

Article

Reproducibility of Aerobic Granules in Treating Low-Strength and Low-C/N-Ratio Wastewater and Associated Microbial Community Structure

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Abstract: Long-term stability of the aerobic granular sludge system is essentially based on the microbial community structure of the biomass. In this study, the physicochemical and microbial characteristics of sludge and wastewater treatment performance were investigated regarding formation, maturation, and long-term maintenance of granules in two parallel sequencing batch reactors (SBR), R1 and R2, under identical conditions. The aim was to explore the linkage between microbial community structure of the aerobic granules, their long-term stability, as well as the reproducibility of granulation and long-term stability. The two reactors were operated with a COD concentration of 400 mg/L and a chemical oxygen demand to nitrogen (COD/N) ratio of 4:1 under anoxic–oxic conditions. It was found that although SVI₃₀, sludge size, and distributions in R1 and R2 were different, aerobic granules were formed, and they maintained long-term stability in both reactors for 320 days, implying that a certain level of randomness of granulation does not affect the long-term stability and performance for COD and N removal. In addition, a significant reduction in the richness and diversity of microbial production was observed after the sludge was converted from inoculum or flocs to granules, but this did not negatively affect the performance of wastewater treatment. Among the predominant microbial species in aerobic granules, Zoogloea was identified as the most important bacteria present during the whole operation with the highest abundance, while Thauera was the important genus in the formation and maturation of the aerobic granules, but it cannot be maintained long-term due to the low food-to-microorganisms ratio (F/M) in the system. In addition, some species from Ohtaekwangia, Chryseobacterium, Taibaiella, and Tahibacter were found to proliferate strongly during long-term maintenance of aerobic granules. They may play an important role in the long-term stability of aerobic granules. These results demonstrate the reproducibility of granulation, the small influence of granulation on long-term stability, and the robustness of aerobic granulation for the removal of COD and N. Overall, our study contributes significantly to the understanding of microbial community structure for the long-term stability of aerobic granular sludge in the treatment of low-COD and low-COD/N-ratio wastewater in practice.

Keywords: aerobic granular sludge; microbial community structure; reproducibility; low-strength COD; low COD/N ratio



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1. Introduction

Aerobic granules are dense and compact aggregates immobilized by microorganisms themselves without a carrier medium. Due to the high compactness and large size, the

aerobic granules have high settling capacity, biomass retention, and tolerance to toxic and shock loads during treatment of various wastewaters. However, aerobic granular sludge is still far from widespread in practice due to a concern of low stability in long-term operation, during which the main reasons for the instability of aerobic granules could be core hydrolysis [1,2], overgrowth of filament bacteria [3,4], and reduction in the excretion of functional extracellular polymer substrates [5]. In recent years, many efforts have been made to address this problem, but it remains a challenge because the factors involved are interrelated, manipulation capabilities are limited, and trials for long-term studies are very time-consuming. The stability of the aerobic granules in practice still needs to be investigated thoroughly.

The stability of aerobic granular sludge is essentially determined by the structure of microbial populations residing in granules, such as predominant bacteria groups, the richness and diversity of microbial community, and microbial dynamics in response to operational and environmental changes in wastewater treatment. The structure of microbial populations in aerobic granules is greatly dependent on influent wastewater compositions, operation parameters, and environmental conditions. For example, Cydzik-Kwiatkowska et al. (2015) found that the bacterial structure of aerobic granules is significantly affected by the aeration mode and nitrogen loading in a nitrogen removal system [6]. Specifically, Swiatczak et al. (2018) proved that the variables, i.e., temperature, time of aerobic granular sludge adjustment at start-up, and reactor conditions, contributed 92.3% to the variability of the microbial community of a full-scale granular sludge system [7]. However, all the above adjustable factors cannot be used to directly and precisely regulate the microbial structure, because some uncontrollable factors due to theoretical and technical limitations, such as size distribution, biomass growth, and microenvironment in aerobic granules, also affect the microbial structure. The growth of microbial species inside the aerobic granules is controlled by the microenvironment, which is affected by the granule size, compactness, and other physical properties of the granules, resulting in different performances and stabilities of the aerobic granules during the long-term operation period, even under the same operating conditions. Currently, there is limited information on the effects of these uncontrollable conditions on the microbial community in aerobic granular sludge, which hinders a deep understanding and effective control of the aerobic granular sludge system for long-term stable operation. Cultivation of aerobic granules using feeds with high ammonia concentration and low carbon-to-nitrogen (C/N) ratio under anoxic–oxic conditions has been proven to be effective in increasing the stability of aerobic granules [8]. The high ammonia concentration favors the enrichment and accumulation of slow-growing nitrifying bacteria in the granules, while the anoxic–oxic conditions inhibit the overgrowth of ordinary heterotrophic organisms (OHO) to promote floc formation [9]. Liu et al. (2004) reported that the nitrifying granules at a C/N ratio of 100/30 had the smallest average size, i.e., about 0.3 mm, compared to the other granules cultured at higher C/N ratios [10]. Cha et al. (2021) reported that the mean size of aerobic granules in the system with a C/N of 4 was less than 0.5 mm during operation for over 300 days [8]. It has also been reported that the diffusivity of the substrate directly affects microbial competition for the substrate and thus granulation [11,12]. When the size of the aerobic granules is less than 0.7 mm, the substrate and oxygen distribute more evenly in the granules than in the large granules because the mass transfer resistance is lower due to the size [13]. Therefore, it could be assumed that the microbial population structure in smaller aerobic granules is mainly affected by the population of microorganisms and their mutual interactions, rather than by the physical properties of the granules such as size and compactness. This would greatly simplify the mechanism of microbial community formation and facilitate the understanding of microbial development in practical aerobic granule applications.

In this study, aerobic granular sludge treating synthetic wastewater with COD of 400 mg/L and ammonia nitrogen of 100 mg N/L under anoxic–oxic conditions was cultured to investigate the microbial structure of the aerobic granular sludge during the

long-term operation period. During more than 300 days of operation, the wastewater treatment performance and the physical and microbial properties of the aerobic granules were monitored at selected stages. The specific objective of this study was to characterize the relationships between the performance, microbial structure, and stability of the aerobic granular sludge during start-up, maturation, and continued long-term maintenance of the aerobic granular sludge operation. The results of the study provide insight into the key factors affecting the long-term stability of an aerobic granular system and clues for better control of the system in practical applications.

2. Materials and Methods

2.1. Experimental Setup and Operation

The experiment was carried out in two parallel bubble columns (R1 and R2) with an internal diameter of 5 cm and a working volume of 2 L. The two reactors were operated sequentially with a cycle time of 4 h, including 5 min of anaerobic feeding, 55 min of static condition without mixing, 145 to 170 min of aeration, 30 to 5 min of settling, and 5 min of effluent discharging. The feed was pumped into the reactors from the top port, while the effluents were discharged from the middle port of the reactor with a volumetric exchange ratio of 50%. During the aeration phases, fine air bubbles were pumped into the reactors through an air sparger at the bottom of the reactors with a flowrate of 2 L/min. The inoculated activated sludge was collected from a local domestic wastewater treatment plant with a sludge volume index (SVI₃₀) of 106.2 mL/g. The experiment took place at a temperature ranging from 18 to 25 °C.

2.2. Medium

The influent was synthetic wastewater with compositions of NaCH₃COO, (NH₄)₂SO₄, KH₂PO₄, NaHCO₃, and micronutrients. NaCH₃COO and (NH₄)₂SO₄ provided carbon and nitrogen sources, respectively, while NaHCO₃ provided inorganic carbon and pH buffer for nitrification. The experiment was conducted in two stages: the first stage was from day 1 to 32 with influent concentrations of COD and NH₄⁺-N of 400 mg/L and 50 mg N/L, respectively; while the second stage lasted from day 32 until the end of the operation, i.e., day 320, with influent concentrations of COD and NH₄⁺-N of 400 mg/L and 100 mg N/L, respectively. With these compositions of synthetic wastewater, OLR was constant at 1.21 kg COD/m³d^{−1} during the two stages, while the ammonia loading rate increased from 0.15 to 0.3 kg N/m³d^{−1} from the first to the second stage. pH was controlled in the range of 7.5–8.5 during the whole operation period. Micronutrients were supplied: CaCl₂·2H₂O 25 mg/L, MgSO₄·7H₂O 20 mg/L, FeSO₄·7H₂O 10 mg/L, EDTA-2Na 10 mg/L, MnCl₂·4H₂O 0.12 mg/L, ZnSO₄·7H₂O 0.12 mg/L, CuSO₄·5H₂O 0.03 mg/L, (NH₄)₆Mo₇O₂₄·4H₂O 0.05 mg/L, NiCl₂·6H₂O 0.1 mg/L, CoCl₂·6H₂O 0.1 mg/L, AlCl₃·6H₂O 0.05 mg/L, and H₃BO₃ 0.05 mg/L.

2.3. Analytical Method

COD, NH₄⁺-N, NO₃[−]-N, NO₂[−]-N, SVI, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were analyzed according to standard methods (APHA) [14]. Average particle size was determined by a laser particle size analysis system with a measuring range from 0 to 2000 µm (Malvern MasterSizer Series 2600, Malvern Instruments Ltd., Malvern, UK). Morphometry of the aerobic granules was observed by an optical microscope equipped with a digital camera (Leica Microsystems Wetzlar GmbH, DM100.DEU, Wetzlar, Germany). Surface profile was evaluated by means of a scanning electron microscope (Hitachi SU-8010, Tokyo, Japan).

2.4. Microbiological Analysis of Granular Sludge

For biomolecular analyses of the microorganisms, sludge from the two reactors was collected at different days of operation and stored at −80 °C. Sample DNA was extracted using a PowerSoil[®] DNA Isolation kit. The purity and concentration of the isolated

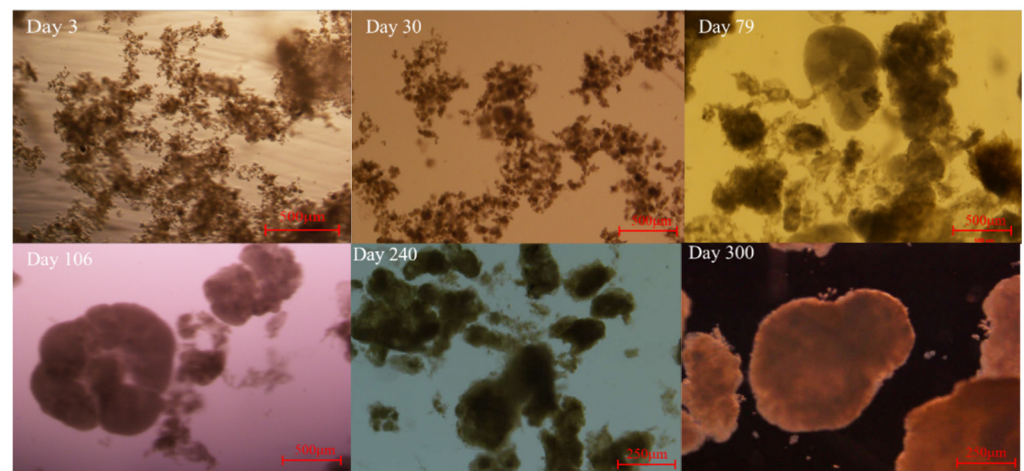
DNA was measured by a Qubit2.0 DNA kit (Life Technologies, Carlsbad, CA, USA). The 515F/805R primer set (GTGCCAGCMGCCGCGGTAA/GACTACHVGGGTATCTAATCC) was used to amplify the V4 regions of the bacterial 16SrDNA gene. Amplification began with an initial denaturation at 95 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s and extension at 72 °C for 30 s. It ended with a final extension step at 72 °C for 5 min. The PCR products were then subjected to 1.8% agarose gel electrophoresis for detection. Samples with a bright main band of approximately 450 bp were chosen and mixed in equidensity ratios. The mixture of PCR products was purified using a VAHTSTM DNA Clean Beads (Vazyme Biotech Co., Ltd., Nanjing, China). After quantification, the amplicons were sequenced using the MiSeq platform (Illumina, LA, USA). The sequences were validated by software cutadapt (version 1.2.1), pear (version 0.9.6) and Prinseq (version 0.20.4), among which low-quality sequences were treated by Prinseq. Chimeras were detected and removed by software Usearch (version 5.2.236) and identified by Uchime (version 4.2.40). An identity of 97% was adopted to cluster the normalized sequences into operational taxonomic units (OTUs), which were annotated based on the Silva taxonomic database. The diversity was assessed within a community (α -diversity) and was calculated and displayed with Mothur (Version 1.30.1). The Chao-1, Shannon, and Simpson indices were analyzed according to Hill et al. [15], and the abundance-based coverage estimator (ACE) was analyzed according to Westphal et al. [16].

3. Results and Discussion

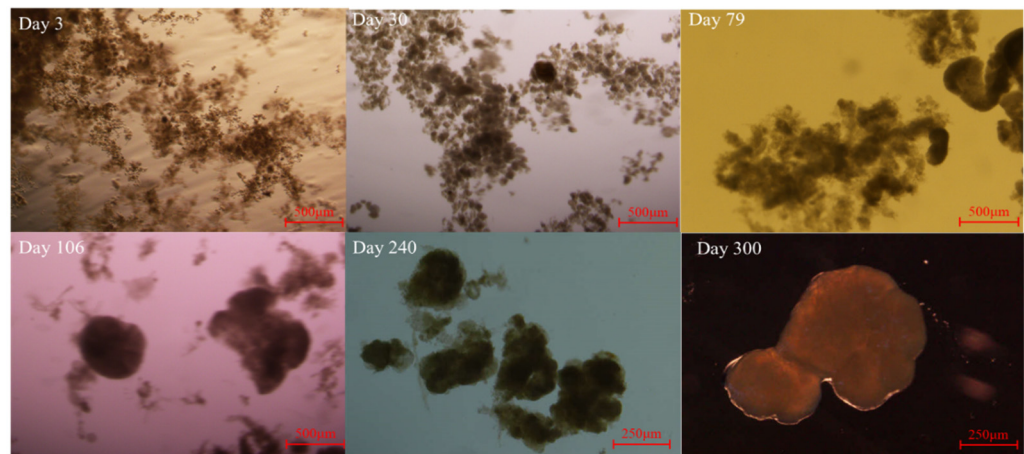
3.1. Characteristics of Aerobic Granular Sludge during Long-Term Operation

Figure 1 shows the morphology of sludge in R1 and R2, respectively, during the operation time. It can be seen that tiny aggregates appeared on day 30 in the two reactors and mixed with flocs. On day 79, the aerobic granules formed in R1 with clear-cut outlines and fewer flocs, while in R2 there were only small granules with a large number of flocs. On day 106, aerobic granules of similar size were present in both reactors and gradually developed into large and dense granules with small flocs by day 300. Figure 2 shows the surface morphometry of the aerobic granules in R1 and R2 examined by SEM. It can be seen that the surface of the aerobic granules with cocci, bacilli, and filaments are quite similar.

The size of aerobic granules throughout the whole operation is shown in Figure 3, which reveals a different development of granules in R1 and R2. The mean size of the aerobic granules in R1 increased with a large fluctuation, while that in R2 increased stably, until the end of the operation. In R1, the mean size of aerobic granules increased to 380 μm on day 136, while it decreased to 276 μm on day 240, which was combined with variations in the settling ability of aerobic granules. On day 294, the size appeared to recover and increased to 462 μm . In R2, the mean size of aerobic granules gradually increased to 662 μm on day 294. Meanwhile, the size distribution of aerobic granules was found to be highly variable, with a narrower size distribution in R1 than in R2. These discrepancies in the size development and morphometry of the sludge in R1 and R2 over time indicate that the formation and growth of aerobic granules are different even under seemingly identical operating conditions, suggesting some degree of randomness unrelated to the apparent operating conditions related to granule formation and development. In addition, it should be noted that the mean size of the aerobic granules in the two reactors was less than 700 μm , implying that the overall mass transfer resistance in the granules may be negligible.



(A)



(B)

Figure 1. Morphology of the sludge on different days of the operation. (A) in R1; (B) in R2.

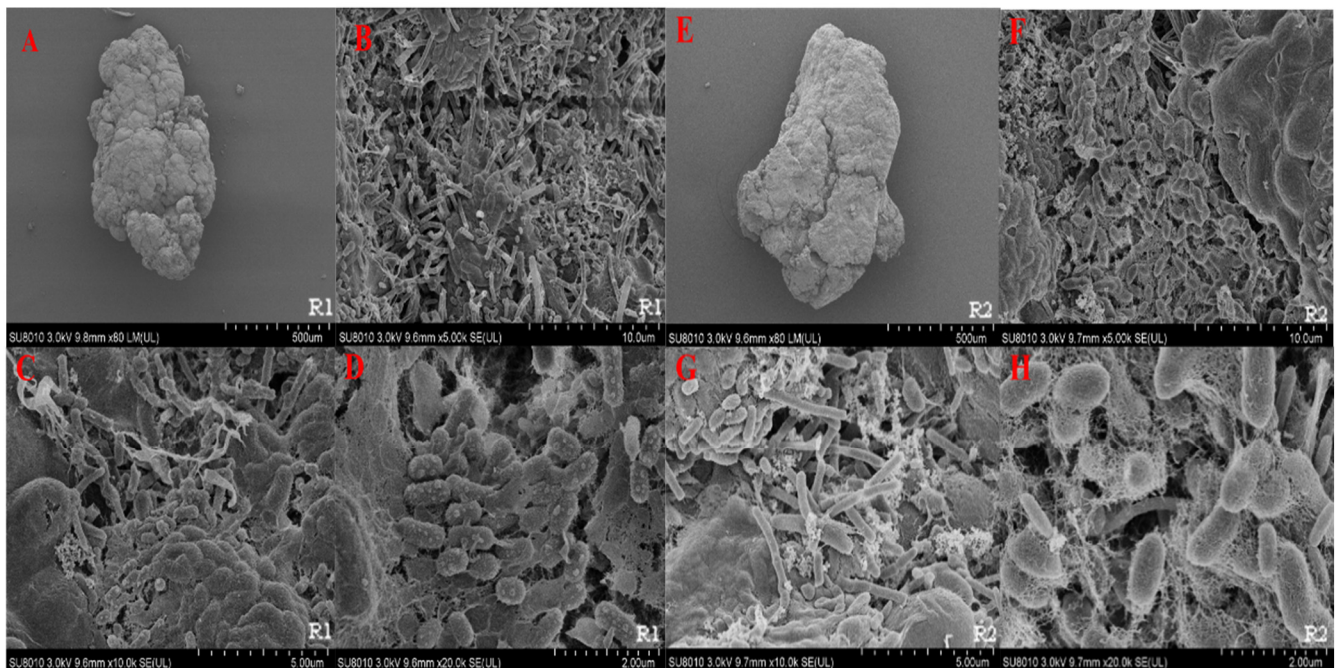


Figure 2. SEM of the aerobic granules in R1 and R2. (A–D), in R1; (E–H), in R2.

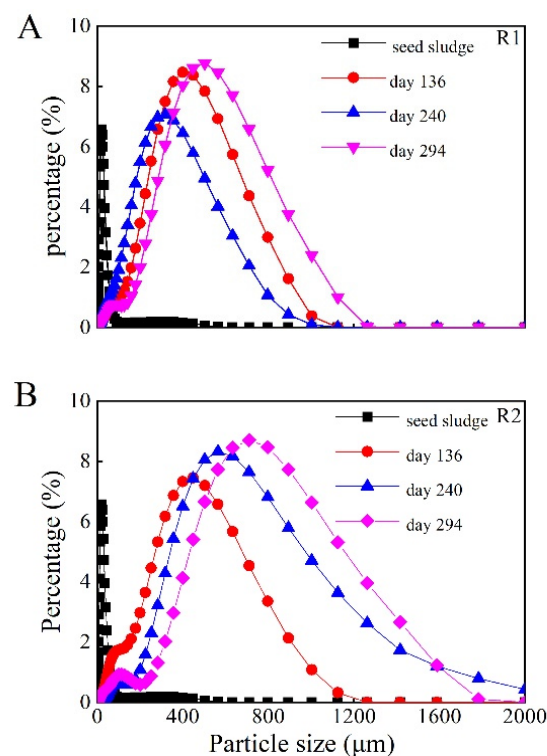


Figure 3. Size distribution and development of aerobic granules in R1 and R2 over the operating time. (A) R1; (B) R2.

SVI is normally used as a direct indicator of both sludge settling ability and aerobic granule stability. An SVI_{30} below 80 mL/g is usually considered indicative of increased sludge stability with good settling ability. At the same time, the ratio of SVI_{30} to SVI_5 close to 1 is often used as an indicator of aerobic granule dominance. Figure 4A,B shows the development of SVI in R1 and R2 during the operating period. It can be seen that the sludge in both reactors was subject to large variations in settling ability and stability during the formation period, i.e., from the inoculation of the activated sludge to the formation of the aerobic granules. In R1, SVI_{30} decreased sharply in the first 20 days and then increased abruptly to 148.6 mL/g within the next 20 days due to sludge bulking, indicating low settling ability and stability of the sludge. Thereafter, SVI_{30} began to decrease and reached 50 mL/g on day 65, which was considered to be the time when a dominant aerobic granule formed in R1, as SVI_{30}/SVI_5 was close to 1 at this time. Similarly, SVI_{30} in R2 first decreased and then increased due to sludge bulking until day 90, showing a twofold increase and decrease. Thereafter, aerobic granules began to form in R2 with SVI_{30}/SVI_5 close to 1.

After the formation of the aerobic granules in each reactor, the SVI_{30} remained in the range of 60–80 mL/g until day 140, which can be considered as the maturity stage of the aerobic granules in the two reactors. From day 140 to the end of the operation period on day 320, the SVI_{30} of the aerobic granules in both R1 and R2 remained around 40 mL/g, and no floc competition was observed, as reported by Liu et al. (2012) on the real wastewater treatment during the long-term operation period [17], demonstrating the high stability of the aerobic granules.

Again, the difference in the change of SVI_{30} in two identical reactors indicates some randomness of granule formation especially during the granule formation phase. In contrast to the size development of aerobic granules, it is noted that the settling ability and stability of aerobic granules in R1 and R2 were quite similar during maturation and long-term maintenance, regardless of the differences in the mean size and distribution of aerobic granules in the two reactors. This indicates that aerobic granules with different granule formation with respect to SVI_{30} can still achieve similar settling ability and stability after maturation.

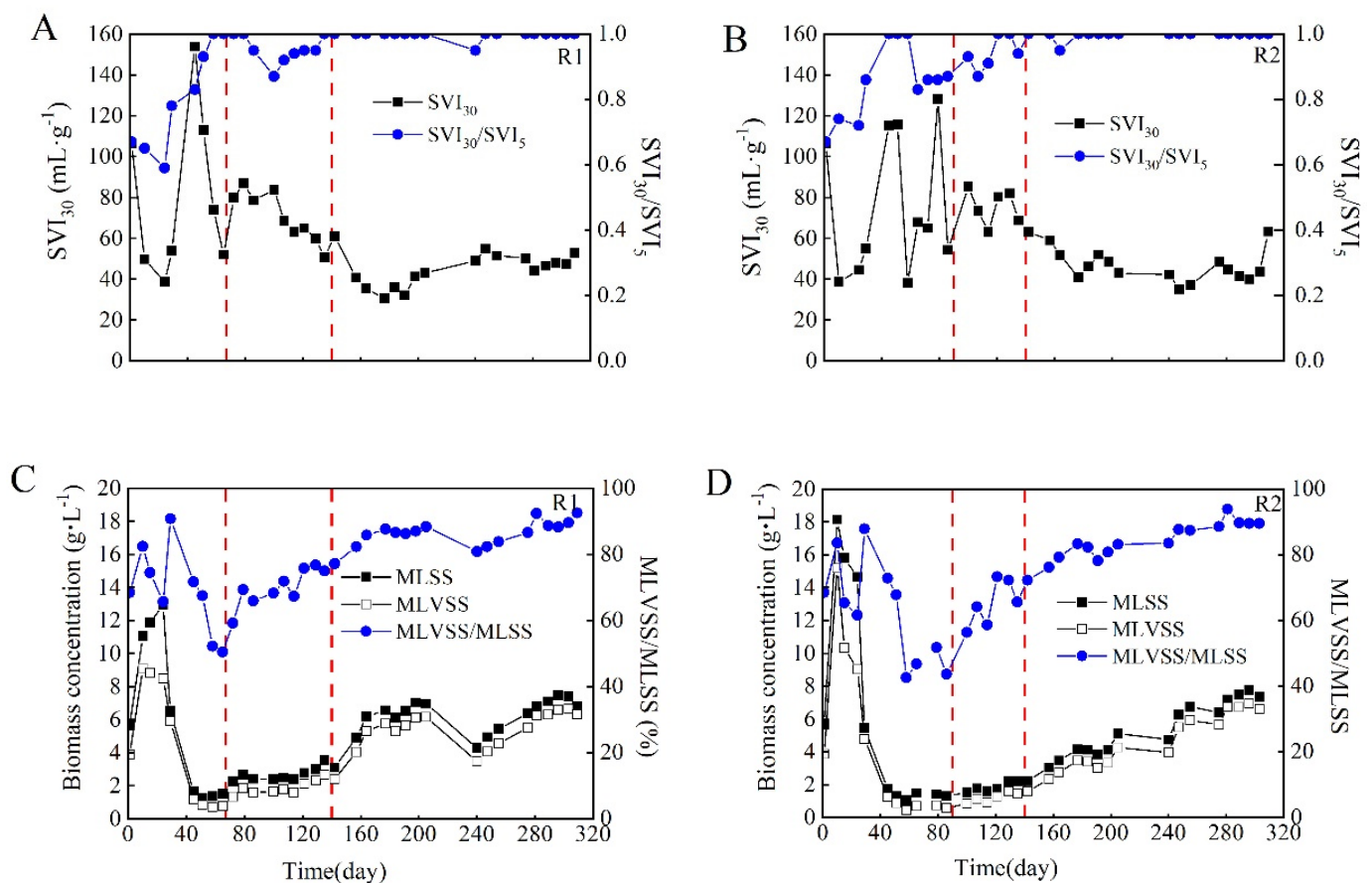


Figure 4. Profiles of SVI and MLSS during the operation period. (A,C), in R1; (B,D), in R2.

The biomass concentration, indicated by MLVSS/MLSS over the whole operation time, is shown in Figure 4C,D, which depicts the growth and accumulation of aerobic granules as a whole. MLSS in the two reactors initially decreased, stagnated at the lowest concentration after the granules had formed for a period of time, and increased again by the end of the operation. In R1, the MLSS gradually decreased from 5.6 g/L of inoculum to 0.7 g/L on day 58, which was caused by the washout of the flocs due to the increased SVI and the shortened settling time. After this low MLSS was maintained for about 7 days, aerobic granules began to form and MLSS began to increase due to retention and growth of high-settling biomass. In R2, the only difference in MLSS development from R1 was that the low biomass period (0.69 g/L) lasted longer, approximately 40 days. This was due to the longer sludge bulking and SVI₃₀ fluctuating twice in R2, resulting in less biomass retention than in R1. After aerobic granules formed in R2 on day 90, MLSS began to increase. After the aerobic granules were formed in both reactors, the MLSS gradually increased to 6.8 g/L in R1 and 5.1 g/L in R2 at the end of the operation.

At the same time, it is observed that MLSS shows an inverse trend with SVI, i.e., lowest MLSS in the formation phase, slightly increased MLSS in the maturation phase, and further increased MLSS in the long-term maintenance of aerobic granules. The MLVSS can be used to detect the amount of microbial organisms in the biomass, which represents the organic composition of the MLSS. During the development of the aerobic granules, the MLVSS/MLSS changed according to the MLSS: it decreased from 80% to 60% and 50% in R1 and R2, respectively, after the formation of the aerobic granules, and then gradually increased to 80% in both reactors during the maturation of the aerobic granules. Subsequently, the MLVSS/MLSS remained in the range of 80–90% until the end of the operation, indicating high microbial richness and activity in the long-term maintenance of the aerobic granules in R1 and R2.

3.2. Performance of the Aerobic Granular Sludge during the Long-Term Operation Period

Figure 5 shows the performance of the aerobic granules in R1 and R2, respectively. It can be seen that the COD removal efficiency in both reactors was above 90% throughout the operating period, although sludge bulking and extremely low biomass concentrations occurred in the first operating phase. This indicates that the heterotrophic microorganisms responsible for organic matter removal were very active in the aerobic granules to handle the added COD. In comparison, the efficiency of ammonium removal fluctuated when the aerobic granules were formed, which can be attributed to the low biomass concentration at the beginning due to the leaching of the nitrifying bacteria.

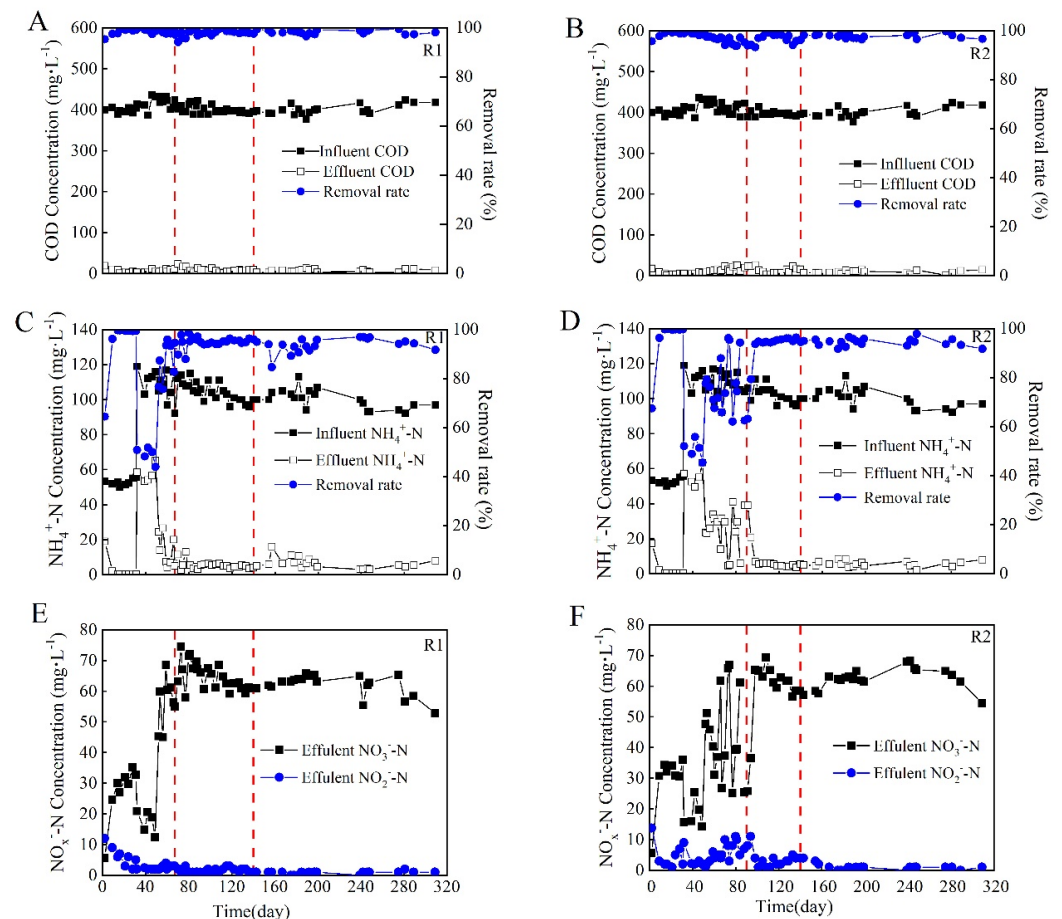


Figure 5. Performance of the aerobic granules over the operation period in R1 and R2. (A,B), COD removal in R1 and R2, respectively; (C,D), ammonium removal in R1 and R2, respectively; (E,F), NO_x formation in R1 and R2, respectively.

After the formation of aerobic granules in the two reactors, the ammonia removal efficiency increased to over 90% as the nitrifying bacteria in the granules re-accumulated with increasing biomass. Throughout the operating period, ammonium was mostly converted to nitrate in R1 and R2, except during the period of aerobic granule formation with fluctuations in ammonium removal. The similar performances of the aerobic granules in R1 and R2 indicate that the different average size and size distribution of the aerobic granules does not have a large effect on the ability to biodegrade when the granule size is smaller and there is no obvious mass transfer resistance.

3.3. The Richness and Diversity of Microbial Community in the Aerobic Granular Sludge

The richness and diversity of the microbial population throughout the operating period are presented in Figure 6. The number of operational taxonomic units (OTUs), the abundance-based coverage estimator (ACE) index, and the Chao1 index are used to indicate the richness of the microbial population.

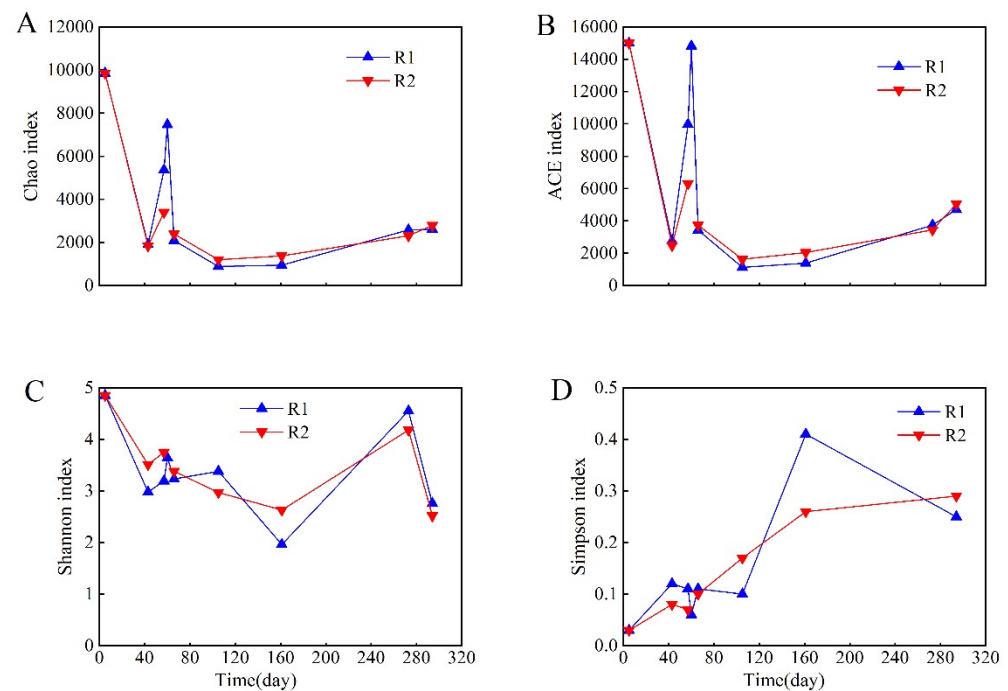


Figure 6. Richness and diversity indices of microbial populations in sewage sludge over the entire operating period in R1 and R2. (A) Chao1; (B) ACE; (C) Shannon; (D) Simpson.

It can be seen that there was no obvious difference in terms of the richness of the microbial population in both reactors during the different phases of operation. In addition, it was found that the richness of the microbial population decreased sharply after the formation of the aerobic granules, although there was a small fluctuation before the aerobic granules became dominant. The richness of the microbial population remained at its lowest level during the maturation period of the aerobic granules. After the maturation period, microbial richness increased slightly, but was still far below that of the original microbial population in the inoculum.

The Shannon and Simpson indices, which are positively and negatively related to diversity, respectively, are standard indicators of microbial diversity [15]. The Shannon index decreased during aerobic granule formation and maturation and recovered somewhat after maturation, while the Simpson index showed the opposite trend. All the indices shown in Figure 4 indicate that the richness and diversity of the microbial population decreased sharply during the formation of aerobic granules due to the selection of granule-favoring microbial populations and the leaching of flocs-favoring microbial populations, while it increased slightly during long-term maintenance due to the embedding and growth of more species in the granules. The coverage rate of the detected genera was 0.99 for both reactors, indicating the high credibility of the statistical data obtained from the microbial analysis of the aerobic granule samples.

3.4. Microbial Population Dynamics and the Predominant Functional Groups

Tracking the microbial composition of aerobic granules at various stages of growth and development can provide important information for identifying key microbial organisms and their associated specialized functions in aerobic granules, which could be used as meaningful indicators for monitoring and controlling the operating process. Tables 1 and 2

show the main composition of the microbial populations in R1 and R2, respectively, during the entire operating period. A significant shift in microbial structure from the inoculum to the stable granule was observed, which was consistent with the report of aerobic granule microbial population dynamics during the start-up and steady state [18]. In addition, it is clear that the dominant genera in the two reactors are quite similar on the different days of operation, although the abundance of each genus is different. This indicates that the microbial structure in aerobic granules with small average size is determined by the operating conditions, which can be duplicated by setting the same operating parameters. Of all the genera of aerobic granules identified in Tables 1 and 2, most microbial organisms have at least one of the functions of EPS excretion, nitrification, denitrification, and organic matter degradation. EPS excretion is one of the most important microbial properties in granules, which can help microbial organisms aggregate and maintain a stable structure during long-term operation. Denitrifying bacteria or slow-growing autotrophic nitrifying bacteria promote a dense structure of aerobic granules by minimizing overgrowth of more general heterotrophic organisms that hinder aerobic granule formation and performance by promoting loosely structured flocs rather than compact granules [11]. A comparison with the predominant genera in the inoculum also shows that most of the bacteria in the aerobic granules originated from the genera in the inoculum during the formation and growth periods, and only 5 of the total 30 genera in the aerobic granules were new. This could show that most of the microbial genera in the aerobic granules are not exclusive and can be selected and enriched from the inoculated activated sludge.

Table 1. Predominant microbial genera in R1.

Genus	Operation Day						
	1	43	74	105	161	273	294
<i>Alkanindiges</i>	6.88%						
<i>Dechloromonas</i>	4.57%	26.43%	1.95%	0.45%	0.15%	0.05%	0.11%
<i>Acidovorax</i>	1.52%			0.31%	0.66%	0.86%	1.53%
<i>Stenotrophomonas</i>	2.72%	0.03%					
<i>Propionivibrio</i>	1.94%						0.04%
<i>Nitrosomonas</i>	1.63%		4.25%	3.09%	0.15%		0.23%
<i>Flavobacterium</i>	1.61%	0.42%	0.09%	0.1%	0.06%	0.22%	0.05%
<i>Thauera</i>	0.13%	21.6%	5.69%	16.06%	5.20%	0.04%	0.12%
<i>Zoogloea</i>	0.38%	8.15%	18.32%	26.00%	64.26%	11.9%	50.13%
<i>Sideroxydans</i>		3.99%		0.02%		0.14%	
<i>Bdellovibrio</i>	0.11%	1.69%	0.96%	0.26%	0.08%	0.10%	
<i>Ferruginibacter</i>	0.26%	1.07%	0.66%	0.39%	0.11%	1.58%	0.95%
<i>Simplicispira</i>	0.05%	0.64%	0.05%			0.01%	0.03%
<i>Phreatobacter</i>		0.12%	0.14%	0.06%	0.01%		
<i>Prostheco bacter</i>			3.43%	2.57%	1.16%	2.76%	1.1%
<i>Aquimonas</i>		0.83%	1.24%	0.37%	0.05%		
<i>Sphingopyxis</i>			1.8%	1.02%		0.04%	0.01%
<i>Chryseolinea</i>	0.05%	0.33%	2.64%	2.26%	0.18%	0.83%	0.42%
<i>Terrimonas</i>	0.45%	0.57%	0.75%	0.3%	0.32%	1.03%	0.48%
<i>Lacibacter</i>	0.05%			0.06%	0.04%	0.01%	
<i>Azoarcus</i>		0.06%	0.74%	2.08%	0.03%	0.72%	0.09%
<i>Phaeodactylibacter</i>	0.04%			4.83%		0.12%	
<i>Ohtaekwangia</i>	0.02%		0.11%	0.55%	1.09%	8.67%	2.61%
<i>Aggregicoccus</i>	0.11%	0.01%	0.02%	0.04%	0.13%	0.04%	
<i>Chryseobacterium</i>	0.04%		0.26%	0.01%	0.36%	2.65%	5.63%
<i>Nitrospira</i>	0.47%		0.01%	0.01%	0.41%	4.06%	0.76%
<i>Taibaiella</i>	0.98%				0.36%	2.43%	10.68%
<i>Tahibacter</i>	0.01%	0.01%	0.13%	0.14%	0.42%	4.21%	2.33%
<i>Sediminibacterium</i>	0.01%	0.13%	0.49%	0.27%	0.01%	1.32%	0.49%
<i>Luteimonas</i>	0.06%	0.04%				0.1%	0.38%

Table 2. Predominant microbial genera in R2.

Genus	Operation Day						
	1	43	74	105	161	273	294
<i>Alkanindiges</i>	6.88%						
<i>Dechloromonas</i>	4.57%	26.01%	2.2%	1.11%	0.43%	0.02%	0.10%
<i>Acidovorax</i>	1.52%			0.64%	1.13%	0.76%	1.22%
<i>Stenotrophomonas</i>	2.72%	0.02%					
<i>Propionivibrio</i>	1.94%						0.03%
<i>Nitrosomonas</i>	1.63%		5.45%	5.84%	0.66%		0.24%
<i>Flavobacterium</i>	1.61%	0.22%	0.24%	0.45%	0.29%	0.76%	0.05%
<i>Thauera</i>	0.13%	3.9%	4.96%	6.59%	2.19%	0.01%	0.07%
<i>Zoogloea</i>	0.38%	8.53%	26.11%	38.93%	60.09%	21.13%	54.16%
<i>Sideroxydans</i>		3.81%		0.01%		0.06%	
<i>Bdellovibrio</i>	0.11%	2.09%	0.26%	1.10%	0.06%	0.13%	0.01%
<i>Ferruginibacter</i>	0.26%	1.19%	0.38%	0.08%	0.04%	1.26%	0.86%
<i>Simplicispira</i>	0.05%	2.11%	0.08%			0.01%	0.01%
<i>Phreatobacter</i>		1.53%	0.16%	0.16%	0.01%	0.02%	
<i>Prosthecotheca</i>					1.89%	2.4%	1.01%
<i>Aquimonas</i>		0.07%	1.5%	0.81%	0.51%		
<i>Sphingopyxis</i>			0.44%	0.10%	0.07%	0.07%	
<i>Chryseolinea</i>	0.05%	1.17%	3.97%	2.30%	1.04%	0.14%	0.03%
<i>Terrimonas</i>	0.45%	0.81%	1.43%	0.97%	0.1%	2.34%	0.43%
<i>Lacibacter</i>	0.05%			3.82%	3.64%	0.05%	
<i>Azoarcus</i>		0.44%	0.31%	1.92%	0.06%	0.29%	0.1%
<i>Phaeodactylibacter</i>	0.04%	0.01%		0.14%		0.06%	
<i>Ohtaekwangia</i>	0.02%			0.02%	0.28%	2.96%	2.71%
<i>Aggregicoccus</i>	0.11%	0.17%	0.01%		1.04%		
<i>Chryseobacterium</i>	0.04%		0.19%	0.29%	0.02%	5.26%	6.02%
<i>Nitrospira</i>	0.47%	0.07%	0.01%		0.01%	2.08%	0.55%
<i>Taibaiella</i>	0.98%				0.09%	2.45%	10.58%
<i>Tahibacter</i>	0.01%	0.03%	0.36%	0.03%	0.1%	0.01%	1.79%
<i>Sediminibacterium</i>	0.01%	0.07%	0.56%	0.35%	0.02%	0.07%	0.34%
<i>Luteimonas</i>	0.06%	0.06%				2.54%	0.38%

The microbial genera from the inoculum that persisted and thrived in the aerobic granules can be characterized by their good aggregation ability combined with a short settling time. Among these species, *Zoogloea*, *Thauera*, and *Dechloromonas* were predominant and had a high percentage in the different stages of the sludge in both reactors (R1, R2). *Zoogloea* is much more abundant in the aerobic granules than *Thauera* and *Dechloromonas*, which increased significantly from 0.38% in the inoculum to over 50% in the granules at the end of the operation in both reactors, although there were fluctuations in the long-term maintenance of the aerobic granules. *Zoogloea* is a filamentous denitrifier that improves sludge adhesion and hydrophobicity by secreting EPS, thereby promoting microbial aggregation and granule stability [19]. Consequently, it becomes obvious that *Zoogloea* in aerobic granules play an important role in the formation and long-term stability of aerobic granules. Thus, *Zoogloea* can be considered as one of the major indicators of the stability of long-term operation of aerobic granule system for wastewater treatment with low strength and C/N ratio. *Thauera* is also a typical denitrifier with the ability to excrete EPS in an aerobic granule system [20]. The abundance of *Thauera* gradually increased from 0.13% in the inoculum to maxima of 21.6% in R1 and 6.59% in R2, respectively, during the formation and maturation phases of the aerobic granule, but declined sharply to a level similar to that of the inoculum during long-term maintenance of the aerobic granules. Therefore, it can be hypothesized that *Thauera* is a very important functional genus for the formation and maturation of aerobic granules, but an unimportant functional genus for maintaining the long-term operation of the aerobic granular system. From the comparison of the abundance of *Thauera* in the two reactors, it can be seen that the abundance of *Thauera* in the granules in R2 with a much looser structure and a longer formation time was much lower than

in R1. This confirms that *Thauera* plays a key role in the formation and maturation of the aerobic granules, the abundance of which determines the compactness of the aerobic granules and the rate of formation of the aerobic granules. In addition, *Dechloromonas* was found to be transiently abundant at 26.43% in R1 and 26.01% in R2 on day 43, when sludge bulking occurred in both reactors. After the aerobic granules were formed, the abundance decreased sharply and remained at a much lower level than in the inoculum (4.57%) during the remaining days of operation. *Dechloromonas* is a type of denitrifier with the ability to reduce nitrate to nitrite. The huge accumulation of *Dechloromonas* in the sludge bulking and the decrease in the established aerobic granule system indicate that *Dechloromonas* is closely related to the sludge formation and plays an unimportant role in the formation and maintenance of aerobic granules.

It should be noted that some genera, including *Ohtaekwangia*, *Chryseobacterium*, *Taibaiella*, and *Tahibacter*, had very low abundance during most of the operating time, but had relatively higher abundance during longer operation of the mature aerobic granules. The abundance of these bacteria ranged from 2 to 10% in both reactors, compared to the low abundance of 0.01 to 0.5% during most of the operating time. *Ohtaekwangia* has been described as a type of structure-forming bacteria in aerobic granules that excrete EPS and whose abundance increases with the growth of aerobic granules [7]. As reported, *Chryseobacterium* can live in a nutrient-poor environment [21]. Although a low organic concentration and a high ammonium concentration were used in this study, the low F/M in the long-term maintenance phase of the aerobic granules actually resulted in a nutrient-poor environment. This could explain the existence of *Chryseobacterium* in the aerobic granules. *Tahibacter* and *Taibaiella* are both aerobic, and it has been reported that they can reduce nitrate to nitrite [22] and nitrate to gaseous nitrogen [23], respectively. Their increased abundance may indicate that they play an important role in denitrification in the long-term maintenance phase of aerobic granules. Thus, from the above, it is clear that these genera perform different functions in aerobic granules, and their structure supports their proliferation and accumulation in the granules during the long-term maintenance phase.

In addition, there were also some microbial genera such as *Acidovorax*, *Bdellovibrio*, *Ferruginibacter*, *Terrimonas*, and *Chryseolinea* which showed very low abundance throughout the entire operating period. Their abundances were less than 2%, indicating that they may play an unimportant role in the aerobic granules. Upon closer inspection, it turns out that these bacteria are all heterotrophic, but have several adaptive characteristics that allow them to live in aerobic granular sludge. Specifically, *Acidovorax* is a type of facultative denitrifier that occurs in low-COD conditions [24,25] and is able to self-aggregate and produce EPS. *Bdellovibrio* is aerobically heterotrophic and occurs in well-treated wastewater where it is able to prey on Gram-negative bacteria and utilize COD such as polymers, and ammonium [26]. *Ferruginibacter* and *Terrimonas* can degrade organic matter and excrete EPS for better sludge aggregation [27,28]. *Chryseolinea* can degrade small organic molecules as well as large polymers such as proteins and polysaccharides [29]. Of all the genera identified in Tables 1 and 2, *Nitrosomonas* and *Nitrospira* are the ammonia-oxidizing bacteria in R1 and R2 that originated from the inoculum and remained in the aerobic granules throughout the operating period. The abundance of the two types of bacteria showed opposite development trends during operation. *Nitrosomonas* was more abundant in the newly formed aerobic granules, with a maximum of 4.25% in R1 and 5.84% in R2, respectively, while its abundance was very low in the long-term maintenance phase. Conversely, the abundance of *Nitrospira* was higher in the long-term maintenance phase, with a maximum of 4.06% in R1 and 2.08% in R2, respectively, but a very low abundance in the granule formation phase. This is due to the different ammonium affinity of the two bacteria, which determines their proliferation and accumulation in the stages with different available ammonium concentrations. *Nitrosomonas* tends to grow at high ammonium concentrations but is inhibited by low ammonium concentrations, while *Nitrospira* is adaptive only to conditions of low ammonium concentration. In both R1 and R2, although the ammonium concentrations in the influent were constant throughout the operating period, the F/M

of ammonium changed greatly due to the development of biomass, with MLSS ranging from less than 1 g/L to about 6 g/L. During the formation phase of the aerobic granules, the F/M of ammonium was comparatively high with extremely low MLSS, while after the maturation phase, the F/M of ammonium was comparatively low due to the highly accumulated biomass in the reactors. This explains well why *Nitrosomonas* predominated during the period of aerobic granule formation when the ammonium concentration was relatively high for a long time, while *Nitrospira* predominated after the maturation of aerobic granules when the ammonium concentration in the microenvironment was lower.

In addition to the above genera that originated from the inoculum, there were some bacteria that were not detectable in the inoculum but that formed during granule formation. These were *Sideroxydans*, *Phreatobacter*, *Prostheobacter*, *Aquimonas*, *Sphingopyxis*, and *Azoarcus*. The abundance of these bacteria was less than 3.5% in R1 and R2, but they were present in different stages of aerobic granule formation, showing the possible role of each bacterium in the corresponding stages. In particular, *Phreatobacter* accounted for 3.43% and 2.76% in aerobic granules at days 76 and 273 in R1, respectively, while it accounted for 2.4% at day 273 but did not appear in R2 until after 161. *Phreatobacter* is a strictly aerobic, oligotrophic denitrifier capable of excreting extracellular protein for sludge aggregation [30,31]. Its presence in the maturation and long-term maintenance phases of aerobic granules in R1 and R2 may indicate that it is particularly adapted to the long-term maintenance environment. Moreover, *Azoarcus* occurred in all stages of aerobic granules since its first appearance on day 43 in both R1 and R2. *Azoarcus* is a denitrifier similar to *Thauera* [32,33], but may play only a minor role in aerobic granules at such low abundance.

3.5. Food to Microbial Biomass Ratio (F/M) and its Relationship with the Predominant Genus in the Aerobic Granules

Food-to-microbial biomass ratio (F/M) is the biomass loading rate, which refers to the substrate loading per unit biomass in a unit time for wastewater treatment. In the aerobic granular sludge system, F/M affects the average available substrate in the microenvironment, which could influence the microbial growth of the aerobic granules. From Figure 7, F/M is high during aerobic granule formation, while it decreases sharply during maturation and remains at a very low level in the long-term maintenance phase. Specifically, F/M increased to 1.83 g COD/g VSS-d at the maximum in formation and remained in a narrow range of 0.2–0.4 g COD/g VSS-d in the long-term maintenance of aerobic granules in R1; whereas F/M increased to 2.90 g COD/g VSS-d at the maximum in formation and remained in the same range as in R1 in the long-term maintenance of aerobic granules in R2. The changing trends of F/M are consistent with the previous assumption that high F/M stimulates the formation while low F/M maintains the long-term stability of aerobic granules [8]. In addition, it is noted that the much higher F/M in R2 causes a much longer formation time than in R1, which may indicate that there may be a suitable range of F/M for a better balance between formation stimulation and formation rate of aerobic granules.

F/M was the main variable in the operation as other adjustable operating parameters were set unchanged in this study. The change of F/M in the operation period may lead to changes in the microenvironment of the bioprocess and thus to the response of the predominant bacteria. In order to clarify the role of the predominate bacteria in the bioprocess, the relationship between F/M and the predominant bacteria during the whole operation period was analyzed as shown in Figure 8. From Figure 8A,B, it can be seen that denitrifier *Zoogloea* is one of the most predominant genera throughout the whole F/M range while it is an absolutely dominant genus when the F/M was below 0.6 in both R1 and R2. Denitrifier *Thauera* and *Dechloromonas* are adaptive to the high F/M range but cannot survive in the low F/M range, especially for *Dechloromonas*, which presented as a transit. Based on the characteristics of these genera, it can be assumed that *Zoogloea* has a fundamental role in the aerobic granules, whereas *Thauera* plays important roles at high F/M values due to their great ability for EPS excretion. *Dechloromonas* may play

unimportant roles in aerobic granules due to inability for EPS excretion, though they are active at high F/M values. Conversely, *Ohtaekwangia*, *Chryseobacterium*, and *Taibaiella* are only adaptive in the low-F/M ranges, which might be connected with their ability for EPS excretion, adaption to nutrient-deficient environment, and aerobic denitrification, respectively. Besides, ammonia-oxidation and nitrification are also very important metabolism processes in the aerobic granules, with *Nitrosomonas* in dominance in the high F/M ranges while *Nitrospira* in dominance in the low F/M ranges. In addition, from Figure 8C,D, it can be seen that some genera with very low abundance could contribute to the diversity of the microbial community in the aerobic granules, but their microbial functions are unclear.

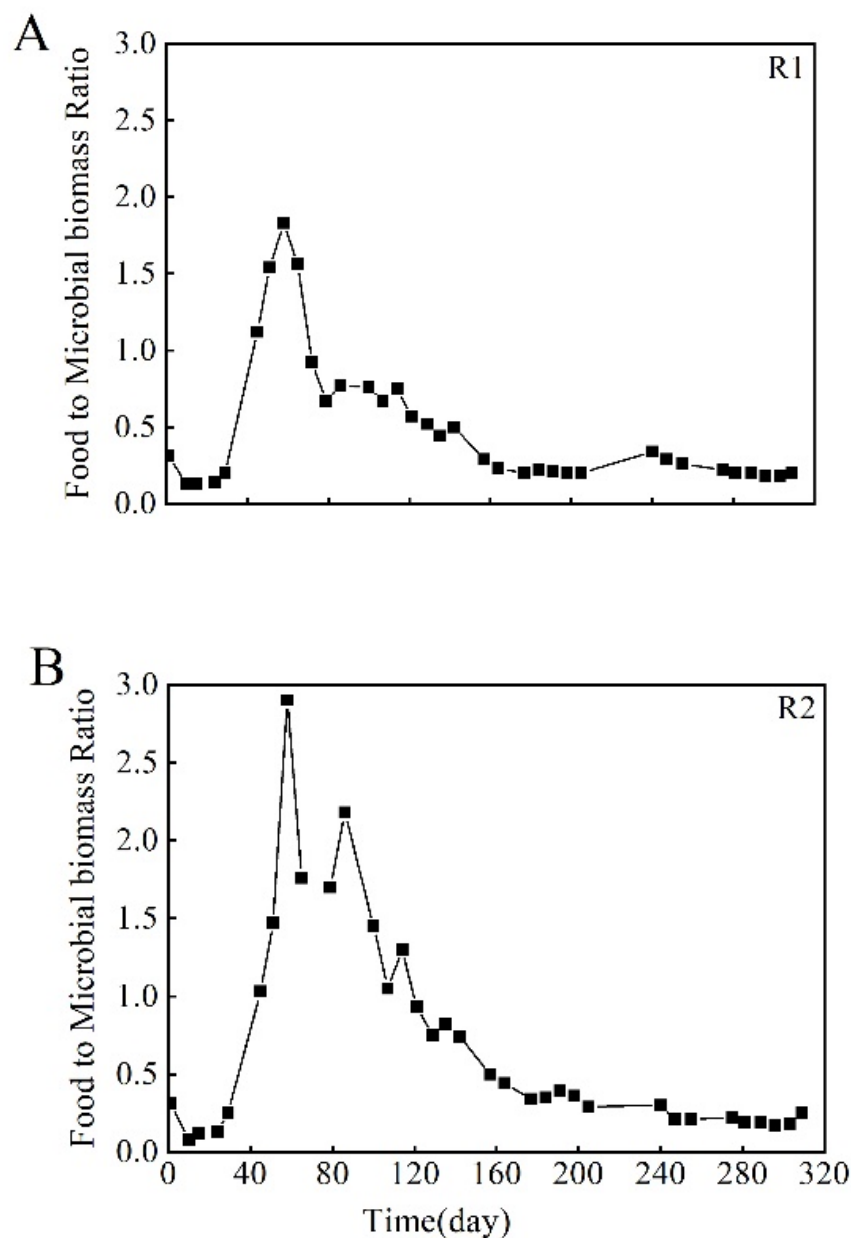


Figure 7. Food-to-Microbial Biomass Ratio in the whole operation period. (A) R1; (B) R2.

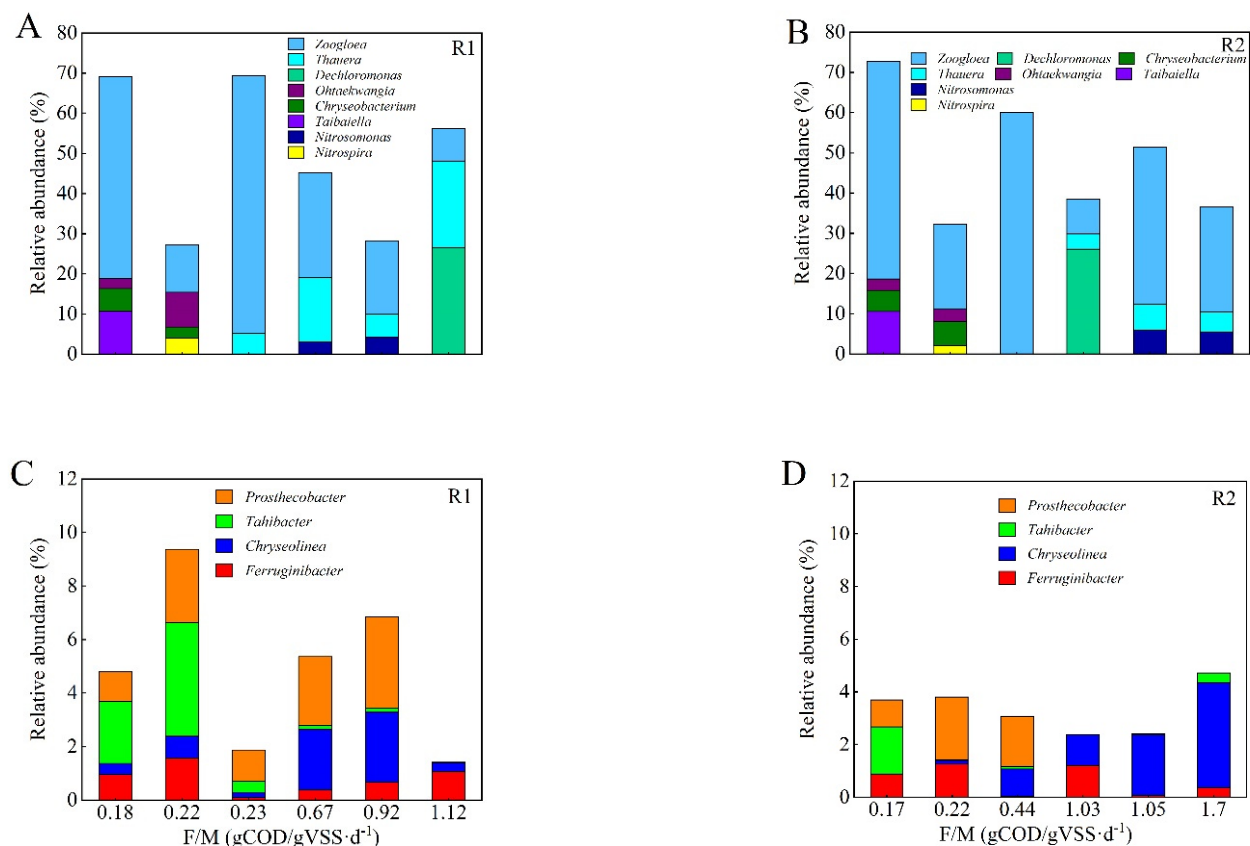


Figure 8. The relationship between F/M and the relative abundance of the predominant genus in the aerobic granules in R1 and R2. (A,B), genera with maximum relative abundance higher than 5% in R1 and R2, respectively; (C,D), genera with maximum relative abundance lower than 5% in R1 and R2, respectively.

4. Conclusions

In this study, two parallel aerobic granular sludge SBR reactors, R1 and R2, were operated for the treatment of synthetic wastewater with a COD concentration of 400 mg/L and a chemical-oxygen-demand-to-nitrogen (COD/N) ratio of 4:1 under anoxic–oxic conditions. All other operating conditions were also identical. The main conclusions are as follows.

- The SVI_{30} before and during granulation was different in the two reactors, although it reached similar values after granule maturation. In addition, the mean size and size distribution of the sludge were quite different in the two reactors, although both reactors were operated under exactly the same conditions. These differences indicate some degree of randomness in granule formation and size development even under identical conditions. However, the similarity of the physicochemical and microbial properties of the granules, as well as the performance of the wastewater treatment after granule maturation, indicate that the operating conditions can produce consistent results, implying predictability of stable operation in practice.
- A high F/M value promotes the formation of aerobic granules, while a low F/M value in the range of 0.2–0.4 g COD/g VSS-d facilitates the long-term stability of aerobic granules.
- The richness of the microbial population of the granules was much lower than that of the inoculum and the flocs with bulking, although the richness may increase slightly during the long-term operation period. The diversity of the microbial structure decreased over time. However, the reduction in the richness and diversity of the microbial population due to the conversion of flocs to granules did not affect the wastewater

treatment performance and long-term stability of the sludge, indicating the robustness of different microbial structures to achieve the same function in wastewater treatment.

- Among the dominant genera of the sludge, *Zoogloea* played a key role in maintaining the stable structure of aerobic granules throughout the operation period, while *Thauera* is an important genus for the formation and maturation of aerobic granules, but not for long-term maintenance. In addition, *Ohtaekwangia*, *Chryseobacterium*, *Taibaiella*, and *Tahibacter* can play an important role in the long-term stability of aerobic granules.

These results demonstrate the reproducibility of granulation, the small influence of granulation on long-term stability, and the robustness of aerobic granulation for the removal of COD and N. Overall, our study contributes significantly to the understanding of microbial community structure for the long-term stability of aerobic granular sludge in the treatment of low-COD and low-COD/N-ratio wastewater in practice.

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References

1. Wang, L.; Liu, X.; Lee, D.-J.; Tay, J.-H.; Zhang, Y.; Wan, C.-L.; Chen, X.-F. Recent advances on biosorption by aerobic granular sludge. *J. Hazard. Mater.* **2018**, *357*, 253–270. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Lee, D.-J.; Chen, Y.-Y.; Show, K.-Y.; Whiteley, C.G.; Tay, J.-H. Advances in aerobic granule formation and granule stability in the course of storage and reactor operation. *Biotechnol. Adv.* **2010**, *28*, 919–934. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Liu, Y.; Liu, Q.S. Causes and control of filamentous growth in aerobic granular sludge sequencing batch reactors. *Biotechnol. Adv.* **2006**, *24*, 115–127. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Bengtsson, S.; de Blois, M.; Wilen, B.-M.; Gustavsson, D. Treatment of municipal wastewater with aerobic granular sludge. *Crit. Rev. Environ. Sci. Technol.* **2018**, *48*, 119–166. [\[CrossRef\]](#)
5. Adav, S.S.; Lee, D.-J.; Show, K.-Y.; Tay, J.-H. Aerobic granular sludge: Recent advances. *Biotechnol. Adv.* **2008**, *26*, 411–423. [\[CrossRef\]](#)
6. Cydzik-Kwiatkowska, A.; Wojnowska-Barya, I. Nitrogen-converting communities in aerobic granules at different hydraulic retention times (HRTs) and operational modes. *World J. Microbiol. Biotechnol.* **2015**, *31*, 75–83. [\[CrossRef\]](#)
7. Swiatczak, P.; Cydzik-Kwiatkowska, A. Performance and microbial characteristics of biomass in a full-scale aerobic granular sludge wastewater treatment plant. *Environ. Sci. Pollut. Res.* **2018**, *25*, 1655–1669. [\[CrossRef\]](#)
8. Cha, L.; Liu, Y.-Q.; Duan, W.; Sternberg, C.E.W.; Yuan, Q.; Chen, F. Fluctuation and Re-Establishment of Aerobic Granules Properties during the Long-Term Operation Period with Low-Strength and Low C/N Ratio Wastewater. *Process* **2021**, *9*, 1290. [\[CrossRef\]](#)
9. Pronk, M.; de Kreuk, M.K.; de Bruin, B.; Kamminga, P.; Kleerebezem, R.; van Loosdrecht, M.C.M. Full scale performance of the aerobic granular sludge process for sewage treatment. *Water Res.* **2015**, *84*, 207–217. [\[CrossRef\]](#)
10. Yang, S.F.; Tay, J.H.; Liu, Y. Effect of substrate nitrogen/chemical oxygen demand ratio on the formation of aerobic granules. *J. Environ. Eng.* **2005**, *131*, 86–92. [\[CrossRef\]](#)
11. de Kreuk, M.K.; Kishida, N.; Tsuneda, S.; van Loosdrecht, M.C.M. Behavior of polymeric substrates in an aerobic granular sludge system. *Water Res.* **2010**, *44*, 5929–5938. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Layer, M.; Adler, A.; Reynaert, E.; Hernandez, A.; Pagni, M.; Morgenroth, E.; Holliger, C.; Derlon, N. Organic substrate diffusibility governs microbial community composition, nutrient removal performance and kinetics of granulation of aerobic granular sludge. *Water Res.* **2019**, *160*, 100033. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Liu, Y.Q.; Liu, Y.; Tay, J.H. Relationship between size and mass transfer resistance in aerobic granules. *Lett. Appl. Microbiol.* **2005**, *40*, 312–315. [\[CrossRef\]](#) [\[PubMed\]](#)
14. APHA, *Standard Methods for the Examination of Water and Wastewater*, 23rd ed.; American Public Health Association, American Water Works Association, and Water Environmental Federation: Washington, DC, USA, 2017.

15. Hill, T.C.J.; Walsh, K.A.; Harris, J.A.; Moffett, B.F. Using ecological diversity measures with bacterial communities. *FEMS Microbiol. Ecol.* **2003**, *43*, 1–11.
16. Westphal, C.; Bommarco, R.; Carré, G.; Lamborn, E.; Morison, N.; Petanidou, T.; Potts, S.G.; Roberts, S.P.M.; Szentgyörgyi, H.; Tscheulin, T.; et al. Measuring bee diversity in different European habitats and biogeographical regions. *Ecol. Monogr.* **2008**, *78*, 653–671. [\[CrossRef\]](#)
17. Liu, Y.-Q.; Tay, J.-H. The competition between flocculent sludge and aerobic granules during the long-term operation period of granular sludge sequencing batch reactor. *Environ. Technol.* **2012**, *33*, 2619–2626. [\[CrossRef\]](#)
18. Liu, Y.Q.; Kong, Y.H.; Zhang, R.; Zhang, X.; Wong, F.S.; Tay, J.H.; Zhu, J.R.; Jiang, W.J.; Liu, W.T. Microbial population dynamics of granular aerobic sequencing batch reactors during start-up and steady state periods. *Water Sci. Technol.* **2010**, *62*, 1281–1287. [\[CrossRef\]](#)
19. Wang, L.; Zhan, H.; Wang, Q.; Wu, G.; Cui, D. Enhanced aerobic granulation by inoculating dewatered activated sludge under short settling time in a sequencing batch reactor. *Bioresour. Technol.* **2019**, *286*, 121386. [\[CrossRef\]](#)
20. He, Q.; Chen, L.; Zhang, S.; Chen, R.; Wang, H. Hydrodynamic shear force shaped the microbial community and function in the aerobic granular sequencing batch reactors for low carbon to nitrogen (C/N) municipal wastewater treatment. *Bioresour. Technol.* **2019**, *271*, 48–58. [\[CrossRef\]](#)
21. Yoon, J.-H.; Kang, S.-J.; Oh, T.-K. *Chryseobacterium daeguense* sp. nov., isolated from wastewater of a textile dye works. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 1355–1359. [\[CrossRef\]](#)
22. Makk, J.; Homonnay, Z.G.; Keki, Z.; Lejtovicz, Z.; Marialigeti, K.; Sproeer, C.; Schumann, P.; Toth, E.M. *Tahibacter aquaticus* gen. nov., sp. nov., a new gammaproteobacterium isolated from the drinking water supply system of Budapest (Hungary). *Syst. Appl. Microbiol.* **2011**, *34*, 110–115. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Chhetri, G.; Kim, I.; Kim, J.; Kang, M.; Seo, T. *Taibaiella lutea* sp. nov., Isolated from Ubiquitous Weedy Grass. *Curr. Microbiol.* **2021**, *78*, 2799–2805. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Gonzalez-Gil, G.; Holliger, C. Dynamics of microbial community structure of and enhanced biological phosphorus removal by aerobic granules cultivated on propionate or acetate. *Appl. Environ. Microbiol.* **2011**, *77*, 8041–8051. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Ehsani, E.; Jauregui, R.; Geffers, R.; Jarek, M.; Boon, N.; Pieper, D.H.; Vilchez-Vargas, R. First Draft Genome Sequence of the *Acidovorax caeni* sp. nov. Type Strain R-24608 (DSM 19327). *Genome Announc.* **2015**, *3*, e01378-15. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Feng, S.; Tan, C.H.; Constancias, F.; Kohli, G.S.; Cohen, Y.; Rice, S.A. Predation by *Bdellovibrio bacteriovorus* significantly reduces viability and alters the microbial community composition of activated sludge flocs and granules. *FEMS Microbiol. Ecol.* **2017**, *93*, fix020. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Han, X.; Zhou, Z.; Mei, X.; Ma, Y.; Xie, Z. Influence of fermentation liquid from waste activated sludge on anoxic/oxic-membrane bioreactor performance: Nitrogen removal, membrane fouling and microbial community. *Bioresour. Technol.* **2018**, *250*, 699–707. [\[CrossRef\]](#)
28. Ummalyma, S.B.; Gnansounou, E.; Sukumaran, R.K.; Sindhu, R.; Pandey, A.; Sahoo, D. Bioflocculation: An alternative strategy for harvesting of microalgae—An overview. *Bioresour. Technol.* **2017**, *242*, 227–235. [\[CrossRef\]](#)
29. Yu, H.; Meng, W.; Song, Y.; Tian, Z. Understanding bacterial communities of partial nitrification and nitrification reactors at ambient and low temperature. *Chem. Eng. J.* **2017**, *337*, 755–763. [\[CrossRef\]](#)
30. Staley, J.T.; Bouzek, H.; Jenkins, C. Eukaryotic signature proteins of *Prostheco bacter de jonegii* and *Gemmata* sp Wa-1 as revealed by in silico analysis. *FEMS Microbiol. Lett.* **2005**, *243*, 9–14. [\[CrossRef\]](#)
31. Baek, K.; Choi, A. Complete Genome Sequence of *Phreatobacter* sp. Strain NMCR1094, a Formate-Utilizing Bacterium Isolated from a Freshwater Stream. *Microbiol. Resour. Announc.* **2019**, *8*, e00860-19. [\[CrossRef\]](#)
32. Raittz, R.T.; de Pierri, C.R.; Maluk, M.; Batista, M.B.; Carmona, M.; Junghare, M.; Faoro, H.; Cruz, L.M.; Battistoni, F.; Souza, E.D.; et al. Article comparative genomics provides insights into the taxonomy of *Azoarcus* and reveals separate origins of *nif* genes in the proposed *Azoarcus* and *Aromatoleum* genera. *Genes* **2021**, *12*, 71. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Xi, L.; Liu, D.; Huang, W. Effect of acetate and propionate on the production and characterization of soluble microbial products (SMP) in aerobic granular sludge system. *Toxicol. Environ. Chem.* **2018**, *100*, 175–190. [\[CrossRef\]](#)