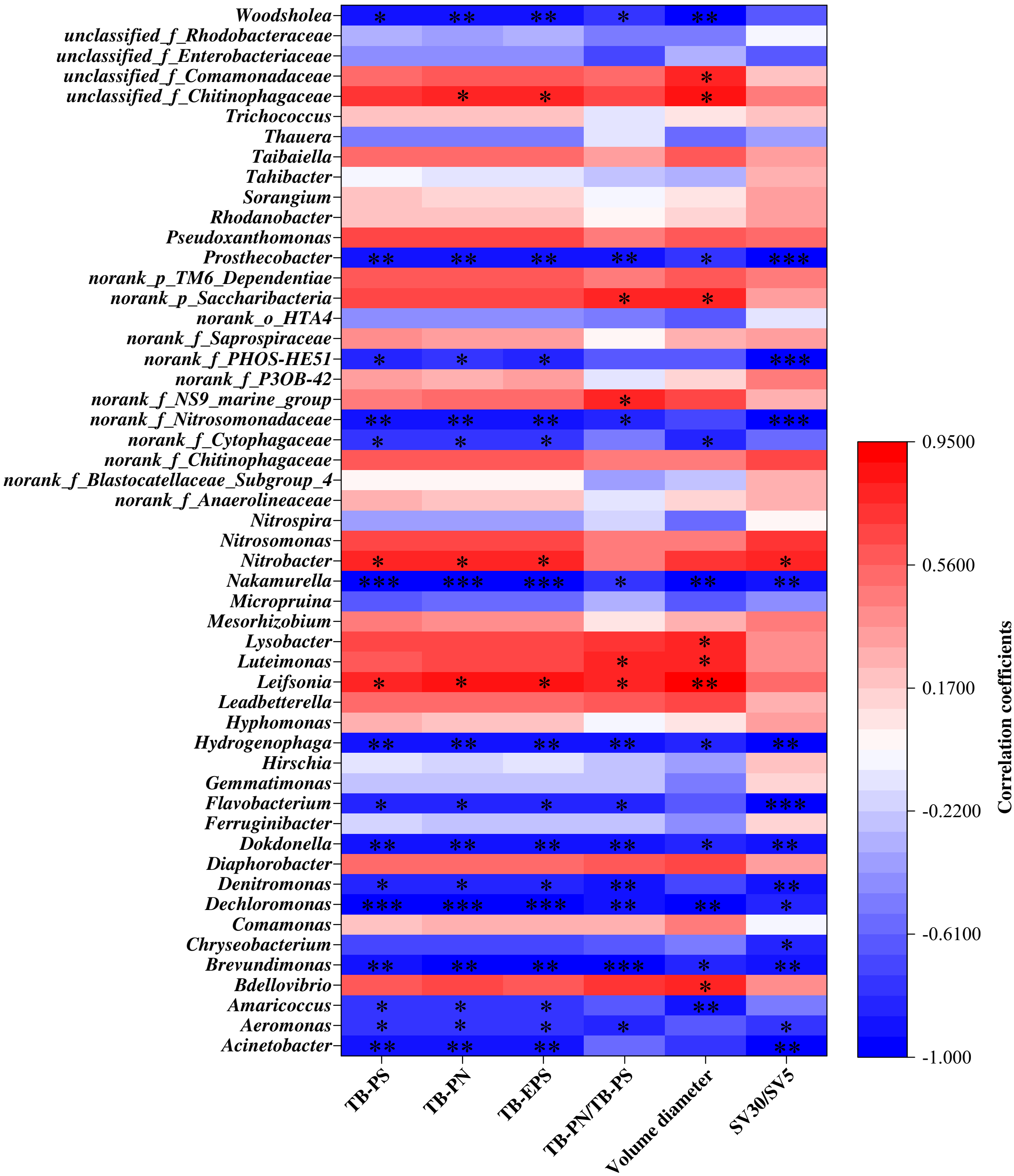

Fig. 8. Pearson correlations of different AGS properties with bacterial genera. (*p < 0.05, **p < 0.01 and ***p < 0.001).

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