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Enhancing Effects of Sludge Biochar on Aerobic Granular Sludge for Wastewater Treatment

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Abstract: Sludge biochar can be used as bio-carrier to enhance aerobic granular sludge, however, its impact on the formation and especially long-term stability of aerobic granules has not been fully investigated. In this paper, aerobic granular sludge was cultivated in two parallel sequencing batch reactors (SBRs), R1 and R2, with and without sludge biochar addition in the activated sludge inoculum, respectively. The sludge characteristics, wastewater treatment performance, and microbial community structure of granular sludge were examined on a 240-day operation, during which aerobic granular sludge in the two reactors experienced dynamic changes including granule formation, maturation, breakage, filamentous proliferation, and recovery. Aerobic granules in R1 with biochar formed two weeks earlier than that in R2, presenting a larger mean size, and higher settling ability and biomass retention in the granule maturation period. Concurrently, aerobic granules in R1 showed higher denitrification ability with over 80% removal efficiency throughout the whole operation period. During the maturation period, the ratio of food to biomass (F/M) in R1 was below 0.5 gCOD/gVSS d while it ranged between 0.5 and 1.0 gCOD/gVSS d in R2 due to lower biomass retention. The elemental analysis showed more Ca and P accumulation in aerobic granular sludge from R1, with 3% Ca and 2.75% P in sludge from R1 and 0.91% Ca and 0.75% P in sludge from R2, respectively. The microbial community in R1 had higher richness, diversity, excretion of extracellular polymer substances (EPSs) and abundance of denitrifying genera than that in R2, supporting its higher stability and denitrification performance. These results demonstrated that aerobic granular sludge formed by using sludge biochar as a carrier for granulation can speed up granule formation, improve denitrification performance, and enhance the long-term stability of aerobic granules. The findings disclosed the enhancing effects of biochar for wastewater treatment by aerobic granular sludge, suggesting the potential of practical application of biochar in aerobic granular sludge-based reactors.

Keywords: aerobic granular sludge; sludge biochar; denitrification; formation; long-term stability; microbial community

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1. Introduction

Aerobic granular sludge technology has demonstrated high performance efficiency and small footprint in wastewater treatment practices. However, long start-up and unexplainable sludge instability sometimes during long-term operations are concerns for wide development of this technology in the wastewater treatment industry. To avoid these two problems, intensive studies have been conducted. Different strategies were used to speed up granulation such as using selective inoculum [1,2], adjusting operating conditions [3,4], and dosing carrier media or chemicals [5–7]. Stability of granules can be enhanced through the manipulation of operating conditions [8], selection of slow-growing microorganisms [9], or selective sludge discharging [10]. However, cost-efficient methods for fast start-up and long-term high performance and reliability stability are desirable in aerobic granular sludge systems.

In wastewater treatment plants, massive excess sludge discharge needs to be treated and managed properly to minimize environmental risks [11]. Among the current treatment methods of the excess sludge, pyrolysis can greatly decrease sludge volume, produce pyrolytic oil, and sequester carbon in the form of biochars, which is a sustainable solution for sludge disposal particularly in the circular economy. Biochars produced from sludge pyrolysis have high specific surface, porous structure, and enriched nutrients, and have great potential in soil mediation or the preparation of engineering materials. Recently, sludge biochar has been studied in aerobic granular sludge systems as a carrier media to enhance process. This opens a new method to enhance treatment efficiency of aerobic granular sludge systems. For example, Wang et al., (2020) used biochar produced from waste petroleum-activated sludge to enhance granulation and degradation of COD in petroleum refinery wastewater by aerobic granular sludge successfully [12]. As carrier media to be used for wastewater treatment by aerobic granular sludge, sludge biochar may exert both positive and negative effects on aerobic granules. On one side, sludge biochar may boost formation and enhance stability of granules by providing surface for the agglomeration of bacteria. Moreover, nutrients in biochar can be favorable to attached microbial organisms. On the other side, some compositions of sludge biochar especially polycyclic aromatic hydrocarbons may be toxic to the microbial microorganisms in aerobic granular sludge [13]. Thus, the functions and mechanisms of sludge biochar on the practical operation of aerobic granular sludge need to be elucidated. In addition, long-term stability of aerobic granular sludge with sludge biochar as a carrier has not been investigated yet, which hinders its further practical applications.

In this study, sludge biochar was applied as a carrier into the aerobic granular sludge system to investigate the formation and long-term stability of aerobic granules. The results from the 240-days operation of aerobic granular sludge reactor sheds light on the effects and mechanisms of biochar on the formation and long-term stability of aerobic granule sludge, providing guidance for the practical application of sludge biochar in aerobic granular sludge systems.

2. Materials and Methods

2.1. Biochar Preparation

The biochar was prepared by pyrolysis in a muffle furnace at 700 °C in a nitrogen atmosphere. The feedstock was municipal waste sludge, which was collected from a wastewater treatment plant in Kunming, Yunnan province of China. The dried sludge was sieved through a mesh of 100-m, which provided a mean size of the produced sludge biochar of 0.15 mm. The characteristics of the sludge biochar are presented in Table 1. The main minerals in the biochar are as follows: Al (7.23%), Fe (6.33%), Ca (2.24%), Mg (0.92%), K (0.91%), Ti (0.43%), and Zn (0.39%).

Table 1. Element analysis and property of the sludge biochar.

Sample	Element Analysis								Ash %	BET m^2/g	pH
	C%	H%	N%	S/%	O/%	H/C	O/C	(N + O)/C			
Biochar	7.09	0.48	0.52	0.32	10.59	0.07	1.49	1.57	90.58	67.45	8.57

2.2. Experimental Setup and Operation

The experiments were carried out in two bubble columns, R1 and R2, with sequential batch operating modes. The working volume of the reactors was 2 L with the inner diameter of 5 cm and the working height of 115 cm, resulting in a ratio of height to diameter (H/D) ratio of 23. The influent was fed from the bottom and the effluent was discharged from the middle with a volumetric exchange ratio of 50%. The cycle time of the SBRs was set as 4 h with 5 min of feeding, 55 min of anoxic condition, 145~170 min of aeration, 5~30 min of settling, and 5 min of discharging. In the aeration phase, the air was pumped into the reactors and diffused with an air sparger at the bottom of the reactors with a flowrate of

2 L/min. In the anoxic phase, the wastewater was circulated from the top to the bottom of the reactors to ensure good mixing. R1 and R2 were seeded with the activated sludge collected from a local wastewater treatment plant. A total of 2 g of sludge biochar was added to R1 as carrier media at the start of the experiment for enhancing the formation of aerobic granules, while R2 without biochar addition was operated as the control. Aerobic granular sludge is determined to form when granule is observed through a microscope and meanwhile SVI_{30}/SVI_5 of the sludge is close to 1.

2.3. Medium

The influent was synthetic wastewater with COD of 500 mg/L and ammonia of 25 mgN/L. COD and ammonia were supplied by $NaCH_3COOH$ and $(NH_4)_2SO_4$, respectively. $NaHCO_3$ was used to adjust pH in the range of 7.5–8.5. In addition, other nutrition was supplied, which included KH_2PO_4 22.5 mg/L, $CaCl_2{\cdot}2H_2O$ 12.5 mg/L, $MgSO_4{\cdot}7H_2O$ 15 mg/L, $FeSO_4{\cdot}7H_2O$ 10 mg/L, $MnCl_2{\cdot}4H_2O$ 0.12 mg/L, $ZnSO_4{\cdot}7H_2O$ 0.12 mg/L, $CuSO_4{\cdot}5H_2O$ 0.03 mg/L, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.05 mg/L, $NiCl_2{\cdot}6H_2O$ 0.1 mg/L, $CoCl_2{\cdot}6H_2O$ 0.1 mg/L, $AlCl_3{\cdot}6H_2O$ 0.05 mg/L, and H_3BO_3 0.05 mg/L.

2.4. Analytical Method

COD, ammonia nitrogen, nitrite, and nitrate were measured by ultraviolet visible light photometer (DR3900, HACH, Loveland, CO, USA). SVI, mixed liquor suspended solids (MLSSs) and mixed liquor volatile suspended solids (MLVSSs) were analyzed according to standard methods (APHA) [14]. Morphometry of the aerobic granules was observed by an optical microscope equipped with a digital camera (Leica Microsystems Wetzlar GmbH.DM100.DEU, Wetzlar, Germany). The mean size of the sludge 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). The elements such as C, H, N, S, and O in the biochar were analyzed by an element analyzer (MicroCube, Elementar, Frankfurt, Germany). Ca^{2+}, Fe^{2+}, and Mg^{2+} in aerobic granules were tested by inductively coupled plasma–optical emission spectroscopy (ICP) (PerkinElmer Avio 200 ICP-OES, Waltham, MA, USA) and scanning electron microscopy (SEM) and energy dispersive spectrum (EDS) (Hitachi SU-8010, Tokyo, Japan).

2.5. Extracellular Polymeric Substances (EPS) Measurement

EPS is composed of two fractions in aerobic granular sludge, including readily extractable EPS fraction termed as loosely bound EPS (LB-EPS) and condensed EPS fraction termed as tightly bound EPS (TB-EPS), respectively. The LB-EPS and TB-EPS were determined by the high-speed centrifugation and thermal extraction method, respectively [15,16]. The total organic carbon (TOC) contents of LB-EPS and TB-EPS were determined by TOC analyzer (vario TOC select, Elementar, Frankfurt, Germany).

Apart from the analysis of the content of EPS, the composition of EPS was further analyzed by a fluorescent photometer to obtain three-dimensional excitation-emission matrix fluorescence spectra (3D-EEM) (Hitach F-700, Tokyo, Japan) [17]. The spectrum was set by 5 nm incremental scanning emission spectroscopy, ranging in 250~550 nm and 200~400 nm. The scan velocity was set at 2400 nm/min. The data obtained from fluorescence spectrum were analyzed by the software of Origin 2019 64Bit.

2.6. Microbial Community Analysis of Granular Sludge

The seed and sludge were collected on different operation days and stored at −80 °C Sample DNA was extracted using a PowerSoil® DNA Isolation kit. The purity and concentration of the isolated DNA were measured by a Qubit 2.0 DNA kit (Life Technologies, Carlsbad, CA, USA). The 27F/1492R primer set (AGRGTTTGATYNTGGCTCAG/TASGGHTACC TTGTTASGACTT) was used to amplify V1-V9 region of the bacterial 16SrDNA gene. After amplification, PCR products were detected and purified. The amplicons were then sequenced using the MiSeq platform (Illumina, LA, USA).

The sequences were analyzed for diversity and taxonomic compositions. The operational taxonomic units (OUTs) clusters (USEARCH, version 10.0) were defined by a 97% identity threshold of the 16S gene sequence variants, and then were annotated based on Silva taxonomic database for OTUs taxonomic assignment. Microbial richness, indicated by Chao1 richness estimator (Chao1) and Ace richness estimator (ACE), and microbial diversity, indicated by Shannon–Wiener diversity index (Shannon) and Simpson diversity indices (Simpson), were assessed within a community (α-diversity), and were calculated and displayed with Mothur (Version 1.30).

3. Results and Discussion

3.1. Characteristics of the Aerobic Granular Sludge

Aerobic granular sludge was successfully cultivated in reactors R1 and R2 which were operated for 240 days. The morphology of the aerobic granules is shown in Supplementary Figure S1. It can be seen that aerobic granules in R1 originally appeared on day 24 and became dominant on day 33, while aerobic granules in R2 became dominant on day 55. At the same time, sludge biochar was observed as cores encapsulated in granules on day 33 in R1. After the maturation of aerobic granules, it was observed that mature granules in both reactors were broken on around day 83 followed by filamentous proliferation on day 140, but the granule deterioration occurred earlier in R2 than R1.

Sludge volume index in 30 min (SVI_{30}) is an important indicator of settling ability of sludge. For aerobic granular sludge with good properties, the ratio of SVI_5 to SVI_{30} (SVI_{30}/SVI_5) is close to 1. Thus, SVI_{30}/SVI_5 can be used to indicate the dominance of aerobic granular sludge. From Figure 1A,B, it can be seen that the settling ability of the aerobic granules in R1 was relatively stable: SVI_{30} gradually decreased to 41.6 mL/g on day 20 and then plateaued at around 75 mL/g to day 120; after that, it maintained in the range between 30 and 60 mL/g until the end of the operation. In contrast, the settling ability of the aerobic granules in R2 was quite fluctuated, i.e., SVI_{30} fluctuated from 30 to 120 mL/g after the first decrease to 50.8 mL/g on day 20. In addition, the value of SVI_5/SVI_{30} reached around 1 on day 33 in R1, and day 52 in R2, indicating that the formation of the aerobic granules in R1 was about 20 days earlier than that in R2. The difference in the evolution of SVI in the two reactors suggested that suspended activated sludge with biochar addition can be more quickly transformed into aerobic granule with better settling stability and maintain long-term stability.

Figure 1C,D show biomass concentration in the two reactors throughout the whole operation period. It can be seen that the biomass concentration can be divided into 3 stages. In the first stage, MLSS decreased to a low level and then increased quickly concurrently with the formation and maturation of aerobic granules until day 60. At the end of the first stage, MLSS in R1 and R2 reached 4.8 and 3.5 g/L, respectively. In the second stage, MLSS leveled at 4.5 g/L for 40 days and then gradually decreased to 2.5 g/L on day 127 in R1, while it fluctuated from 2.0 to 5.0 g/L in R2 until day 120. In this stage, it was observed that aerobic granules in both reactors experienced spontaneous breakage and morphology recovery (Supplementary Figure S1), leading to the fluctuation of MLSS. This indicates that the growth of aerobic granular sludge is a dynamic process, in which aerobic granules change particle size to adapt themselves to the changing environment caused by varied MLSS and F/M ratios [18]. In the third stage, MLSS in R1 fluctuated from 2 to 4 g/L before day 220 and then gradually increased to 4 at the end of the operation; while MLSS in R2 fluctuated at a lower concentration, i.e., around 1.5 g/L, until day 180, and then gradually increased to 4.1 g/L at the end of the operation. In this stage, filamentous bacteria grew to be dominant in both reactors; however, they just diminished without clear reasons. At the same time, it was found that MLVSS had a similar development as MLSS. Though it seems that the changing trends of sludge in both reactors were similar, it was noted that MLVSS in the maturation period of the aerobic granular sludge in R1 was much higher than that in R2, indicating that the presence of sludge biochar can assist to retain more biomass in the reactor.

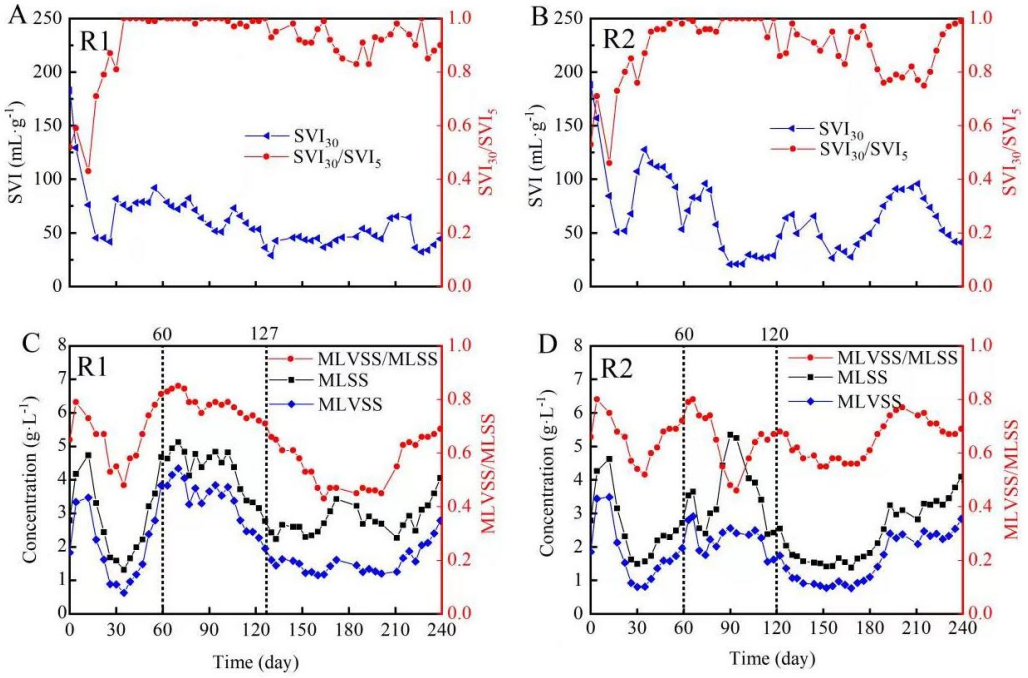

Figure 1. SVI and MLSS profiles in R1 and R2 during the long−term operation period. (**A**,**B**) SVI_{30} and SVI_{30}/SVI_5 and (**C**,**D**) biomass concentration of the aerobic granular sludge.

Food-to-microbial biomass ratio (F/M) is the substrate loading per unit biomass for wastewater treatment, which greatly affects the microbial growth in aerobic granular sludge. It was reported that F/M above 1.8 gCOD/gVSS d may boost the formation of aerobic granules, while F/M below 0.5 may sustain long-term stability of aerobic granules [19]. Figure 2 shows that F/M increased to the maximum of 2.5 gCOD/gVSS d in R1 on day 33 and 2.0 gCOD/gVSS d in R2 on day 35, respectively, due to the wash-out of biomass under short settling time, providing favorable F/M ratios for the formation of aerobic granular sludge. In the maturation period of the aerobic granular sludge, F/M reduced below 0.5 gCOD/gVSS d in R1 due to the retention of well-settled granular sludge, while fluctuated in the range of 0.5~1.0 gCOD/gVSS d in R2, showing that F/M ratio in R1 was more favorable to maintain the stability of mature granules than R2. In the following filamentous proliferation period, F/M was in the range of 0.7~1.3 gCOD/gVSS d in R1, which then greatly decreased due to the biomass wash-out caused by deteriorated settling ability of sludge with filamentous and reached 0.5 gCOD/gVSS d again. With this favorable F/M ratio, filamentous vanished and well-settled granules formed again at the end of the operation. While in R2, F/M was highly fluctuated in the range of 1.0~2.0 gCOD/gVSS d in this period, and there may be newly formed aerobic granular sludge since F/M increased to be as high as the values which are favorable for the formation of granules. These results revealed that R1 with sludge biochar addition operated more stably than R2 with relatively stable F/M ratios throughout the whole operation period.

Figure 3 shows size development of the aerobic granular sludge in R1 and R2, respectively. It can be seen that the mean size of the aerobic granular sludge in R1 was generally larger than that in R2 on most days. From day 44 to 91, the mean size in R1 maintained at around 526 μm, while it was between 345 and 440 μm in R2, smaller than that in R1. On day 170, the mean size in R1 increased to 814 μm, and 762 μm in R2. On day 200, due to the breakage of granules and overgrowth of filamentous bacteria, it decreased to 564 μm in R1 and 632 μm in R2. The discrepancy in the size development in the two reactors indicated that the mean size of the aerobic granular sludge with biochar addition increased more quickly than that of aerobic granular sludge without biochar.

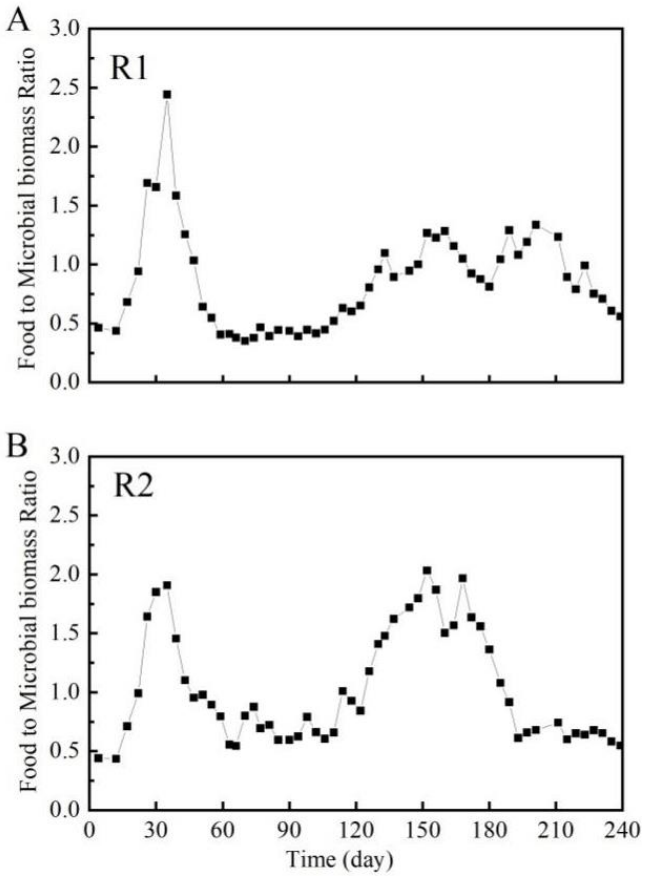

Figure 2. F/M in R1 and R2 throughout the whole operation period. (**A**) for R1; (**B**) for R2.

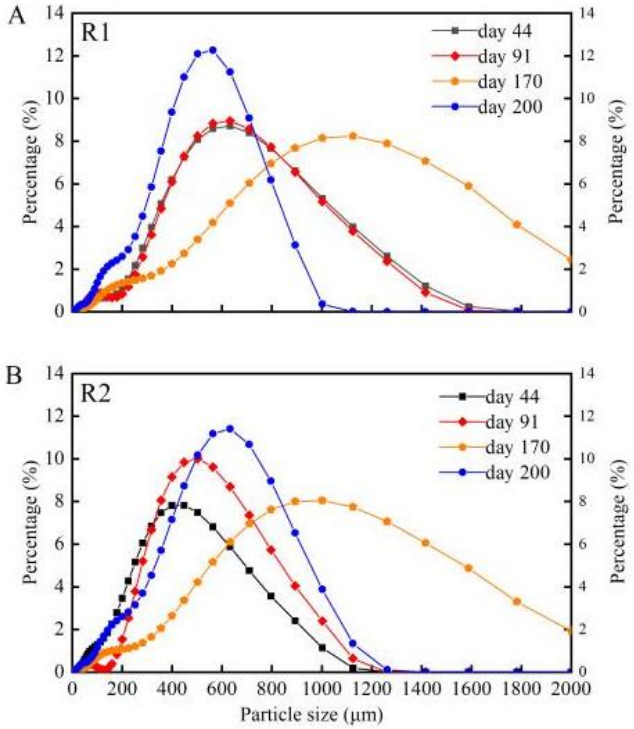

Figure 3. The change in mean sizes of the aerobic granules in R1 and R2 during the long-term operation period. (**A**) for R1; (**B**) for R2.

3.2. Elemental Analysis of the Aerobic Granular Sludge

Elements in the aerobic granules were analyzed after the formation of aerobic granules. Table 2 shows that the carbon content of the aerobic granular sludge in R1 was higher than that in R2. Since sludge biochar added in R1 had much lower carbon content (7.09%), the higher carbon content in the aerobic granules in R1 may be mainly due to the higher amount of microorganisms in the granules. At the same time, EDS analysis (Table 3 and Figure 4) shows that aerobic granular sludge in R1 also had higher Ca and P content than that in R2. Considering the high mineral content in sludge biochar, the high Ca and P contents in R1 may originate from sludge biochar. It has been widely reported that Ca and P are more likely to be observed at the core of aerobic granules. It is thus speculated that the chemical microenvironment in granules is favorable for CaP precipitation due to increased pH, Ca, and phosphate concentrations caused by mass transfer resistance and other reasons [20,21]. At the same time, it can be calculated from Table 3 that Ca/P molar ratio of granules in R1 is around 0.86, much smaller than the theoretical molar ratio (1.67) of hydroxyapatite, suggesting that mineral participants can be other types of CaP rather than hydroxyapatite [20] or calcium carbonate [22] observed in aerobic granules by others. In addition, it has been reported that the large granule size can create a more favorable chemical microenvironment for calcium precipitation [23], which might partly explain why aerobic granules in R1 had higher Ca and P contents. Higher contents of Ca and P in R1 can enhance the stability of aerobic granules due to the increased mechanical strength of granules.

Table 2. Elemental analysis of aerobic granular sludge.

Reactor	C%	H%	N%	S%	O%
R1	35.45	4.41	6.83	1.81	37.73
R2	30.98	4.18	6.80	2.35	38.28

Table 3. Elemental analysis of aerobic granular sludge by EDS.

Reactor	C%	O%	Na%	P%	S%	Ca%
R1	57.38	33.94	2.22	2.65	0.82	3.0
R2	57.72	36.91	3.02	0.75	0.70	0.91

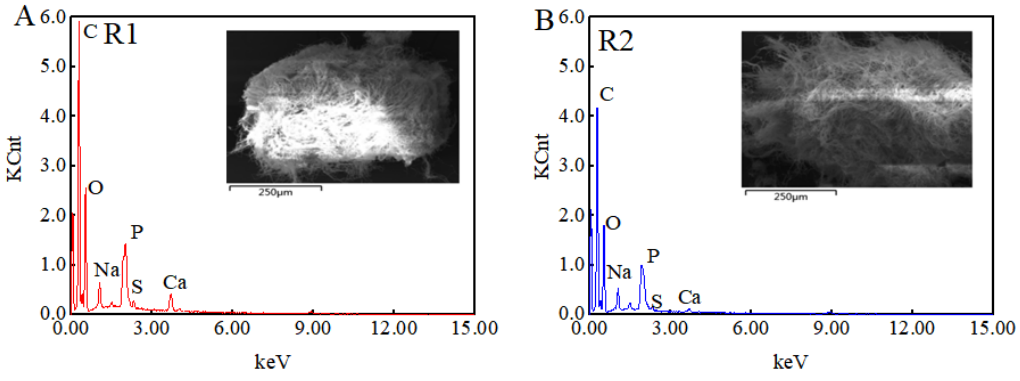

Figure 4. EDS analysis of granules in R1 and R2 on the operation day of 144. (**A**) for R1; (**B**) for R2.

3.3. Performance of Wastewater Treatment by the Aerobic Granular Sludge

Figure 5 shows the performances of wastewater treatment by the aerobic granules in the two reactors. It was found that both reactors had high removal efficiency in most of the operation period, i.e., over 95% for both COD and ammonia, though the aerobic granules experienced breakage and filamentous bacteria proliferation. During the formation of aerobic granular sludge, there was a period with lower ammonia removal efficiency in the two reactors, i.e., 80~90%. This can be attributed to the low nitrifying population in

low biomass concentration caused by the continuous wash-out of slow-settling flocs under selective pressure exerted by short settling time.

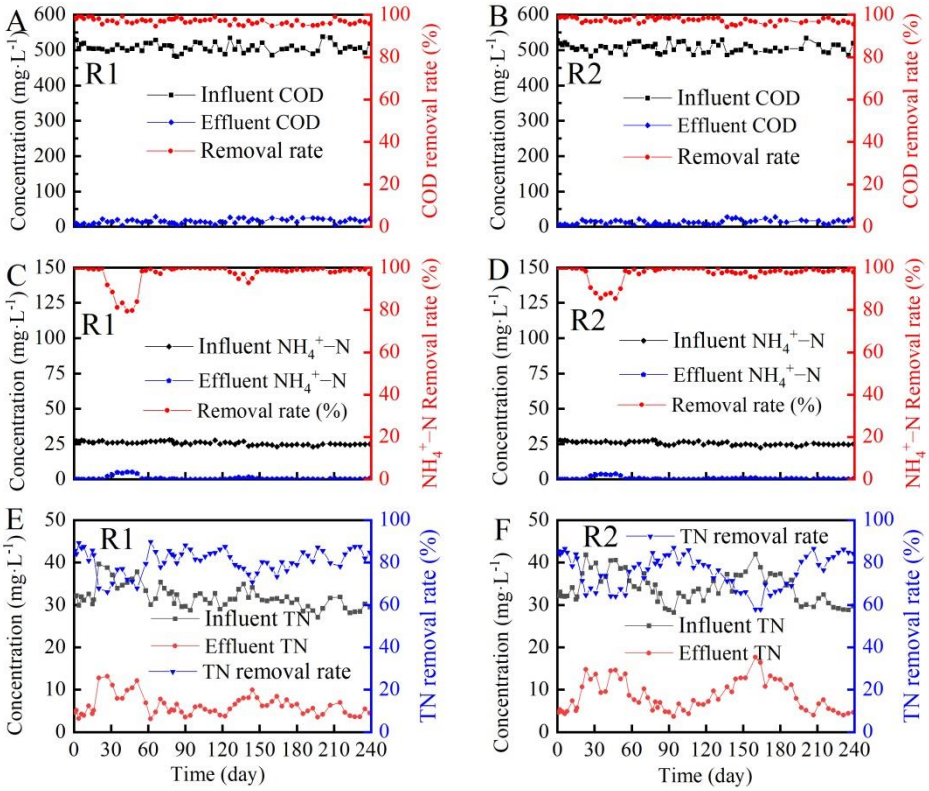

Figure 5. Performance of R1 and R2 with aerobic granular sludge. (**A**,**C**,**E**) for R1; (**B**,**D**,**F**) for R2.

TN removal by aerobic granular sludge is typically restricted by denitrification because of limited available carbon or insufficient anoxic zone in granules for denitrification [24]. From Figure 5, it can be seen that R1 had higher TN removal efficiency than R2. During the maturation period of aerobic granules, TN removal efficiency in R1 averaged at 84.3%, higher than 80.6% in R2. Even in the filamentous proliferation period, the TN removal efficiency in R1 was higher than that in R2, with 80.2% in R1 and 70.0% in R2. This can be partly explained by the larger size of the aerobic granules in R1, which may provide more anoxic zones in the aerobic granules for nitrite/nitrate removal through denitrification. The similar COD and ammonia removal efficiencies in both reactors and the higher TN removal efficiency in R1 suggest that biochar has little negative effect on the microbial activities of aerobic granules for COD and N removal.

3.4. EPS Contents in Aerobic Granular Sludge in R1 and R2

Extracellular polymeric substance (EPS) is the excretion of microbial organisms under certain environmental conditions, which is an important component of aerobic granules and may facilitate granule agglomeration, enhance resistance to unfavorable perturbation, and serve as carbon source when substrates are scarce, in the formation and long-term operation of aerobic granular sludge [25,26]. Normally, EPS integrated with aerobic granular sludge has double-layered structures, consisting of tightly bound EPS tangled with cells and loosely bound EPS extending outwards from cells, respectively. The content and composition of the two EPSs throughout the whole operation period were traced to reveal the role of sludge biochar on EPS of aerobic granular sludge. From Figure 6, it can be seen that the total amount of EPS indicated by total organic carbon (TOC) has similar levels in R1 and R2 during the formation and maturation of aerobic granules from day 75 to day 117. The amount of EPS increased consistently from around 60 to 120 mg/gVSS, facilitating the granulation of aerobic granules in the two reactors. However, during the following

filamentous proliferation period, the amount of EPS in the two reactors show a different trend. In R1, the amount of EPS decreased greatly on day 167 corresponding to the overgrowth of filamentous [27] and then recovered on day 199 and stabilized at high level until the end of the operation. While in R2, the amount of EPS abnormally increased to 140 mg/gVSS on day 167 and then greatly decreased to a lower level, which gradually recovered at the end of the operation. The reason for the abrupt increase in the amount of EPS in R2 after the filamentous proliferation needs further investigation. Notably, the high amount of EPS in R1 on day 199 and 240 shows that sludge biochar may stimulate the secretion of EPS in aerobic granules in filamentous proliferation, ensuring higher stability of aerobic granular sludge in long-term operation.

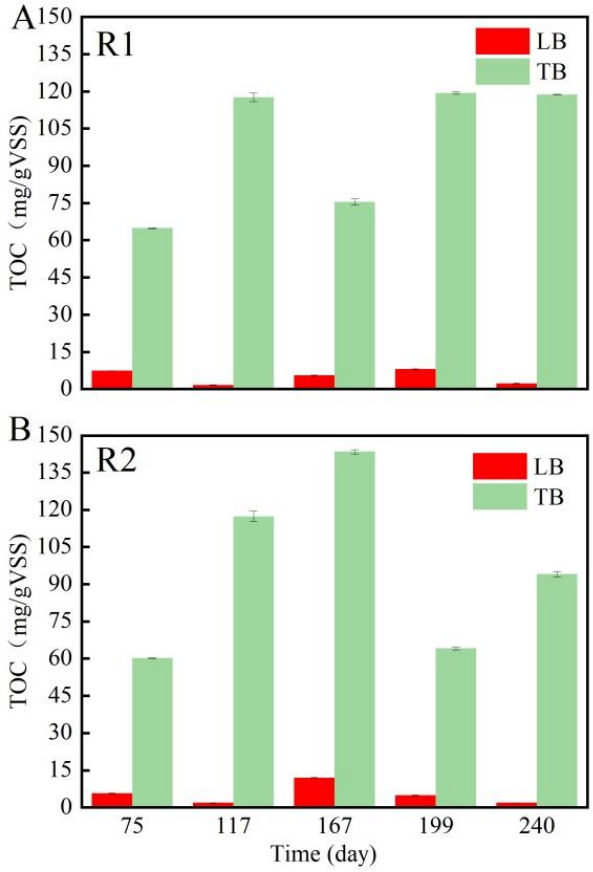

Figure 6. LB-EPS and TB−EPS contents in aerobic granules in R1 and R2 during the long-term operation period. (**A**) for R1; (**B**) for R2.

The composition of EPS in aerobic granular sludge in R1 and R2 were analyzed by 3D-EEM fluorescence spectra of EPS. Based on the classification by Chen et al., (2003) [28], the peaks representing the compositions of EPS can be classified as humic acid-like substances (Ex/Em: 320~380 nm/400~455 nm), tryptophan-like substances (Ex/Em: 260~300 nm/320~380), tyrosine-like substance (Ex/Em: 210~240 nm/300~360 nm), marine humic acid-like substances (Ex/Em: 310~330 nm/360~400 nm), and fulvic-like substances (Ex/Em: 210~280 nm/360~450 nm). Accordingly, the main peaks observed in Figures 7 and 8 corresponded to humic acid-like substances (peak A and F), tryptophan-like substances (peak B), tyrosine-like substance (peak C), marine humic acid-like substances (peak E), and fulvic-like substances (peak D, G, H).

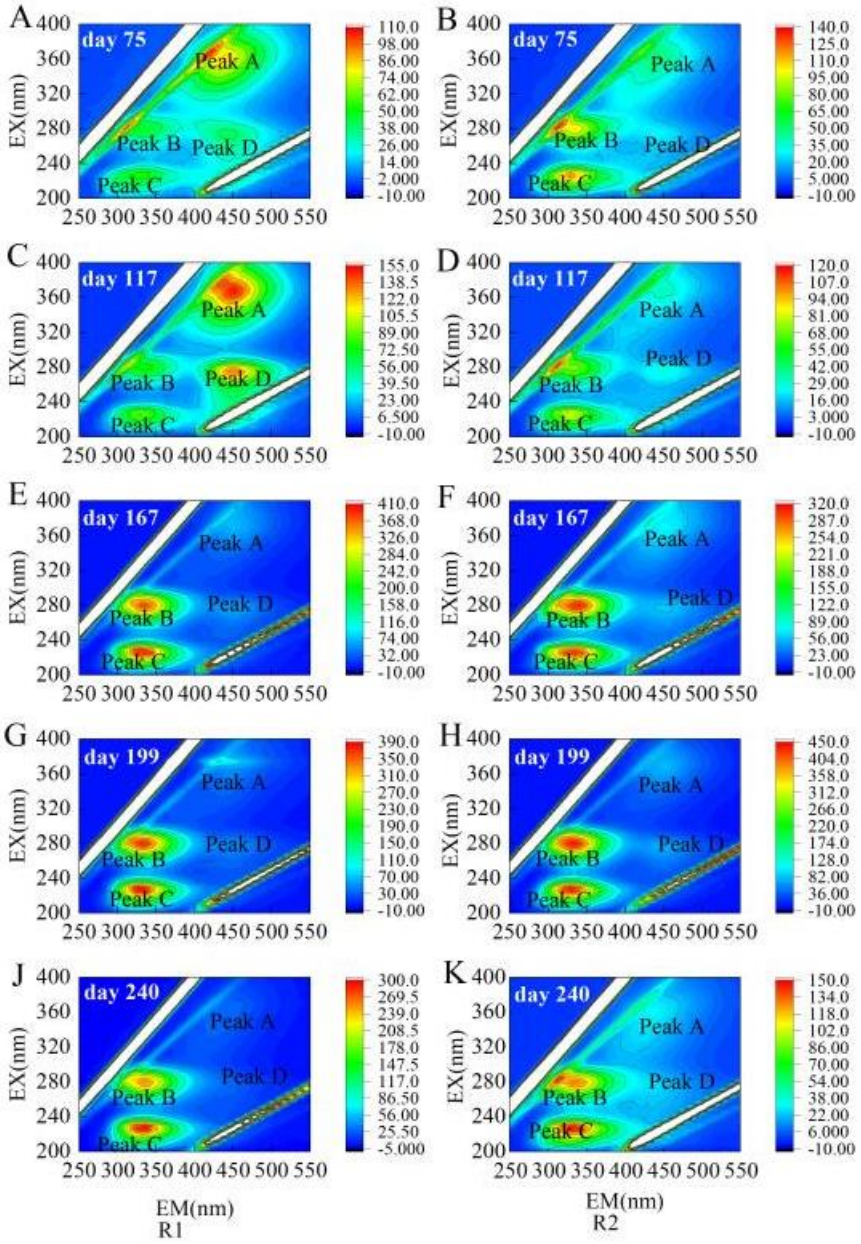

Figure 7. EEM spectra for LB−EPS of aerobic granular sludge in R1 and R2. (**A**,**C**,**E**,**G**,**J**) for R1; (**B**,**D**,**F**,**H**,**K**) for R2.

As shown in Figure 7, even though the peak intensities were different, the compositions of LB-EPS were quite similar in the two reactors, which consisted of humic acid-like substances (peak A), tryptophan-like substances (peak B), tyrosine-like substances (peak C), and fulvic-like substances (peak D). After the formation and maturation of aerobic granules on days 75 and 117, humic acid-like substances and fulvic-like substances reached a high quantity and increased with time in R1, while tryptophan-like substances and tyrosine-like substances had a relatively high quantity in R2. Tryptophan-like substances and tyrosine-like substances are the main components of aromatic protein, which are important components of microbial cells and extracellular enzyme and can be released during the metabolic process of cells. These protein substances were also reported to be important components for both granule formation and maintenance of long-term stability of aerobic granules [29]. Humic acid-like substances and fulvic-like substances are reported to be related to biological activity and dead cells [30]. The high contents of humic acid-like substances and fulvic-like substances in R1 can be correlated with the toxicity of sludge

biochar. After day 117, the tryptophan-like substances and tyrosine-like substance were the main compositions of LB−EPS in both reactors.

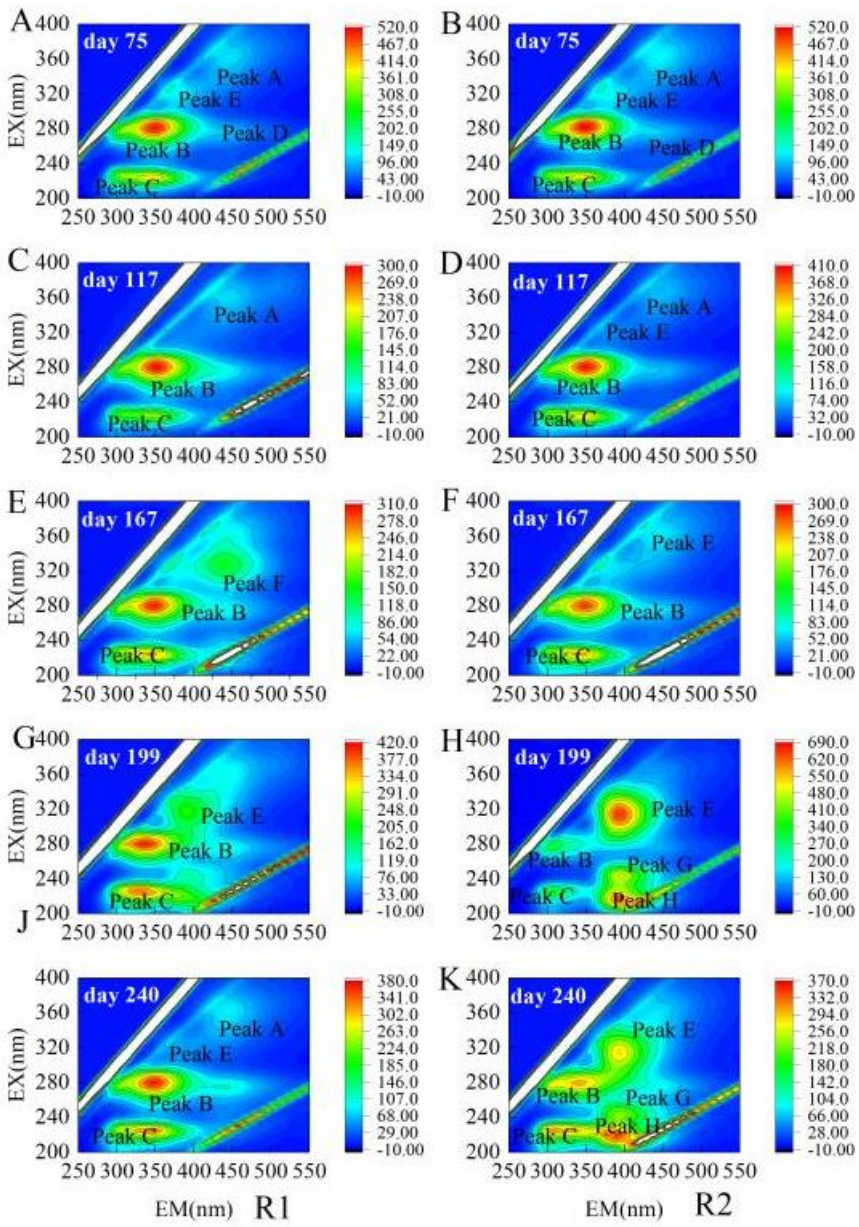

Figure 8. EEM spectra for TB−EPS of aerobic granular sludge in R1 and R2. (**A**,**C**,**E**,**G**,**J**) for R1; (**B**,**D**,**F**,**H**,**K**) for R2.

Figure 8 shows the EEM spectra for TB−EPS of aerobic granular sludge in the two reactors. It can be seen that the main compositions of TB−EPS were both tryptophan-like and tyrosine-like substances in the two reactors before day 167. On day 199, marine humic acid-like substances (peak E) and fulvic-like substances (peak F) gradually increased in R1, while marine humic acid-like substances (peak E) and fulvic-like substances (peak G) increased notably to be the main compositions with greatly declined tryptophan-like substances and tyrosine-like substance in R2. The different degrees of increase in humic acid-like and fulvic-like substances in the two reactors may indicate the high microbial lethality due to toxicity of biochar, during which filamentous proliferation was prevailed in the reactors.

3.5. Microbial Community Structure of the Aerobic Granules in Two Reactors

The microbial population determines the characteristics and performance of aerobic granular sludge for wastewater treatment. Its compositions, amount, and distribution might be changed by the presence of biochar.

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

Table 4 shows the richness and diversity indices of the microbial community in the aerobic granular sludge in R1 and R2. It can be seen that OTUs ACE and Chao1 in the inoculum decreased sharply after granules formed and there was not much difference in different stages in terms of the richness indices between the aerobic granules in the two reactors. This indicates that the species richness is highly dependent on sludge morphology and operating conditions and it was not affected by the presence of biochar. The granulation is a process to select some species with good auto or co-aggregation ability while washing out species with poor aggregation ability. Thus, it is not very surprising that OTUs and richness decreased after granules formed. The species diversity indicated by the Shannon index in sludge showed a similar trend in the two reactors, which decreased greatly after granular sludge formed and then decreased mildly until the end of the operation. Correspondingly, the Simpson index, which is negatively related to the species diversity, showed that the species diversity in the two reactors drastically decreased after the formation of the granules and then recovered a little in the following operation.

Table 4. The alpha diversity indices of the species in aerobic granules in R1 and R2.

	ACE		Chao1		Simpson		Shannon	
Reactor	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
Seed	7422.61	7421.42	5809.65	5810.05	0.03	0.03	5.69	5.70
Day 62	115.65	109.83	115.08	109.27	0.92	0.86	4.81	4.31
Day 130	148.46	138.64	152.07	137.25	0.88	0.78	4.15	3.73
Day 195	80.69	84.23	80.00	85.43	0.78	0.75	3.25	3.16

3.5.2. Microbial Population Dynamics and the Predominant Functional Groups

Figure 9 shows the composition of the microbial population in R1 and R2 at the phylum level. It can be seen that during the formation period of aerobic granules on day 62, the most predominant phyla in R1 and R2 were same, i.e., Proteobacteria, Bacteroidetes, and Firmicutes, which are common in both activated sludge [31] and aerobic granular sludge [19]. Proteobacteria and Bacteroidetes have high capability for the degradation of COD and oxidation of ammonium. *Firmicutes* has high adaptive ability to an extreme environment, which is easier to be enriched under the high selective pressure conditions in an aerobic granular sludge system [32]. However, compared with the high abundance of predominant phylum in R2, R1 had a more evenly distributed predominant phylum. Specifically, the three phyla Proteobacteria, Bacteroidetes, and Firmicutes account for 97.8% in R2, but account for 58.2% in R1 with six other phyla having at least 2% distributions, e.g., Verrucomicrobia and Cyanobacteria account for 5.38% and 4.48%, respectively. This relative higher phylum diversity of the microbial population in R1 may indicate that biochar is able to facilitate the survival of more microbial populations by providing a better niche for the formation of aerobic granules. In the maturation period on day 130 and long-term operation period on day 195, the predominant phylum was similar in the two reactors, with Proteobacteria and Bacteroidetes accounting for over 90% of the microbial community. This result is highly in agreement with the microbial community analysis with the clone library reported by Liu et al. that Proteobacteria and Bacteroidetes were dominant in acetate-fed granules [33].

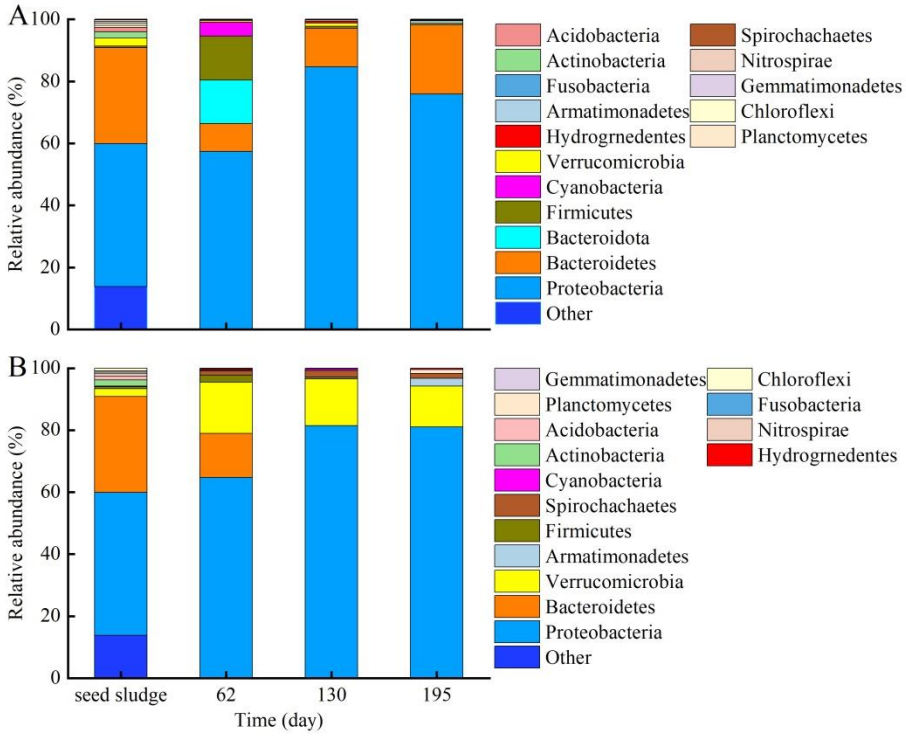

Figure 9. Microbial community structure and distribution at phylum level in R1 and R2 during the long-term operation period. (**A**) for R1; (**B**) for R2.

Figure 10 shows the composition of the microbial populations in R1 and R2 at the genus level. It can be seen that diversity of the predominant genera in R1 was higher than that in R2, especially on day 62 and 195. After the formation of the aerobic granules on day 62, the predominant genera were *Zoogloea*, *Thauera*, *Flavobacterium*, and *Saprospiraceae* in R2. While in R1, besides those four predominant genera, *Lactobacillus*, *Lelliottia*, and *Providencia* also had higher percentages, accounting for 9.81%, 6.74%, and 4.31%, respectively. Among the same predominant genera in the two reactors, *Zoogloea* and *Thauera* are well-known important genera in aerobic granules [33], which normally have high microbial proportions in granules with a good ability to denitrify nitrogen oxides and excrete EPS [34,35]. *Zoogloea* was the most abundant in both reactors, with 24.42% in R1 and 34.12% in R2, while *Thauera* accounted for 4.04% in R1 and 8.37% in R2. Their occurrence with much higher abundance than activated sludge was an obvious sign of successful formation of aerobic granules in the two reactors. Moreover, *Flavobacterium*, accounting for 6.41% in R1 and 7.1% in R2, respectively, is a typical floc-forming bacteria, which can secrete excess EPS and conduct denitrification [36,37]; *Saprospiraceae*, accounting for 2.72% and 4.38%, respectively in R1 and R2, has EPS excretion ability, and may contribute to the fermentation coupling with denitrification process [38]. These genera have functions of matrix structure stabilization and denitrification, facilitating the maintenance of structural stability and wastewater treatment performance of the aerobic granules. The other two predominant genera only present in R1, i.e., *Lelliottia* and *Providencia*, both have denitrification ability through different pathways [39–41]. The higher diversity and abundance of these functional genera in R1 explained better reactor performance of R1 shown in Figure 5. This indicates that the positive impact of sludge biochar on the reactor performance and granule stability is mainly due to the higher diversity and abundance of microbial community enhanced by biochar.

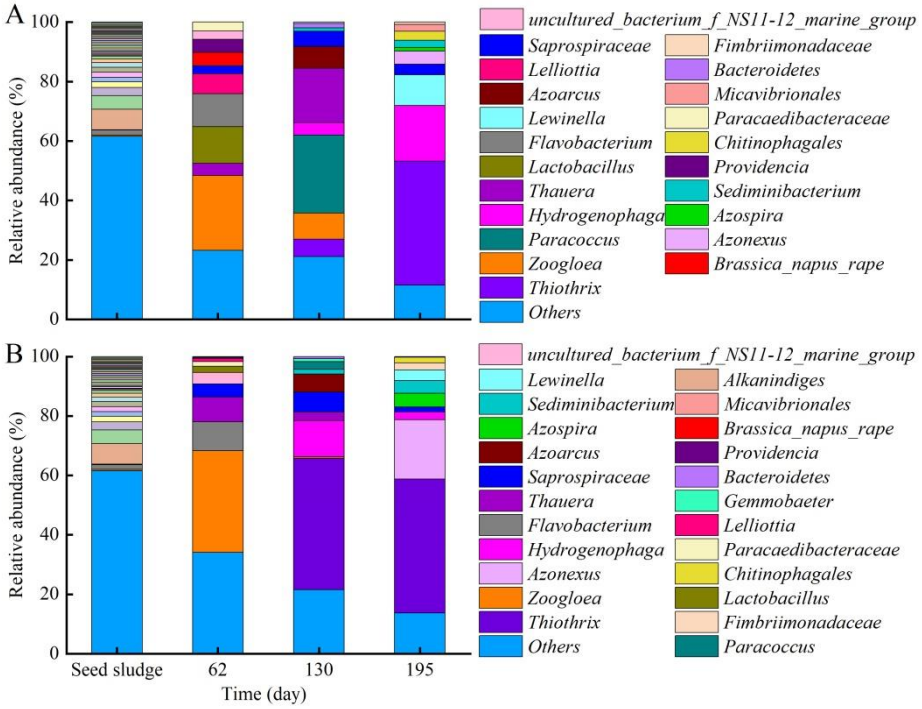

Figure 10. Microbial community structure and distribution at genus level in R1 and R2 during the long-term operation period. (**A**) for R1; (**B**) for R2.

During the maturation period of aerobic granules on day 130, the filamentous genus *Thiothrix* proliferated in R2 with 44.19% content, compared with 5.93% in R1, leading to sludge bulking in R2. As one of the most common genera causing fluffy aerobic granules, *Thiothrix* is very competitive for organic substrates due to its high growth rate, especially in substrate limiting environments [42]. In R2, with the overwhelming abundance of *Thiothrix*, other genera had much lower abundances, with only three genera having a high percentage of over 3%, including *Hydrogenophaga* of 12.12%, *Azoarcus* of 6.67%, and *Saprospiraceae* of 6.06%. In R1, without the over proliferation of *Thiothrix*, more denitrifiers with higher abundances, such as *Paracoccus*, *Thauera*, *Zoogloea*, and *Azoarcus*, existed, accounting for 26.29%, 18.3%, 8.7%, and 7.32%, respectively. By comparison, it was noted that the most abundant genus following *Thiothrix* in R2 was *Hydrogenophaga*, which is an autotrophic bacteria with denitrification ability [43]; while the most abundant genus in R1 was *Paracoccus*, which is a heterotrophic denitrifier, similar to other predominant genera [44]. This may demonstrate that aerobic granules can survive in a filamentous proliferation environment if autotrophic denitrifiers with no organic substrate demand, such as *Hydrogenophaga*, may enrich in the microbial community. In addition, the abundance of *Thiothrix* in R1 was much lower than that in R2, which might be due to the inhibitory effects of biochar on *Thiothrix* proliferation in aerobic granular sludge which led to better settling ability of granules and more stable physical characteristics of granules in R1.

On day 195, *Thiothrix* proliferated in both reactors, with 41.65% in R1 and 45.07% in R2, respectively. Interestingly, *Hydrogenophaga* also became the most predominant genus in R1 besides *Thiothrix*, similar to the conditions in R2 on day 130. This confirmed our previous speculation that the accumulation of *Hydrogenophaga* in filamentous proliferation may be favorable to the stability of aerobic granules. However, it was noted that Azonexus substituted *Hydrogenophaga* as the most abundant genus besides *Thiothrix* in R2, which accounted for 18.69% in contrast with only 2.66% of *Hydrogenophaga*. Azonexus is heterotrophic aerobic denitrifying genus, which greatly depends on organic substrates for energy [45]. Its predominance due to substitution for autotrophic *Hydrogenophaga* indicated that the substrates conditions in the environment that aerobic granules reside may have changed from day 130 to 195, though filamentous proliferation similarly prevailed on the

two days. By the comparison, it was noted that F/Ms of the two days in R2 were very different, which were 1.41 gCOD/gVSS d on day 130 and 0.61 gCOD/gVSS d on day 195, respectively. The higher F/M on day 130 boosted microbial activities and substrate uptake of *Thiothrix*, which inhibited the growth of heterotrophic bacteria due to the food competition, and in turn saved space for the growth of autotrophic bacteria with no organic carbon demand; while the lower F/M on day 195 was not optimal for *Thiothrix,* but suitable for the growth of heterotrophic bacteria, which simultaneously inhibited the growth of autotrophic bacteria [46] and caused a large decrease in the content of *Hydrogenophaga*. Correspondingly, the higher F/M of 1.08 gCOD/gVSS d on day 195 in R1, boosted the growth of *Hydrogenophaga* too, which was the same condition with that on day 130 in R2. These findings demonstrate that the chemical environment in reactors controlled by F/M affects the type of predominant genera and microbial community structure of aerobic granules. Thus, to obtain a more stable reactor operation, F/M should be closely monitored to guide sludge discharge for maintaining a stable and suitable F/M ratio.

4. Conclusions

By investigating the effects of sludge biochar on granule formation, stability, and wastewater treatment performance, the conclusions below could be drawn:

1. Both the formation of and long-term stability of aerobic granules were enhanced by adding biochar into inoculum only at the beginning of the reactor start-up;
2. The granules enhanced by biochar tended to accumulate more Ca and P which might be due to the larger size of granules with the addition of biochar;
3. No noticeable positive effects were observed for the COD oxidization and nitrification with the addition of biochar, but better denitrification with a higher abundance of denitrifying genera was found;
4. The higher microbial richness, diversity, EPS excretion, and steadier chemical environment were achieved in the reactor with the addition of biochar, enhancing long-term stability of aerobic granular sludge.

In summary, sludge biochar is promising to enhance aerobic granular sludge systems for biological wastewater treatment by just being added in the inoculum once to stimulate more healthy microbial community in granules.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10112385/s1, **Figure S1.** Morphology of the aerobic granules in R1 and R2 during the long-term operation period; **Figure S2.** SS in the effluent from R1 and R2; **Figure S3.** Ammonia, nitrite, and nitrate in the effluent from R1 and R2.

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