Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2021.27402

229 (2021) 93–103
July

Rapid start-up of aerobic granular sludge bioreactor for the treatment of automobile coating wastewater performance and analysis of microbial community dynamics

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Received 19 December 2020; Accepted 3 May 2021

ABSTRACT

In this study, by inoculating 65% activated sludge and 35% intact aerobic granular sludge (AGS), the rapid granulation of AGS was achieved to treat automobile coating wastewater (ACW). The results showed that after 15 d of operation, compact AGS which a clear shape was found in the sequencing batch reactor with the sludge volume index is 31 mL/g, and an average particle size of 1.2 mm. When the reactor was operated for about 50 d, the chemical oxygen demand, total nitrogen, total phosphorus, and phenol could be effectively removed from the ACW by AGS, with removal rates of 85%, 82%, 62%, and 90%, respectively. Furthermore, the microbial community dynamics were studied by high-throughput sequencing. The correlation between the pollutant removal efficiency and the level of the microbial community (phylum, class, order, family, and genus) was investigated. The results showed that the most abundant genus *Thauera* played an important role in phenol degradation. Moreover, the abundances of *Zoogloea*, *Azoarcus*, *Stenotrophomonas*, *Sediminibacterium*, *Methyloversatilis*, and *Acidovorax* gradually increased. These functional genera had a significant effect on the biodegradation of phenol and organic matter. This research provides new insights into the rapid start-up of AGS bioreactors and illustrates the practical application potential of AGS technology in the treatment of phenol-containing ACW.

Keywords: Rapid granulation; Automobile coating wastewater; Aerobic granular sludge; Phenol; Sequencing batch reactor; High-throughput sequencing

1. Introduction

In recent years, with the increasing demand for automobile products, a large amount of automobile manufacturing wastewater has been produced. The discharge of automobile manufacturing wastewater considerably affects the natural environment and human health. The primary hazardous wastewater in automobile manufacturing plants is the automobile coating shop, which generates significant environmental strain during the production of vehicles [1]. In addition, various types of wastewater are generated due to the use of different chemical materials in the automotive surface coating process. These wastewater supplies contain rich organic matter, heavy metals, suspended solids, and a low biodegradability index, and thus are harmful to the environment [2]. Meanwhile, traditional biological treatment technologies cannot effectively treat automotive coating wastewater (ACW). ACW treatment has become a priority environmental issue for the coatings industry.

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Phenol is the primary pollutant of concern in industrial wastewater attributed to its high toxicity, high oxygen demand and poor biodegradability [3]. Meanwhile, phenol is also one of the primary pollutants in ACW. Owing to its carcinogenic properties and high toxicity to human health, its removal is also received considerable concerns [4]. For fish, the concentration of phenol over 2 mg/L is toxic, and concentrations of 10 to 100 mg/L will cause the death of aquatic organisms within 96 h [3,5]. The discharge of wastewater containing phenols can have harmful effects on human health and environmental damage if not adequately treated [6]. Therefore, it must be removed from the environment. However, conventional biological wastewater treatment methods are not effective in treating phenol-containing wastewater due to the inhibitory effect of substrates. To date, there are quite a few studies have reported on ACW treatment. The main methods include anaerobic degradation methods [7], advanced oxidation processes [8], anaerobic treatment [9], CF-mFe/Cu/O_3 treatment process [10]. Nonetheless, there are still some shortcomings, including the removal of organic matter in ACW is limited, by-products that may cause secondary environmental problems and high treatment costs are not suitable for practical engineering applications. Therefore, there is an urgent need for practical, environmentally friendly and economically attractive ACW treatment technologies under such disturbing circumstances.

Aerobic granular sludge (AGS) is considered to be the most potential wastewater biological treatment technology in the 21st century due to its high biomass concentration, excellent organic degradation performance, resistance to high organic loads, fast settling performance, and is not easily cause sludge bulking. As previously reported [11], AGS can endure the pressure load of highly toxic substances and concentrations. Previous findings have shown that phenol biodegradation in the AGS bioreactor has been successfully achieved [12]. However, the long granulation time is one of the main bottlenecks in applying this technology when the reactor is injected with real industrial wastewater. The flocculated sludge particles and the large amounts of biomass washed out of the reactor during granulation time has resulted in low pollutant degradation efficiency, thereby limiting its large-scale and widespread application of this technology. Many studies have shown that the cultivation of AGS with synthetic wastewater usually takes approximately 30 d to complete granulation, and when real wastewater was treated, the granulation time was usually more prolonged to greater than 400 d [13]. The main reason is that the real wastewater quality components are complicated, and their concentrations change significantly. Such a long granulation time limits the practical application and development of this technology in wastewater treatment. Therefore, achieving rapid cultivation of AGS is the key to its wide application. Extensive research has been conducted to reduce the granulation time of AGS. The results have shown that the granulation time of AGS is related to the inoculated sludge [14–19]. Previous studies have also demonstrated that the inoculation of crushed AGS or mature AGS plays a vital role in the granulation process of flocculated activated sludge, and can usually reduce the granulation time of AGS [15]. Meng et al. [20] inoculated mature AGS after 30 d of cultivation in a continuous flow reactor to obtain AGS with a size of 0.38 mm to treat saline wastewater. Long et al. [21] achieved rapid AGS granulation within 18 d by inoculation of 25% mature AGS and gradually shortening the precipitation selection pressure. This strategy can minimize the amount of biomass washed out of the reactor during the granulation period and maintain a high pollutant removal performance during the initial stage of the reactor start-up. In addition, there are few reports on inoculating part of the intact AGS in the rapid start-up of an AGS bioreactor to treat real high-concentration industrial wastewater. This study presents for the first time a method that reduces the start-up time of an AGS reactor by adding a mixture of intact AGS and activated sludge to treat phenol-containing ACW.

This work develop a novel strategy for rapid startup of AGS bioreactors to treat phenol-containing ACW by inoculating partially intact AGS and activated sludge. To evaluate the feasibility of this strategy, the sludge morphology, particle size distribution, biomass concentration, sludge sedimentation performance, pollutant removal efficiency, and microbial community structure were monitored during the experimental period. The predominant microbes in the bioreactor for the degradation of phenol-containing ACW were also demonstrated. This work could provide useful information about the rapid start-up AGS bioreactors and encourage the application of AGS technology to treat ACW.

2. Materials and methods

2.1. Experimental setup and operating conditions

The experiment was conducted in the aerobic granular sludge sequencing batch reactor (AGSBR). Fig. 1 presents the schematic of the lab-scale SBR in the experiment. The AGSBR had a working volume of 3 L with 1 m height to 0.07 m diameter, the volumetric exchange ratio was 50% and an air aeration rate of 180–220 L/h. The AGSBR operated 6 cycles per day. One 4 h cycle included 4 min of feeding, 224–231 min of aeration period, 10–3 min of sludge settling, and 2 min of effluent discharge. The initial settling time was set to 10 min, and the stable phase was 3 min. The sludge retention time was not controlled during operation. The system was controlled using a timing controller and operated at ambient temperature (22°C ± 3°C). To cultivate and domesticate the AGS and eliminate the ecological harm of toxic substances in ACW. The entire experimental process was divided into three phases based on the addition of different proportions of ACW. The respective dilution ratios of the raw ACW and synthetic wastewater (v/v) were set at 30:70 (phase I, 1–10 d), 90:10 (phase II, 10–31 d), and 100:0 (phase III, 31–55 d).

2.2. Seed sludge and wastewater composition

The inoculation sludge used a mixture of activated sludge and intact AGS with weight proportions of 65% and 35%, respectively. The activated sludge was obtained from the SBR process pool in the coating wastewater treatment workshop of an automobile factory in Hefei, China. The wastewater treatment workshop mainly deals with phosphating wastewater, electrophoretic wastewater, and spray

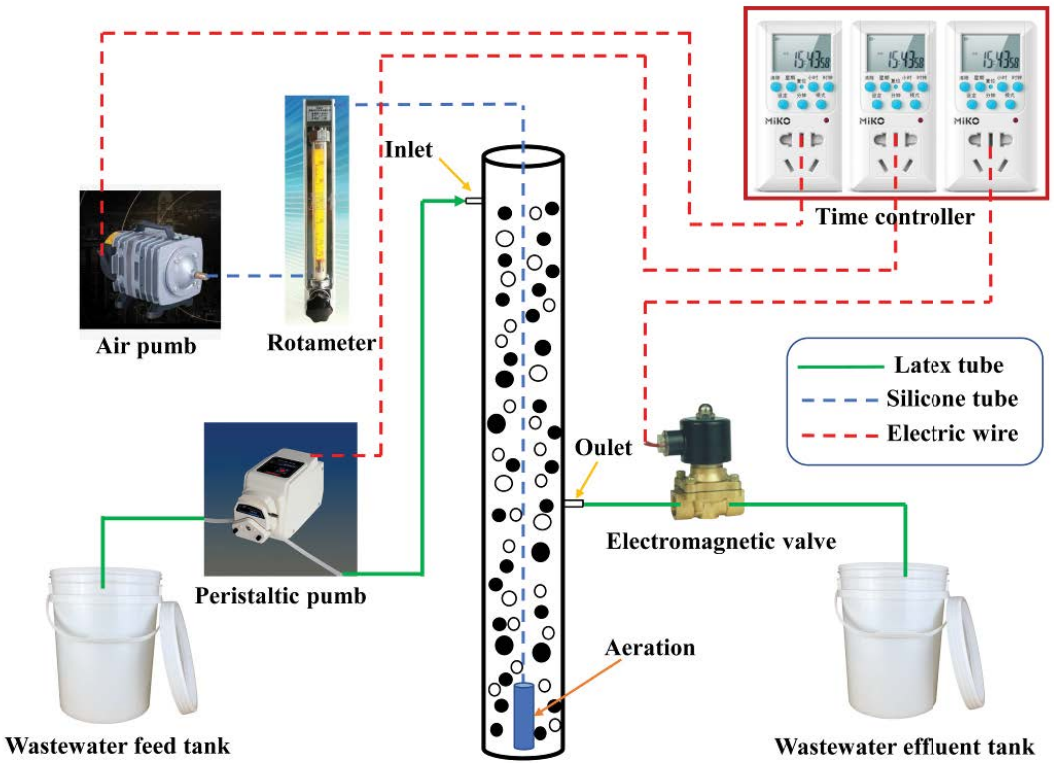

Fig. 1. Experimental setup of the aerobic granular sequencing batch reactor.

paint wastewater. The intact AGS were obtained from the same culture conditions of a 3 L SBR reactor in the laboratory for the treatment of ACW. The particle size of the intact AGS was approximately 1.0–1.2 mm with a smooth surface and compact structure. The initial sludge volume index (SVI) was 40 mL/g, and the mixed liquor suspended solids (MLSS) concentration was approximately 5,500 mg/L.

The synthetic wastewater uses anhydrous sodium acetate as a carbon source, nitrogen (NH_4Cl) and phosphorus (KH_2PO_4) were supplemented, and the trace elements solution contained (per liter): $CaCl_2$ 20 mg, $MgSO_4{\cdot}7H_2O$ 50 mg, $CuCl_2$ 0.05 mg, $NiCl_2{\cdot}6H_2O$ 0.05 mg, $FeCl_3{\cdot}6H_2O$ 0.83 mg, $MnSO_4{\cdot}H_2O$ 0.05 mg, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.05 mg, H_3BO_4 0.05 mg, $CoCl_2{\cdot}6H_2O$ 0.045 mg, $ZnSO_4{\cdot}6H_2O$ 0.11 mg, $Al_2(SO_4)_3{\cdot}18H_2O$ 0.25 mg. The influent pH was controlled at 7 by adding 1 M HCl or $NaHCO_3$. The chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) concentrations in the synthetic wastewater were approximately 500 ± 100 mg/L, 15 ± 5 mg/L, and 5 ± 2 mg/L, respectively.

Raw ACW was collected from a local automobile factory in Hefei, China. The concentrations of COD, TN, TP, and phenol in ACW were approximately 800 ± 200 mg/L, 30 ± 10 mg/L, 15 ± 10 mg/L, and 35 ± 10 mg/L, respectively. During phase I, the COD, TN, TP, and phenol concentrations in the ACW were approximately 800 ± 200 mg/L, 30 ± 10 mg/L, 15 ± 10 mg/L, and 35 ± 10 mg/L, respectively. Those in phase II were approximately 860 ± 100 mg/L, 35 ± 5 mg/L, 16 ± 8 mg/L, and 34 ± 8 mg/L, respectively, and those in phase III were approximately 826 ± 30 mg/L, 34 ± 5 mg/L, 17 ± 7 mg/L, and 35 ± 7 mg/L, respectively.

2.3. Analytical methods

2.3.1. Water quality and sludge property analysis

Samples were regularly collected from the water influent and effluent for MLSS, COD, TN, TP, and SVI analyses according to standard methods [22]. To investigate the removal of pollutants from ACW by AGS, the mixture of the influent and effluent of the bioreactor was collected before and after aeration approximately every 2 d and filtered through a 0.45 mm filter membrane. The filtered water sample was used for water quality analysis [23]. Throughout the experimental period, the phenol concentrations in the influent and effluent of the AGSBR were measured by the GC-MS analysis method [24]. Size distribution was measured by the wet sieving separation method [21]. Scanning electron microscope (SEM) (GeminiSEM500) was used to observe the microscopic behaviour of AGS and pretreatment as described by Adav et al. [25].

2.3.2. Microbial community analysis

To analyze the dynamic changes in the microbial community throughout the experiment, 5 mL sludge samples were collected from the AGSBR operating at 1 (S1), 11 (S2), 21 (S3), 31 (S4), 41 (S5), and 51 d (S6). According to the manufacturer's instruction, the DNA extraction was using the E.Z.N.A.® Soil DNA Kit (MoBio, USA) [26]. The total extracted DNA samples were quantitatively and qualitatively analyzed by a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA). Then, they were frozen at –20°C. The V4–V5 region of the 16S rDNA gene was

amplified by the polymerase chain reaction (PCR) using primers were 520F (5′-GCACCTAAYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) [27]. The composition of the PCR mixture (0.25 μL) included the following agents: 5.00 μL of 5 × FastPfu Buffer, 5.00 μL of 5 GC enhancer, 0.50 μL of 10 mM dNTPs, 1.00 μL of 10 μM primer 520F, 1.00 μL of 10 μM primer 802R, 1.00 μL of DNA template, and 11.25 μL of H_2O. The thermal program was as described by Dai et al. [28]. The PCR products were examined on a 2% agarose gel and further purified. High-throughput sequencing was conducted using the Illumina Miseq platform (Miseq PE300, Illumina, USA) at the Majorbio Bio-Pharm Technology Company (Shanghai, China). All the raw sequences generated were submitted to the NCBI Sequence Read Archive database with accession number SRP215564.

3. Results and discussion

3.1. AGS morphology and properties

Owing to the hydraulic shear damage and the temporary inadaptation of the sludge to the shock of the pollutant concentration in the ACW in the first 5 d of reactor startup, some particles were disintegrated into flocs on day 5 (Fig. 2a), the sludge exists in the form of a loose structure and irregular morphology. This phenomenon was also observed by Wang et al. [16], who found that during the reactor start-up stage, when the aeration rate was high, the number of broken particles increased, many small particles appeared in the reactor, and the effluent contained flocculated sludge. In contrast, the reactor with a lower aeration rate had almost no broken particles. Therefore, the low aeration rate and low hydraulic shear force at the beginning of the start-up phase facilitated aerobic granulation and maintained the sludge concentration in the reactor. Similarly, Long et al. [29] found that when particles temporarily did not adapt to wastewater shocks, some of the large particles disintegrated into small particles or flocculated sludge. When AGS is broken, some of the granules will gather again to form new granules. The broken granules will be attached as flocculated granules, thereby accelerating new granular sludge formation [18,30,31]. As shown in Fig. 2g, the MLSS concentration decreased from 5,500 to 5,010 mg/L, and SVI increased from 35 to 41 mL/g. As there was no sludge return system in the AGSBR system, part of the biomass was flushed out while the reactor was operating, leading to the decline of MLSS in the system. Under the dual reaction of hydraulic shear and sedimentation selective strength, only granular sludge with a faster settling rate is finally retained in the AGSBR. The traditional culture method gradually shortens the settling time and timely discharges flocculent sludge with poor settling performance to provide suitable conditions for the accumulation of AGS, but this will cause a large amount of biomass to be washed out of the reactor, and inoculation of partially intact AGS can effectively avoid these defects [15,32]. Using similar start-up strategies, Coma et al. [14] found that adding partially crushed sludge to flocculation sludge avoided significant biomass loss

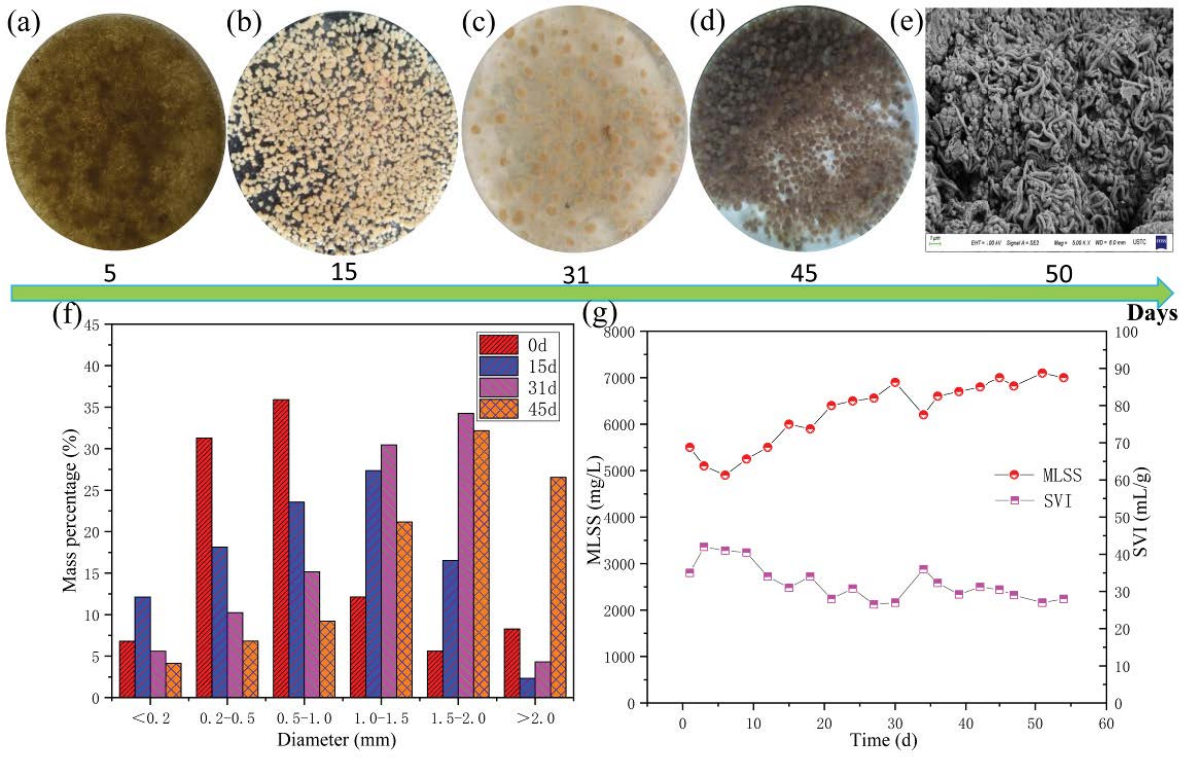

Fig. 2. Variations of the sludge characteristics and morphology in the reactor at different operation periods: (a) 5 d, (b) 15 d, (c) 31 d, (d) 45 d, (e) 50 d granule morphology of AGS using SEM, (f) dynamic changes of granular size and distribution, and (g) the biomass and sedimentation performance of the AGSBR (for 55 d).

and enhanced the start-up capability compared with only inoculating flocs in the start-up reactor.

When the operation time increased, granular sludge was gradually formed, and flocculent sludge in the AGSBR was gradually reduced, and the particle size increased. The MLSS concentration increased from 4,900 mg/L on the 6th day to 6,000 mg/L on the 15th day, and the SVI showed a gradual decrease. SVI decreased to 31 mL/g on day 15 (Fig. 2g). AGS with a relatively regular and compact structure was found at the sampling port on the 15th day. The average particle size of the AGS was 1.2 mm (Fig. 2b). As shown in Fig. 2f, the particle size of the AGS in the reactor is concentrated at 0.5–1.5 mm (accounting for 51%). Similar to previous studies, Long et al. [21] inoculated 25% mature AGS sludge and successfully cultivated AGS with a good pollutants removal performance on the 18th day. This novel strategy of inoculating AGS can shorten the reactor start-up time, reduce the amount of biomass washed out from the reactor, and maintain a high pollutant removal performance during granular sludge formation. On the 31st day, the influent water was adjusted to be all ACW containing a high organic matter content. Therefore, organic matter can diffuse into the particles to promote the growth of deep-layer microorganisms. Increased organic matter concentrations may accelerate the growth of microorganisms and increase the particle size [33,34]. At this phase, the AGS was spherical in shape and had a compact, completely granular structure. The flocs were barely observable in the AGSBR and changed in color from light yellow to yellowish-brown (Fig. 2c). Compared with the 15th day, the average particle size of AGS has increased to 1.5 mm, and the particle size is mainly concentrated in 1–2 mm (Fig. 2f, accounting for 65%), and the surface of the granular sludge was smooth and shiny. Meanwhile, the MLSS concentration increased steadily from 6,200 mg/L at 34 d to 7,000 mg/L at 45 d. It was speculated that the microbial group gradually adapted to the added ACW. These results indicate that the AGSBR can maintain a high biomass concentration and withstand the impact of high organic load and toxic conditions.

After 45 d of operation, the AGS has been fully adapted to the ACW and could maintain a stable form in the AGSBR. Compared with that day 31, the average particle size of AGS has increased to 1.8–2 mm, and the particle size is mainly concentrated in 1.5–2 mm (Fig. 2f, accounting for 59%). The surface of the granular sludge was glossy and all brown in color (Fig. 2d). As shown in Fig. 2g, the MLSS concentration remained stable at 6,900 mg/L, and the SVI gradually decreased and remained at about 28 mL/g. The higher MLSS and lower SVI indicators indicated that after acclimation of ACW, the AGS can maintain a high concentration of microbial biomass in the reactor, which was conducive to the rapid degradation of organic matter and showed that AGS has a good settling performance. Previous studies showed that a large amount of biomass is retained in the reactor owing to the good settling performance of granules, and the high biomass retention capacity improves the system's ability to treat wastewater with high organic loading rates [29,34,35]. The detailed microstructure of AGS on day 50 was observed by scanning electron microscopy (SEM, Fig. 2e). It could be seen that bacilli and cocci dominate the surface of the sludge, and the granules had rich biological species inside. The experimental results are consistent with previous studies, which reported that particle surfaces were associated with many bacilli and cocci in a phenolic pyridine degradation system [36]. Additionally, a small amount of filamentous substance on the surface of the particles plays a crucial effect on the connection between the bacillus. This structure gives the AGS good particle strength. These findings were also confirmed by Long et al. [29], who found that filamentous bacteria were predominantly distributed in the outer layer of the granule with bacillus and coccus closely attached, and that their unique structure improved the ability of the granule to resist outside toxic effects.

3.2. Pollutant removal performance

3.2.1. COD removal performance

The COD, TN, and TP removal performances of AGS in phenol-containing ACW treatment during 55 d were investigated and are shown in Fig. 3.

From Fig. 3a, the COD removal efficiency of the AGSBR system was about 92.7% in the first 10 d. The effluent COD ranged from 27 to 72 mg/L. Then it stabilized briefly until day 24 and then continued to decrease to 74.2% on day 29. Moreover, the effluent COD concentration was higher than that in the previous phase because the AGS was adapting to the ACW. However, the proportion of actual ACW in phase II was higher than that in phase I. The complexity and toxicity of industrial wastewater will decrease the pollutant degradation efficiency of microorganisms; therefore, phase II removal efficiency was lower than that in phase I [23]. Our results are similar to those obtained by Yusoff et al. [37], who found that as the concentration of phenol increased, the complete removal of phenol and COD could not be achieved. Furthermore, when the phenol addition increased from 25 to 100 mg/L, the COD removal rate decreased. After 29 d, the COD removal efficiency rate increased significantly from 74.2% to 87.3% on day 39. It was speculated that the AGS gradually adapted to the water quality of ACW. The AGS contained a large amount of organic matter degrading bacteria. The increase in the proportion of ACW had no significant effect on the COD removal rate. The overall removal effect was stable at about 85% and was not adversely affected by the organic load of the water inflow. These results demonstrate that AGS can degrade organic matter in ACW very well and the high organic removal capacity during the start-up stage further illustrates the progressive nature of the strategy by inoculating intact AGS to start the reactor.

3.2.2. TN and TP removal

As shown in Fig. 3b and c, the influent concentrations of TN and TP ranged from 26.7–38.8 mg/L and 6.8–25.4 mg/L respectively. In the first 10 d of reactor operation, the AGSBR effectively removed 78% of TN and 65% of TP. After 19 d, the TN removal rate decreased rapidly. This might have been related to the quality of influent water from ACW. Similarly, the influent concentration fluctuates wildly, but the removal rate of TP remains stable at

about 60% at this phase. When the ACW content on the 31st day increased to 100%, the effective removal rate of TN and TP increased from 61.5% to 80.1% and from 51.9% to 69.8%, respectively. At 43 d of operation, the TN removal efficiency is maintained at greater than 80%, thereby indicating that the AGSBR reached a stable state with a good TN removal performance. Subsequently, the removal rate of TP was maintained at around 62.6%. The main reason is that prolonged aeration will cause competition between aerobic bacteria and phosphate accumulating organisms (PAOs), which will change the dominant colony in the particles. Moreover, particulate sludge does not provide an ideal environment for PAOs enrichment [38–40]. However, some PAOs will be taken away in the discharged sludge, which significantly reduced PAOs content, thereby resulting in a TP removal rate of only about 62%. The experiment results showed that the AGS had an excellent removal effect on TN in phenol-containing ACW. This might have been attributed to the unique structure of AGS, which provides an ideal environment for the symbiosis of aerobic microorganisms and facultative microorganisms such as various ammonia-oxidizing bacteria and denitrifying bacteria [41].

3.2.3. Phenol removal

The removal rate of the AGSBR on phenol is shown in Fig. 3d. On the first day of inoculation, the removal rate of phenol by AGS was 85%. Besides the small fluctuations in the first five days, they remained at a high level in the first 20 d. This might have been attributed to the good phenol degradation performance of heterotrophic bacteria in AGS during the domestication period [42]. Although the concentration of phenol in ACW in automobile factories has been changing, the removal rate of phenol in AGS tends to be stable and maintains at about 88%. Such a high removal rate indicates that AGSBR may be ideal for the degradation of phenol-containing ACW. These findings were also confirmed by Liu et al. [43], who found that the AGS structure can provide microbial cells with beneficial protection from phenol toxicity, especially when exposed for longer periods. Larger granules also showed a higher tolerance.

3.3. Microbial community structure

3.3.1. Diversity of microbial community

Six sludge samples obtained from the AGSBR system in distinct stages of the biological reactor used high-throughput sequencing to analyze microbial richness and diversity. A total of 255,436 good reads were obtained from six samples, ranging from 36,910 to 45,764 reads per sample. There was further removal of the chimeras and noise in the sequencing readings. In total, 185,808, high-quality reads were obtained.

The richness and diversity indexes of the microbial community are shown in Table 1. The Chao and ACE values indicate the richness of species, whereas the Shannon and Simpson values indicate the diversity of species [16,44]. The results show that the number of operational taxonomic

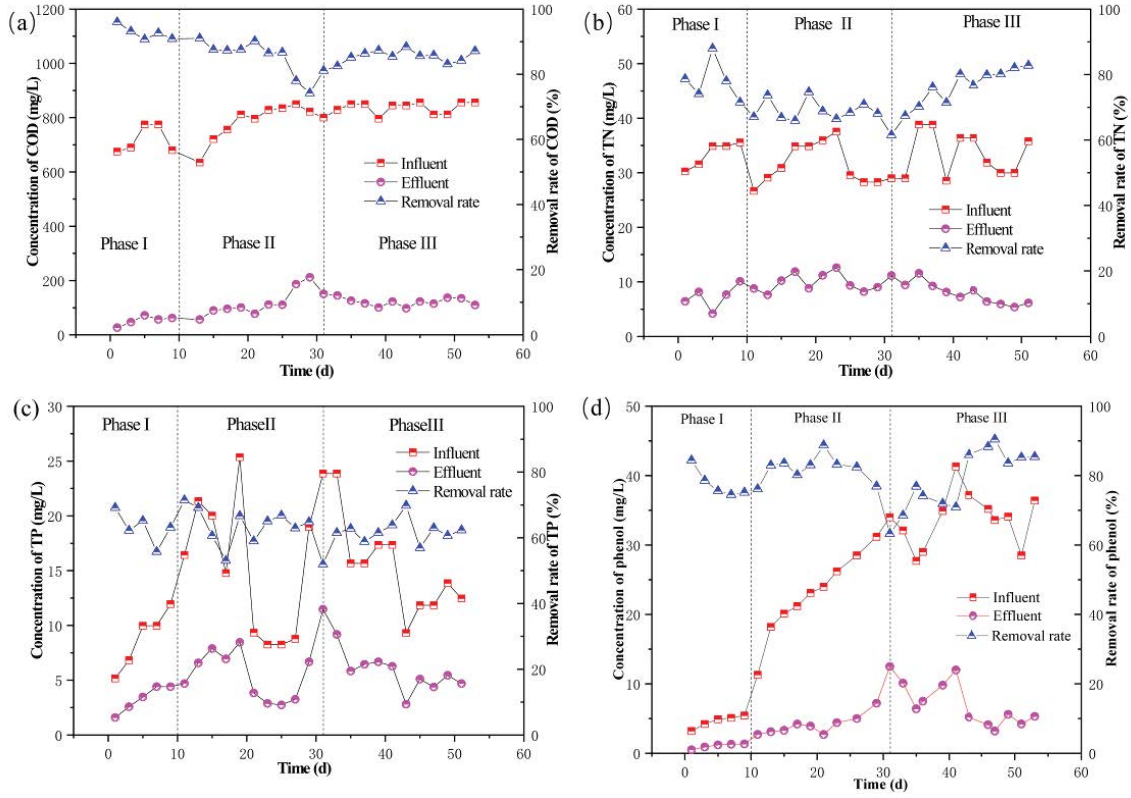

Fig. 3. The pollutant removal performance of the AGSBR during operation: (a) COD, (b) TN, (c) TP, and (d) phenol removal.

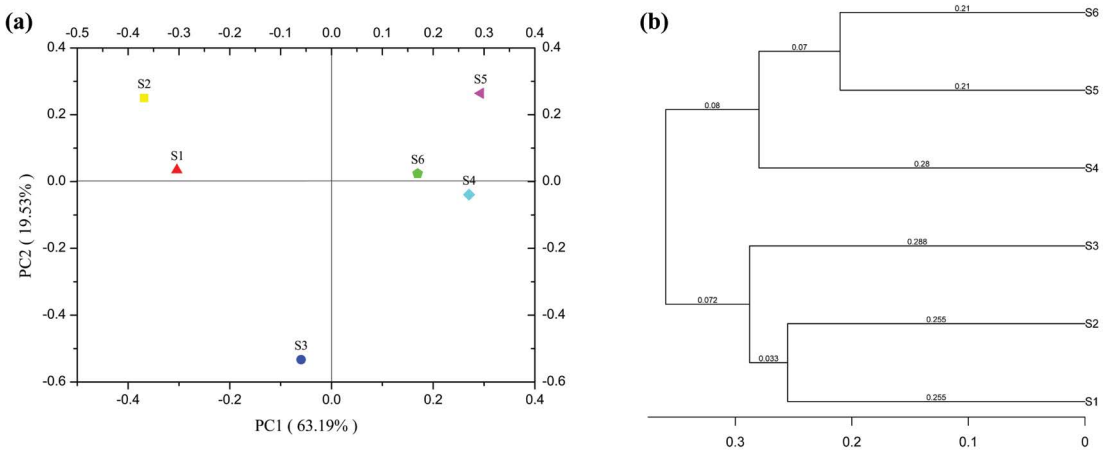

Fig. 4. Beta diversity of the six samples: (a) 2D principal component analysis and (b) phylogenetic tree. Hierarchical cluster analysis of the six sludge samples.

Table 1
Microbial community richness and diversity indices of six samples

Sludge sample	OTUs	Chao[a]	ACE[b]	Shannon[c]	Simpson[d]
S1	4925	1127.7	1210.2	6.08	0.9077
S2	7386	1798.5	1799.4	7.36	0.9592
S3	7597	1920.8	1948.2	7.15	0.9232
S4	9369	2330.1	2372.9	8.52	0.9862
S5	9958	2530.6	2536.1	8.92	0.9916
S6	8068	1771.3	1871.4	7.91	0.9737

[a]community richness. A higher number represents more richness;
[b]community richness. A higher number represents more richness;
[c]community diversity. A higher number represents more diversity;
[d]community diversity. A higher number represents more diversity.

units (OTUs) in these samples was between 4925 and 9958. The OTUs, Chao, ACE, Shannon, and Simpson values of S2–S6 were higher than the inoculated sludge S1, which indicated that the added ACW has changed the microbial community structure and increased the richness and diversity of species. Previous studies have shown that complex influent components promote the growth and reproduction of microorganisms, thereby increasing the number of microorganisms [41]. Simultaneously, compared with those in sample S2, the microorganism abundance in sample S3 increased, but the diversity decreased because the microorganisms were still in the ACW adaptation period. Moreover, part of the flocculent sludge adhered to the granular sludge. The microbial richness would increase and the microbial competition of the granules would gradually decrease the microorganism diversity [45]. Furthermore, the selective pressure discharge of flocculent sludge at this stage reduced the diversity of microorganisms. In addition, S5 (9958 OTUs) had the highest abundance and diversity, whereas S1 (4925 OTUs) had the lowest abundance and diversity. The addition of ACW had a significant effect on the species richness and diversity. However, the microorganisms adapted to the coating wastewater at this time, and had a high growth rate. It is worth noting that the species diversity and abundance of S6 were lower than those of S5. It was speculated that this was due to the influence of the decrease in the influent phenol concentration on the microorganisms [43,46].

As shown in Fig. 4a, principal component analysis revealed that the maximum ratio of the original data difference was 63.19% (PC1) and 19.53% (PC2). It also demonstrated that samples from S1 and S2 tended to cluster together. Because the granular sludge matured and grew steadily from the 15th day of the experiment, and the microorganisms adapted to the system environment, the three sludge samples S4, S5, and S6 were very similar. Further comparison of them between the six samples showed that the bacterial communities from S3 were different from those in the other samples. It was clear that there would be major variations between the AGS at different domestication stages. The microbial community composition of AGS may be affected by ACW. Moreover, the similarity tree was obtained through the hierarchical cluster analysis, as shown in Fig. 4b. The cluster analysis showed that the bacterial communities in the six samples can be clustered into four groups: (1) Group I consist of sample S1 and S2; (2) Group II consist of sample S5 and S6; (3) Group III included sample S3; (4) Group IV contains sample S4.

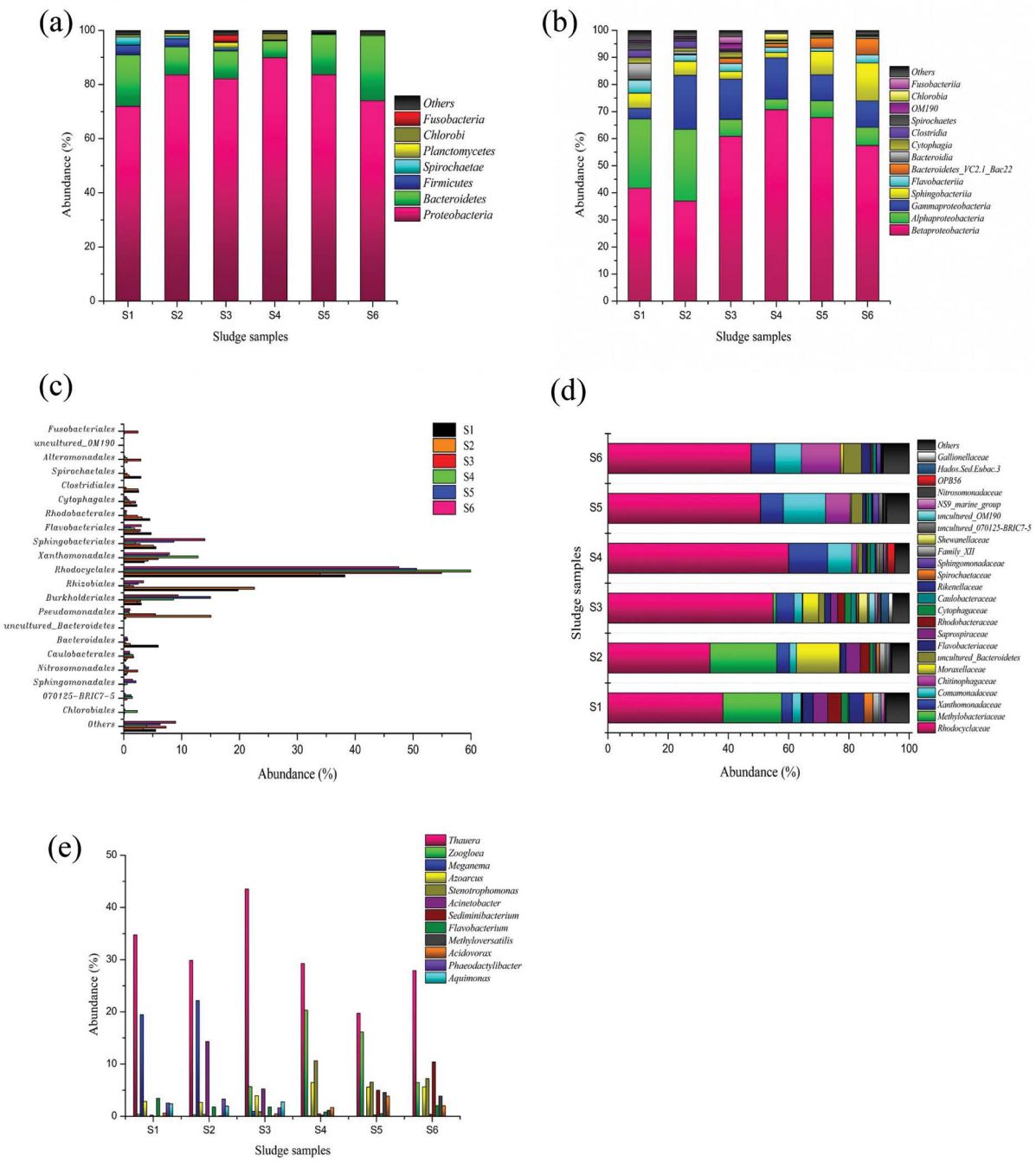

Fig. 5. The microbial community structure of sludge samples at different levels: (a) phylum, (b) class, (c) order, (d) family, and (e) genus

Besides, S3, S4, S5, and S6 were highly similar. These results indicate that the microbial community structure differs significantly at different experimental operation stages affected by ACW addition, but the overall structure becomes more similar in the later stages and gradually stabilizes.

3.3.2. *Microbial community analysis*

Dynamic analysis of the microbial community is crucial for the formation of AGS and the study of wastewater treatment efficiency. The changes in the typical characteristics of the microbial flora structure of each sample can be characterized by different classification levels (from phylum to genus). *Proteobacteria* was the most predominant phylum in all the samples (Fig. 5a), with 80.82% of the sequences on average, the relative abundance of S1 was the lowest proportion of 71.93%, whereas that in S4 relative abundance of the largest accounting for 89.82%. In addition, it was also found that in other studies, *Proteobacteria* was the most abundant phylum in AGS from an A/O/A sequencing batch

reactor [47] and anaerobic sludge from full-scale wastewater treatment plants [48], and biosolids [49]. Furthermore, *Bacteroidetes* (6.31%–19.00%, averaging 14.16%) displayed a considerably higher abundance in S1, S5, and S6. From Fig. 3a, it can be seen that the COD removal efficiency rate was significantly higher than other days on 1, 41, and 51 d. Therefore, *Bacteroidetes* can play a crucial role in removing organic matter, which agreed with the results of previous research [50]. The abundance of *Firmicutes* (0.02%–3.63%, averaging 1.20%) was the third most dominant phylum in the six sludge samples and its relative abundance decreased significantly after 11 d. It had been reported that the during the granulation process, the phylum of *Firmicutes* are quickly effluent discharge from the reactor under the influence of hydraulic shear force and selective precipitation pressure [47], thereby resulting in *Firmicutes* appeared at a superficial levels in S3 (0.54%), S4 (0.03%), S5 (0.02%) and S6 (0.04%). Similar to the results of previous studies [48], six sludge samples of the above three phyla dominate the bacterial communities (92.93%–98.50%), in addition, several other major (>1% in at least one samples) phyla were also present, including *Spirochaetae* (0.09%–2.95%), *Planctomycetes* (0.21%–2.04%), *Chlorobi* (0.04%–2.33%) and *Fusobacteria* (0%–2.46%).

The microbial community structure was further investigated at the class level, and the 13 main microorganisms are shown in Fig. 5b. The relative abundance of these main classes accounted for 96.23%–99.00% of the classified sequences. *Betaproteobacteria* was the most predominated class among the six sludge samples (36.96%–70.68%, averaging 55.90%), followed by *Alphaproteobacteria* (3.85%–26.47%, averaging 12.50%) and *Gammaproteobacteria* (3.96%–19.95%, averaging 12.25%). A previous study [51] had demonstrated that the dominance of *Betaproteobacteria* in the phenol-degrading aerobic granules. As the previous report [52], *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* can degrade nitrate and nitrite. Furthermore, there are also reports that *Betaproteobacteria* and *Gammaproteobacteria* can resist hydraulic shear forces and play a crucial role in AGS formation [47,53]. In addition to these three subgroups, other major classes of bacteria included *Sphingobacteriia* (2.01%–14.01%), *Flavobacteriia* (1.18%–4.74%), *Bacteroidetes_VC2.1_Bac22* (0.26%–6.02%), *Bacteroidia* (0.08%–6.00%), and *Cytophagia* (0.43%–2.27%), which belong to subdivisions of *Bacteroidetes*.

At the order level, as shown in Fig. 5c, *Rhodocyclales* was the most abundant and most extensive order among the six samples, accounting for 47.52% of the sequences on average, the relative abundance of S2 was the lowest proportion of 33.94%, S4 relative abundance of the largest accounting for 59.97%. The following main orders were *Rhizobiales* (0.98%–22.58%), *Xanthomonadales* (3.55%–12.85%), *Burkholderiales* (2.33%–14.99%), and *Sphingobacteriales* (2.01%–14.01%). The results were similar to those obtained by Cydzik-Kwiatkowska [54], which concluded that relatives of *Rhodocyclales*, *Xanthomonadaceae*, and *Rhizobiales* can promote the secretion of extracellular polymeric substances (EPS). AGS can achieve granulation in the ACW environment. EPS play an essential role in the formation and structure maintenance of microorganisms aggregation [55–59]. Other studies have also shown that microorganisms that can secrete EPS play an important role in the long-term stability of industrial wastewater treatment [60]. Furthermore, as shown in Fig. 5d, *Rhodocyclaceae*, *Xanthomonadaceae*, and *Comamonadaceae* were the most abundant families among the six samples, accounting for 33.94%–59.97% (averaging at 47.52%), 3.55%–12.85% (averaging at 7.01%), and 2.30%–14.01% (averaging at 6.46%), respectively. Beyond that, *Methylobacteriaceae* was the dominant group in S1 (19.46%) and S2 (22.15%), whereas its average abundance was only 0.30% in S3, S4, S5, and S6. It is speculated that *Methylobacteriaceae* is not suitable for the water quality and toxicity of ACW.

As shown in Fig. 5e, the average relative abundances of the genera *Thauera*, *Zoogloea*, and *Meganema* were greater than 5% at the genus level. The above three genera have been reported in different AGS bioreactors operated for organic matter removal [60] and nitrogen removal [61,62]. Besides, Szabo et al. and Larsen et al. [63,64] reported that *Thauera*, *Zoogloea*, and *Meganema*, which were putative EPS producers, were found in laboratory-scale SBR. *Thauera* had the highest abundance in all samples, accounting for 19.73%–43.52%, as shown in Fig. 5e. *Thaurea* can degrade phenol [65] and is considered to have potential nitrogen removal [66] and phosphorus removal [67] capacities. Moreover, a previous study [54] reported that *Thauera* plays a crucial role in granule formation under constant aeration. *Zoogloea* was detected in six samples, but its abundance in previous periods was lower than that in later periods (S1 and S2 were only 0.41% and 0.32%, respectively) and increased significantly in S3–S6. It is speculated that *Zoogloea* is a phenol-degrading bacteria, so it had clear advantages in the later stage. On the contrary, *Meganema*, which is a member of *Methylobacteriaceae*, accounted for 19.46% of the genera in S1, 22.15% of that in S2, and no more than 1% of that in other samples. This decreasing microbial population structure revealed that *Meganema* might not have adapted to the toxicity of phenol. Except for these three genera mentioned above, other main genera included *Azoarcus*, *Stenotrophomonas*, *Sediminibacterium*, *Methyloversatilis*, and *Acidovorax*. Their abundances were lower in the early phase than in the later phase, thereby indicating that the phenol concentration significantly influenced these genera. Based on the above analysis, certain phenol-degradation microbes allowed the AGS to survive and grow with phenol addition. Meanwhile, changes in the microbial community during contaminants degradation can result from synergistic effects in which one microbial community promotes the growth of another organism. The interaction between different microbial communities plays a significant impact on the stability of the system and removal of contaminants [68].

4. Conclusion

This study provided a method for the rapid start-up of AGSBR for treating raw ACW through seeding a mixture of intact AGS and activated sludge. The compact AGS had excellent stability, and settleability was formed in 15 d of operation. The AGS exhibited high COD, TN, and phenol removal rates of 85%, 82%, and 90%, respectively, but the TP removal rate was only 62%. Moreover, the diversities

of the microbial communities and the dominant microorganisms changed with ACW addition. Furthermore, the high-throughput sequencing analysis demonstrated that *Proteobacteria* was the most abundant phylum. At the class, order, and family levels, *Betaproteobacteria*, *Rhodocyclales*, and *Rhodocyclaceae* were the main functional bacteria, respectively. At the genus level, *Thauera* which can degrade phenol, was the most abundant. Besides, *Zoogloea*, *Azoarcus*, *Stenotrophomonas*, *Sediminibacterium*, *Methyloversatilis*, and *Acidovorax* gradually increased. The functional genera had a significant effect on the biodegradation of phenol and organic matter. These results could encourage the application of AGS technology in ACW treatment.

Acknowledgments

This study received funds from the National Natural Science Foundation of China (No. 51578003 and 51668062).

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