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**N-Acyl-homoserine lactone-mediated quorum sensing of aerobic granular sludge system in a continuous-flow membrane bioreactor**

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**ABSTRACT**

The investigation aimed at revealing the N-acetyl-homoserine lactone (AHL)-mediated quorum sensing (QS) of aerobic granular sludge (AGS) system in a membrane bioreactor (MBR) with internal circulation. During the experimental period, the MBR was operated under a continuous-flow mode, in which, AGS was cultivated directly. The specific AHL molecules were determined and the correlation between the AHL-mediated QS, extracellular polymeric substance (EPS) production and microbial community was explored. Two kinds of specific signaling molecule, N-octanoyl-homoserine lactone (C8-HSL) and N-tetradecanoyl-homoserine lactone (C14-HSL), were detected in the cultivated AGS, but C14-HSL was the major one, which was detected to be ranging from 86.58 ng/gMLSS to 594.00 ng/gMLSS among all samples. Most importantly, the concentration of C14-HSL was positively correlated to the production of LB-EPS and TB-EPS (r > 0.906, P < 0.034, minimum 'r' and maximum 'p'), which demonstrated it’s a crucial component in the formation of AGS and determined its stability. The results indicated that the structure of microbial community showed weak correlation with the content of AHls and EPS if without the pressure of microbial selection. But, the conditions within the bioreactor determined the microbial community, and the contained functional bacteria released signaling molecules to promote the granulation.

**1. Introduction**

Aerobic granular sludge (AGS) is a kind of self-aggregated microorganisms formed under suitable hydrodynamic and nutritional conditions, and the first appearance of AGS was reported in 1991 [1]. So far, the merits of AGS, including high content of biomass, high resistance to shock of organic loading rate (OLR), rapid settling velocity and rich biodiversity, have been verified to be capable of treating both industrial and domestic wastewater with less energy consumption and space requirement by so many lab-, pilot-, and even full-scale studies [2,3]. Moreover, the special structure of AGS combines coupling anaerobic and aerobic conditions into a single sludge granule, which enables it possible to remove organics and nutrients simultaneously [4,5]. In previous studies, AGS was used to be cultivated in a sequencing batch reactor (SBR), in which, an alternating feast-famine feeding mode and the periodic drainage pattern created a unique external environment for microorganisms, which provided suitable selection pressure for promoting the primary growth of AGS to be mature with compact structure and smooth shape [6]. However, SBRs, actually are operated under a batch mode and capable of treating relatively low-volume wastewater, and there are generally high suspended solids (SS) containing in the effluent. Therefore, it’s quite essential to develop a new process to cultivate AGS under a continuous-flow mode to meet the practical requirements for treating high-volume of wastewater.

The membrane bioreactor (MBR) technique, combining CAS and membrane filtration, is generally operated under a high concentration of biomass, which has been verified to be an efficient process for treating both industrial and domestic wastewater [7]. The most dominant advantage of MBRs is their high-quality effluent with less foot-print, but the energy consumption of MBRs is about 50 % higher than that of a CAS process [8]. Additionally, previous investigation also indicated that membrane fouling was a major “bottleneck” in the scientific research and engineering application of MBRs due to the necessity of frequently physical and chemical cleaning for the used membrane modules, which

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increased the energy consumption of the whole MBR process [9]. Recent years, lots of work focused on the mechanisms leading to membrane fouling from different perspectives, and the results indicated that the degree of membrane fouling was mainly related to the size of bio-flocs [10]. In this regard, changing the size of bio-flocs in an MBR might be a promising approach, such as introducing AGS into an MBR, to mitigate the membrane fouling for a long-term operation and reduce energy consumption.

Actually, combining the advantages of AGS and MBR has aroused widely interests, for example, Li et al. [11] first introduced mature AGS into an MBR and got excellent results for nutrients removal and membrane fouling mitigation. So far, seeding AGS from a stably operated SBR into an MBR is a commonly adopted approach [12]. However, it’s quite difficult to maintain the long-term stability of AGS in an MBR due to its lacking of suitable conditions as that in SBRs [13], thus, it has to add mature AGS continuously into the MBR to keep it stable running. In a previous study, an MBR with an internal circulation was operated under a continuous-flow mode, in which, mature AGS was directly cultivated and then kept stable for a long time [14]. Though, the unique hydraulic conditions and filamentous bacteria were considered to play important roles to the process of aerobic granulation, the underlying molecular biological mechanism is still not well-understanding and also should be further explored.

In recent years, a cell-to-cell communication system, occurred among different functional bacteria termed as quorum sensing (QS), was verified to be an exact mechanism responsible for microbial aggregation [15]. Generally, the functional bacteria can produce and release signaling molecules, named as an auto-inducer (AI), to regulate the behavior and gene expression of the bacteria to adapt the environmental pressure [16]. Such a result provided lots of new opportunities for further understanding the essential mechanism of the granulation of AGS from a molecular biological perspective. N-acetyl-homoserine lactone (AHL) is a kind of signaling molecule that is produced and utilized by Gram-negative bacteria to communicate within intra-species [17]. During a granulation process, numbers of microorganisms gradually aggregate to form a special microbial community, in which, the secreted extracellular polymeric substance (EPS) adheres those microorganisms together [18]. Moreover, the AHL-mediated QS was so far demonstrated to be related with the formation of AGS in SBRs [19]. Chen et al. [20] found that the bacteria utilized specific signaling molecules to regulate the production of tightly bound (TB)-EPS during an AHL-mediated aerobic granulation process, and Zhang et al. [21] also indicated that AHL-mediated QS was essential in the process of microbial aggregation. Moreover, Tan et al. [22] identified that there was a close correlation among AHLs, EPS production and microbial community. Besides, it was notable that the operating conditions would affect the AHL-mediated QS system within the AGS during its aerobic granulation process [23–25]. Nevertheless, almost all the reported AGS was cultivated in SBRs, and it was very difficult to cultivate them directly in an MBR under a continuous-flow mode, thus, the granulation of AGS in MBRs from the perspective of AHL-mediated QS was rarely reported.

The aim of this work mainly focused on revealing the AHL-mediated QS within AGS in an MBR, therefore, an MBR with internal circulation was operated under a continuous-flow mode to cultivate AGS directly, in which, several related aspects were mainly considered: 1) variation of EPS content during the granulation process; 2) identification of the specific AHLs and their concentrations; 3) variation of different functional bacteria during the granulation of AGS; 4) correlation among of the signaling molecules, EPS production and microbial community. It hopes to reveal the molecular biological mechanism for the granulation of AGS in an MBR under a continuous-flow mode, which is expected to provide a useful reference for the large-scale application of this novel process in the future.

2. Materials and methods

2.1. Sample collection

An MBR with internal circulation was continuously operated for more than two years under a continuous-flow mode, whose effective working volume was 36 L with a rectangular shape, and it was divided into a mixing and an aeration zone by a baffle. A membrane module was set in the middle of aeration zone, while a rotator was mounted in the middle of mixing zone, and an internal hydrodynamic circulation would be formed by the upward flow in the aeration zone and downward flow in the mixing zone. With the aid of some functional bacteria with special structure (such as filamentous bacteria), biomass gradually aggregated, and they were also squeezed and polished by the internal circulation, then became AGS with clear and smooth edge [14]. Before the present experiment, the MBR was continuously operated under the same operating conditions with the previous study, in which, AGS actually has formed for a long time. The procedure to collect AGS samples included the following steps: 1) in a five-day period, 1 L mixed sludge were collected every day from the same location in the MBR; 2) a series of standard stainless steel screens were used to divide the obtained AGS samples into five categories according to their granule size (<0.2 mm, 0.2–0.5 mm, 0.5–0.7 mm, 0.7–1.0 mm and >1.0 mm); 3) the classified granules were then labeled by their size as S1 (<0.2 mm), S2 (0.2–0.5 mm), S3 (0.5–0.7 mm), S4 (0.7–1.0 mm) and S5 (>1.0 mm), respectively. The morphology of biomass was observed with an optical microscope (CX41, Olympus, Japan), and the images were snapped with an attached digital camera.

2.2. Extraction of signaling molecule

The AHLs of AGS was extracted by following the procedure of Ma et al. [26]. First, a certain amount of biomass was re-suspended in 40 mL of ethyl acetate solvent, and an ultrasonic cell disruptor (SM-900A, Chen et al. [20]. First, a certain amount of biomass was re-suspended in 40 mL of ethyl acetate solvent, and an ultrasonic cell disruptor (SM-900A, Japan) was employed to disrupt the cells. The culture supernatant was collected and filtrated with 0.45 μm filter and then taken and stored at −80 °C for future analysis.

2.3. Identification and quantification of the specific AHL molecules

The qualitative and quantitative analysis of AHL was conducted by referring to a published report [27] with a slight modification, and its procedure was shown as follows: 1) an UPLC-MS/MS (Thermo Fisher, TQ Endura, USA) with a Hypersil GOLD C18 column (50 × 2.1 mm, 1.9 μm) was conducted to analyze the samples, and the flow rate was 0.3 mL/min; 2) the mobile phase was comprised of solvent B (methanol) and solvent A (water with 0.1 % formic acid) with a gradient elution (10–90 %); 3) electrospray ionization source was used to ionize effluents under positive mode; 4) the characteristic of putative AHLs, including retention time, precursor ion and transition ion, were identified by conducting the matrix-matched multiple reaction monitoring experiment. In all the experiments, the standard substances, including N-hexanoyl-DL-homoserine lactone (C6-HSL), N-octanoyl-DL-homoserine lactone (C8-HSL), N-decanoyl-DL-homoserine lactone (C10-HSL), N-(3-oxododecanoyl)-D-homoserine lactone (3OC12-HSL) and N-tetradecanoyl-DL-homoserine lactone (C14-HSL), were purchased from Sigma-Aldrich.

For AHLs quantification, a quantitation transition ion was employed to draw the matrix-matched standard curves in the range from 0.5 to 100 ng/mL. Thermo Scientific Xcalibur 4.1, 31 software was used to integrate the analyte peak areas. The limits of detection and quantification for all AHLs were calculated with a signal-to-noise ratio of 3 and
10, respectively. In terms of avoiding the overload of samples, blank injections were conducted between the sample injections, and the detailed detection parameters of the different AHLs are listed in the Supporting Information (SI, Table S1).

2.4. EPS extraction

EPS extraction process was performed by citing a published method [28] with some modifications: 1) certain amounts of biomass were placed in a centrifugational tube, 40 mL NaCl solution (0.05 %) was added into the tube at 60 °C for 10 min, then it was vortexed for 1 min, centrifuged at 6000 × g, 4 °C for 15 min, the obtained supernatants were termed as the light-band EPS (LB-EPS); 2) after that, adding 0.05 % NaCl solution to the initial volume, and it was put in a water bath at 80 °C for 30 min, vortexed for 1 min, centrifuged at 6000 × g, 4 °C for 15 min, the obtained supernatants were termed as the tightly-band (TB-EPS); 3) the polysaccharide (PS) component in EPS was measured by the phenol-sulfuric acid method with glucose as a standard [29], the protein (FN) component of EPS was determined by a modified Lowry colorimetric method with bovine serum albumin (BSA) as a standard [30].

2.5. High-throughput sequencing analysis of the microbial community

The composition of the microbial community of each sample was revealed by high-throughput sequencing method (HTS). The E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) was used to extract total DNA of each sample on the basis of its manufacturer’s protocol. The quantitative and qualitative analysis of extracting total samples was both conducted by using a NanoDrop 2000U−v is spectrophotometer (Thermo Scientific, Wilmington, USA) and 1 % agarose gel electrophoresis. Then a thermocycler polymerized chain reactions (PCR) system (GeneAmp 9700, ABI, USA) was used to amplify the extracted DNA with the primers of 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), the detail of the PCR amplification was performed as described by following a published report [31]. Moreover, a 2 % agarose gel was conducted to check the obtained PCR products after PCR amplification, then the obtained PCR amplification was purified by the method of a published method [32]. Finally, an Illumina MiSeq PE250 platform from Majorbio Biopharm Technology (Shanghai, China) were used to analyze the purified products.

2.6. Statistical analysis

The software, IBM SPSS (version 20) and Prism (GraphPad), was conducted to analyze the Statistical data. The Pearson correlation coefficients were calculated to evaluate the relationship among different variables, and the significant difference of the obtained data was indicated as follows: *P < 0.05, **P < 0.01 and ***P < 0.001.

3. Results and discussion

3.1. Distribution of specific AHLs

During the whole experimental period, the organic load and substrate in the influent was almost kept constant, and the bioreactor was operated continuously and stably, as shown in SI, the effluent was almost kept at a high quality, excepting several points, especially the removal of TN, it’s speculated to be caused by the abrupt fall of temperature at that time. Simultaneously, the AGS samples were collected and sieved, whose optical images are shown in SI. It was observed that the morphology of AGS in S2 was irregular, and along with the granulation process, the shape of AGS gradually became clear and regular (from S2 to S4), and then some fracture at the edge of the AGS appeared when AGS developed from S4 to S5. Moreover, as observed in the optical images, S1 was defined as the floc sludge, and S2-S5 were determined to be the AGS samples according to their size distribution and morphological observation. Meanwhile, in terms of the granulation process, S1-S5 could be regarded as the different granulation phases [33]. After the samples were collected, the contained various AHLs were measured according to the methods as described in Section 2, and the results are shown in Fig. 1.

C8-HSL and C14-HSL were detected in all samples, while, the other AHLs were not found due to their low concentration. Actually, different microbial species are able to produce and secrete various types of signaling molecule, which are also influenced by their surrounding conditions, thus, it is assumed that the existence of AHLs within the AGS system is determined by the microbial community and operational conditions in this MBR. Moreover, it was worthwhile to note that the concentration of C8-HSL was too low to be quantified accurately, but on the contrary, the concentration of C14-HSL showed high enough to be detected in all samples (Fig. 1), which indicated that C14-HSL was the major signaling molecule that produced and utilized by the microorganisms in the study. It was observed that the C14-HSL concentration of S1 was lower than the other samples, and the C14-HSL concentration of S2 was the highest level. However, the C14-HSL concentration decreased when granule developed from S2 to S3. Interestingly, the second rising occurred in the C14-HSL level from S3 to S4. Finally, the C14-HSL concentration decreased once again with the granule changing from S4 to S5. It was notable that Tan et al. [22] also found that some specific signaling molecules showed the similar variation trend with the C14-HSL profile in the present study during the granulation process.

It is well known that the AHL-producing bacteria produce AHLs, but the AHL-quenching bacteria play the opposite role to degrade AHLs [34], and in a CAS process, the AHL-producing and -quenching bacteria generally co-exist [35]. Tan et al. [36] also suggested that AGS was the harbor for both QS and Quorum Quenching (QQ) organisms, and the overall concentration of AHLs was actually a balancing result between the microorganisms of QS and QQ [23]. The results in Fig. 1 were actually caused by the balanced distribution of different functional bacteria and their population. In brief, the type of signaling molecule was determined by microorganism species [37], and its concentration was heavily influenced by the population of functional microbes inside [20]. Besides, it was confirmed that AHL-mediated Q5 was essential to the formation of AGS, after which, the denitrification was gradually enhanced.

3.2. EPS analysis

EPS is a kind of macromolecule polymers, which is produced by the functional bacteria [38], and it has been demonstrated to be essential for AGS granulation process and maintaining its stability [39]. For the sake of Fig. 1. Content of C14-HSL in different samples.
of revealing the variation of EPS during the process of forming and developing AGS, the content of proteins (PN) and polysaccharides (PS) in AGS with different sizes was determined and the obtained results are shown in Fig. 2.

The contents of LB-EPS and TB-EPS in S1 were much higher than that in other samples, and there was a dramatic reduction in the content of LB-PN and TB-PN from S1 to S2. Subsequently, the contents of LB-PN and TB-PN increased from S2 to S3, and then kept a relatively stable level even along with the further development of AGS. Moreover, the LB-PS content continuously decreased along with the growth of AGS size among the AGS samples, and the contents of LB-PS and TB-PS declined to a relatively lower level in S5. Besides, the PS to PN ratio (including LB-PS/PN and TB-PS/PN) increased rapidly from S1 to S2, then a dramatic reduction was observed from S2 to S3, and the PS/PN (including LB-PS/PN and TB-PS/PN) ratio of S5 finally decreased to the value closing to that of S1.

Under the influence of an external hydrodynamic force, microorganisms generally produce more EPS for self-protection, and when floc sludge transforms into AGS, the contained EPS is degraded to produce energy for AGS to survive [40]. The excessive LB-EPS and TB-EPS in S1 seemed to be caused by forming AGS in the present study, but the excessive LB-EPS might be adverse to the formation process of granules [41], thus, the LB-EPS within AGS samples were much lower than that of floc sludge. Moreover, the results showed that PN was the main component in all samples except in S2 (Fig. 2), which implied that microorganisms were inclined to use PN as an energy source, and the variation of LB-PS and LB-PN might be caused by the needs of maintaining the stability of larger AGS [42].

3.3. Correlation of AHL-mediated QS and EPS production within AGS system

Previous studies have found that the microorganisms could sense the signaling molecules and then use them to regulate their gene expression, besides, the content and composition of the EPS play a significant role in forming aerobic granular sludge, and it was also found that EPS production was related to the concentration of AHLs within AGS [19, 39, 43]. Based on the above results, the relationship between EPS production and AHL concentrations should be further revealed, and in here, the calculated Pearson correlation coefficients were shown in SI (Table S2). Despite, there are no direct evidences to show the correlation between the signal molecule and the size of AGS. However, the results showed that there was a strong positive correlation between the AHL concentration with LB-PS/PN and TB-PS/PN (\( r > 0.906, P < 0.034 \), minimum ‘r’ and maximum ‘p’). Therefore, it was considered that the production of LB-EPS and TB-EPS was both regulated by AHL-mediated QS within the AGS system, and the size of AGS might be indirectly influenced by the AHL-mediated QS through the EPS production [42]. In summary, the AHL-mediated QS utilized the signaling molecule C14-HSL to regulate the production of EPS during the formation and development of AGS.

3.4. Microbial community analysis

3.4.1. Diversity of microbial community

The production of AHL is the regular metabolic activity of a certain kind of microorganisms, whose consequence may cause the formation of AGS, and has a close relationship with the composition of their microbial community. In this section, the HTS method was used to reveal the microbial community of each sample, and the results are shown in SI (Table S3).

As shown in SI, total of 35365-42146 sequences (37561, 35365, 42146, 38947, and 39211 for S1, S2, S3, S4 and S5, respectively) were obtained from the samples, which were assigned to 372-654 operational taxonomic units (OTUs) (372, 567, 654, 601 and 606 for S1, S2, S3, S4 and S5, respectively) at a 97 % sequence identity threshold, indicating that the relative abundance of bacterial community gradually increased when floc sludge (S1) transformed into mature AGS (S3), and the relative abundance of bacterial community decreased with the further development of AGS (S3 to S5).

Additionally, according to the results of HTS, the indexes of Ace and Chao were used to characterize the species richness, and the Shannon and Simpson index were used to evaluate the biodiversity of the microbial community within the MBR, and the calculated results are shown in SI (Table S3). The microbial richness and diversity drastically increased when floc sludge transformed into small-sized AGS, after that the microbial richness continuously increased along with the small AGS developed to mature AGS, while the microbial diversity decreased accordingly. Finally the microbial richness and diversity decreased as the further development of mature AGS. Such results were in accordance with the rarefaction curves and the relative abundance curves shown in Fig. 3(A) and (B)). Moreover, the high coverage values (SI, Table S3), the plateaued rarefaction curves and the Shannon index curves (Fig. 3 (A) and (C)) indicated the used samples were available enough.

Without microbial selection pressure, the granulation process was considered to be similar to that of Zhou et al. [44], who used a complete mixing bioreactor to cultivate AGS. When the floc sludge transformed into small granules, the microbial richness and diversity actually increased because of the formed coupling anoxic/anaerobic zones. Besides, with the further development of small granules (S2 to S3), the microbial richness increased due to the attachment of flocs, but the microbial competition within the granules would gradually decrease the microbial diversity. Moreover, the microbial competition and mass transfer limitation inside the granules decreased the microbial richness and diversity. In the final phase (S5), the death of bacteria might cause the decreasing of microbial richness, but the microbial diversity seemed

![Fig. 2. EPS contents in different samples: (A) LB-EPS; (B) TB-EPS.](image-url)
not change obviously. The Venn diagram (SI, Fig. S3) indicated that although the microbial compositions of S1-S5 were different, some microorganisms indeed participated in the granulation process. Additionally, the shared OTUs among the samples also indicated continuous variation really occurred during the granulation of AGS.

3.4.2. Microbial population dynamics

To further investigate the variation of microbial population during the granulation of AGS, the microbial communities at the phylum and genus level based on the HTS results were obtained and are shown in Fig. 4.

In Fig. 4(A), six phyla, including *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Acidobacteria*, *Firmicutes*, *Verrucomicrobia*, were identified, in which *Proteobacteria* and *Bacteroidetes* were the dominant phyla in all samples. Compared with S1, *Bacteroidetes*, *Chloroflexi*, *Acidobacteria*, *Firmicutes* and *Verrucomicrobia* were enriched within the samples of S2 to S5, excepting a reduction of *Proteobacteria* in their relative abundance, implying the co-existence of anoxic/anaerobic and aerobic zones might be beneficial to the enrichment of these phyla [22]. Moreover, the relative abundance of *Proteobacteria*, *Acidobacteria* and *Verrucomicrobia* was enriched when the smaller AGS (S2) developed to the larger sample (S4), while the relative abundance of *Bacteroidetes*, *Chloroflexi* and *Firmicutes* decreased with the enlargement of AGS size, but *Proteobacteria*, *Acidobacteria* and *Firmicutes* decreased in contrast, and at the same time, the relative abundance of *Bacteroidetes*, *Chloroflexi* and *Verrucomicrobia* increased from S4 to S5. The relative abundance of *Proteobacteria* in the bioreactor was much higher than the results of a previous study [45], which might be caused by the different configuration of bioreactor and corresponding operational conditions. The relative abundance of *Proteobacteria* showed a negative Pearson correlation with that of *Bacteroidetes* ($r = -0.975$, $P = 0.005$) and *Chloroflexi* ($r = -0.893$, $P = 0.041$), which implied that there was a strong competition among these bacteria.

Twenty-two main genera were detected at the genus level (the relative abundance $> 0.01\%$) (Fig. 4(B)), in which, an obvious increasing was observed in the number of dominant bacteria. The relative abundance of some genera gradually decreased with the formation and development process of AGS, while on the contrary, some other genera enriched during the same granulation process. However, most genera fluctuated during the granulation process in their values of relative abundance. It was observed that *Thiothrix* accounted for 57.70 % within S1, which were the dominant filamentous bacteria, and played an important role in initiating the formation of AGS [14]. When floc sludge (S1) transformed into small AGS (S2), the relative abundance of *Thiothrix*, *Haliangium*, *Stella* and norank_f__Chitinophagaceae decreased, while the other 18 kinds of genera were enriched. Especially, the relative abundance of *Thiothrix* decreased markedly (from 57.70 % to 16.99 %), while unclassified_f__Polyangiaceae significantly enriched from 0.02 % to 16.99 %, and *Thiothrix* and unclassified_f__Polyangiaceae were the dominant genera within S2. Subsequently, *Sorangium* became the dominant genus after a dramatic enrichment within S3 and S4, and unclassified_d_o__Xanthomonadales continuously increased in their relative abundance, while the relative abundance of *Thiothrix* and unclassified_f__Polyangiaceae decreased along with the maturation of the bioreactor.
AGS, thus, in S3 and S4, *unclassified_o_Xanthomonadales*, *unclassified_f_Polyangiaceae* and *Thiothrix* became the dominant genera. Moreover, it should be noted that once AGS was in the dispersed phase, the relative abundance of most genera actually decreased, while *unclassified_o_Xanthomonadales* were enriched and they accounted for 19.82 % in S5, and became the dominant genera. Compared with the granulation process in SBRs, there was no obvious microbial selection pressure in the used MBR, in which, the microbial composition seemed more complex, and it might be attributed to the complex hydrodynamic conditions within the bioreactor.

It should be noted that some functional bacteria involving in the AHL-mediated QS were actually detected in the presented works, such as *Sphingomonas*, *Rhodobacter*, *Zoogloea* and *Sorangium*. According to the previous studies, *Zoogloea* played a significant role in initiating the granulation of AGS, and especially they were significantly enriched when floc sludge converted into small AGS, but they also decreased with the further development of AGS. Interestingly, the variation of the relative abundance *Zoogloea* was observed to be accorded with a published result [46]. Previous studies also verified that *Sphingomonas* and *Rhodobacter* were able to produce AHLs [36,47], but *Sorangium* were the genus with AHLs-hydrolase and acylase genes, and they could degrade AHLs [23]. Such results implied that the microbial community in AGS might be correlated to the EPS production and AHL-mediated QS, which will be further investigated in the next section. Moreover, these functional bacteria rarely existed in S1, but their relative abundance was enriched in S2–S5. It was well known that AHL-mediated QS was related to the threshold value of signaling molecule concentration and functional bacterial population, thus, it was assumed that filamentous bacteria, which was regarded to be a skeleton to gather the microbes together and provided a suitable position for the development of microbes. Therefore, it might be an effective approach to increase the density of functional bacteria, and when the density increased to a threshold value, the functional bacteria would be able to sense the signaling molecule and induce the AHL-mediated QS. Subsequently, the aerobic granulation process was initiated after the initial stage of granulation, then the competition of microorganisms would be responsible for the variation of microbial population and AHL concentration.

Fig. 4. Microbial communities at different levels: (A) phylum; (B) genus.
3.5. Correlation of AHL-mediated QS and EPS with the functional bacterial genera

EPS is an important component in forming AGS and keeping it stable, which was also found to be regulated by AHL-mediated QS. As mentioned above, the specific signaling molecule C14-HSL was detected to be present at a high level, which implied that it might be a major signaling molecule in the AHL-mediated QS system. For further revealing the correlation between the microbial community with the AHL-mediated QS and EPS production, the Pearson correlation coefficients were calculated by comparing the proportion of the top 50 genera with the C14-HSL concentration and EPS content, and the results are shown in Fig. 5.

As shown in Fig. 5, the detected genera, including unclassified_f_Burkholderiaceae, norank_f_Saprospiraceae, Zoogloea, AAP99 and Methylibium, exhibited a significant correlation with the C14-HSL concentration ($r > 0.884, P < 0.047$, minimum $r'$ and maximum $p'$), and 20 kinds of genera had significant correlation with the EPS contents ($P < 0.05$), such as Thiobrix, Sphingomonas and norank_f_Chitinophagaceae. Additionally, most of the genera in AGS didn’t show any significant correlation with the contents of C14-HSL and EPS. However, it should be indicated that some AHL-producing and AHLT-quenching bacteria, such as Pseudomonas, Sorangium and Sphingomonas, showed no significant correlation with the C14-HSL concentration, and some EPS producers, including Zoogloea and Rhodobacter, were also not significantly correlated with the EPS contents. According to the previous studies, Sphingomonas were determined to be AHLs producers, and Zoogloea identified as EPS producers, which were involved in the AHL-mediated QS processes [22,23]. However, in the presented results, Zoogloea showed a strong correlation with the content of C14-HSL rather than that of EPS, while Sphingomonas was significantly correlated to LB-PS rather than C14-HSL. Some AHL-irrelevant genera showed a strong correlation with AHLT concentration by the AHL-mediated QS, which was induced by other genera, and the EPS contents were not always correlated to the EPS producers [20], thus, it should be noted that without the microbial selection pressure, the more complex microbial community structure might result in a weak correlation between AHLT concentrations, EPS production and microbial community and population. But, the fluctuation of these genera and microbial activity was contributed to the changes of AHLT concentrations, which might be the reason of the competition among different microbial species. Thus, it could be concluded that even without an existing microbial community, once the AHL-mediated QS was initially induced, the microbial community and population within the AGS system would be regulated, and the EPS contents were actually regulated by the microorganisms through the AHL-mediated QS system.

4. Conclusion

This study investigated the function of specific AHL molecules on the granulation process of AGS in a continuous-flow MBR, and the correlation between signaling molecule, EPS production and microbial community within the obtained AGS. C14-HSL was found to be the critical signaling molecule in the studied system and the production of LB-EPS and TB-EPS were regulated by the AHL-mediated QS during the whole granulation process of AGS, which indicated that the AHL-mediated QS system played an important role in this process. The results also indicated that even without the microbial selection pressure, the conditions within the bioreactor determined the microbial community and the contained functional bacteria might compete within the microbial community and release signaling molecules to promote the granulation.

CRediT authorship contribution statement

Zhan Yue: Methodology, Investigation, Writing - original draft. Ping Li: Project administration, Supervision, Software. Liying Bin: Resources. Shaosong Huang: Methodology. Fenglian Fu: Resources. Zhiwen Yang: Data curation. Bangqiao Qiu: Data curation. Bing Tang: Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2020.107801.

References

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