Microbial community structure of aerobic granular sludge in automobile coating wastewater as revealed by Illumina sequencing

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ABSTRACT

Controlling pollution from automobile coating wastewater is an important issue in the field of water treatment. Aerobic granular sludge, as a special biological aggregate, performs well in the treatment of wastewater owing to its unique structure and characteristics. Using aerobic granular sludge to treat automobile coating wastewater has achieved good results and has a high removal rate of pollutants. At the time when granular sludge was stable, the removal rates of COD, NH_4^+-N and PO_4^{3-}-P in the automobile coating wastewater reached 85%, 95% and 65%, respectively. Via Illumina sequencing, 12 sludge samples from automobile coating wastewater treatment with aerobic granular sludge were studied. As the proportion of automobile coating wastewater and the granulation of sludge increased, the species, quantity, and structure of the microbial community constantly changed, eventually resulting in a stabilized microbial community structure. By means of taxonomic analysis, at the phylum level, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the most abundant groups in all samples, and *Betaproteobacteria* and *Alphaproteobacteria* were very competitive at the class level. The microbial community structure was also constantly changing at the genus level, although unclassified *Comamonadaceae* was dominant at all times. Results showed that the change in microbial community structure corresponded to the degradation efficiency of pollutants.

Keywords Aerobic granular sludge; Automobile coating wastewater; Illumina; Microbial community structure

1. Introduction

Automobile coating is an indispensable part of the automobile manufacturing process. In terms of automobile manufacturing, the coating process has the most significant impact on the environment [1]. Automobile coating wastewater may contain a variety of toxic and refractory materials such as oil, grease, dyestuff, organic and inorganic matter (e.g., phenol, polycyclic aromatic hydrocarbons, and cyanide), and heavy metals (e.g., Cr, Ni, and Zn) [2,3]. Thus the content of organic matter in wastewater is very high and complex, the treatment of automobile coating wastewater is difficult.

Aerobic granular sludge has been widely received favorably by researchers since it was first reported, and research into aerobic granular sludge technology applications using real industrial wastewater was initiated by previous researchers. Results showed aerobic granular sludge had excellent settling ability and exhibited exceptional performance in the removal of organics and nutrients from rubber wastewater [4]. Moreover, the COD removal efficiency was as high as 96.5%, while the removal efficiencies of ammonia and total nitrogen reached 94.7% and 89.4%, respectively. Aerobic granular sludge also was used to treat the wastewater that fish processing industries produce [5], with COD and BOD removal efficiencies of over 90%. The

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results showed that aerobic granular sludge had excellent adaptability under harsh environmental conditions.

High-throughput sequencing technology can be employed to simultaneously determine millions of DNA molecular sequences, more accurately determine the species distribution and abundance in the samples, and solve the problems encountered during comprehensive exploration of microbial colony structure in the samples. According to the developmental history and principles of sequencing, high-throughput sequencing mainly includes the following: massively parallel signature sequencing, polony sequencing, 454 pyrosequencing, Illumina (Solexa) sequencing, Ion semiconductor sequencing, and DNA nanoball sequencing. Among them, Illumina sequencing has been applied in various scientific research fields, such as viral sequencing [6], gut microbiota analysis [7], microbial community analysis in aquaculture systems [8] as well as bacterial community analysis in natural river distribution systems [9].

To date, there is no reported study on the use of an aerobic granular sludge system for the treatment of automobile coating wastewater. In this study, automobile coating wastewater and synthetic wastewater were used to cultivate aerobic granular sludge, and high-throughput sequencing via an Illumina MiSeq was used to analyze the microbial community structure of the sludge throughout the cultivation process. This work could be significant for deeper understanding of aerobic granular sludge as well as for the development of the automotive industry.

2. Materials and methods

2.1. Experimental setup and operation

The Plexiglas reactor was 120 mm in inner diameter and 770 mm in height, giving a working height of 530 mm, an effective volume of 6 L and a ratio of height to diameter (H/D) of 4.4(Fig. 1). The timing for water intake, aeration, sedimentation, and drainage in the operation process was controlled by a microcomputer-controlled switch.

The reactor was operated in successive cycles of 4 h each. One cycle consisted of 10 min of feeding, 203–227 min of aeration phase, 25–1 min of settling time and 2 min of effluent. Effluent was withdrawn at 265 mm from the bottom of the reactor. The sedimentation time was gradually shortened from an initial 25 min to a final 1 min and stabilized at 1 min. The corresponding aeration time also increased from an initial 203 min to 227 min.

In the granulation process, we used a mixed feed of synthetic wastewater and real automobile coating wastewater that varied proportionally, as shown in Table S1. The synthetic wastewater contained a mixture of an equal ratio (based on the COD concentration) of sucrose and sodium acetate as the carbon sources. Other components including Na_2CO_3, NH_4Cl, Na_2HPO_4, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, EDTA and trace solution 1 mL/L were also added in the media solution.

2.2. Seed and measurements

The seed sludge used in the experiment was taken from the aeration tank at the Jianghuai Wastewater Treatment Plant in Hefei, China. The mixed liquid suspend solid
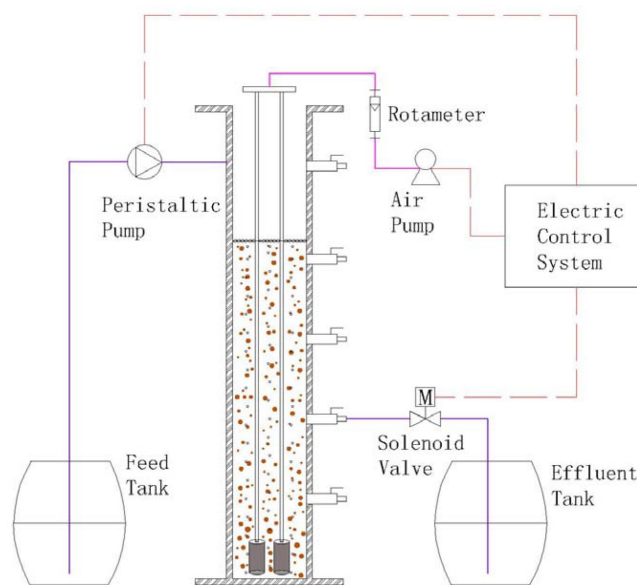

Fig. 1. Experimental setup of the SBR reactor operated at 22 ± 3ºC.

(MLSS) of seed sludge was around 3.5 g/L and sludge volume index (SVI) was 98 mL/g. Samples were periodically collected and analyzed for COD, ammonia nitrogen, TP, MLSS and SVI according to the standard method [10].

2.3. DNA extraction and PCR amplification

Research on the microbial ecology of sludge granulation was based on high-throughput sequencing on the Illumina MiSeq platform. The V4–V5 region of 16S rRNA was amplified and then sequenced. Then, 0.3 g of sludge was removed from the centrifuge tube and its microbial DNA extracted using the E.Z.N.A® Soil DNA Kit. The extracted DNA was then subjected to mass detection and quantification using 0.8% agarose gel electrophoresis and UV spectrophotometry, respectively, followed by storage at –20°C.

Primers were designed based on conserved regions in ribosomal RNA, specific Barcode sequences were added, and then the highly variable V4–V5 region of the bacterial 16S rRNA gene was sequenced. The PCR amplification primers were 520F and 802R. The former primer sequence was 5'-barcode + GCACCTAAYTGGGYDTAAAGNG-3' and the latter primer sequence was 5'-TACNVGGG-TATCTAATCC-3'. The PCR reaction comprised 0.25 µL of Q5® High-Fidelity DNA Polymerase, 5 µL of 5X Reaction Buffer, 5 µL of Q5® High GC Enhancer, 0.5 µL of dNTPs (10 mM each), 1 µL of template DNA, 1 µL of forward primer (10 uM), 1 µL of reverse primer (10 uM), and 11.25 µL of water.

2.4. High-throughput sequencing

Amplified PCR products were detected using 2% agarose gel electrophoresis, and Axygen's gel recovery kit was used to recover the target fragments. Fluorescence quantification was performed using the Quant-iT PicoGreen dsDNA Assay Kit as a fluorescent reagent and

a microplate fluorescence reader (FLx800, BioTek) as a quantification instrument. After the fluorescence quantification was completed, the requirements for the amount of sequencing varied for each sludge sample. The sequencing library was constructed using the TruSeq Nano DNA LT Library Prep Kit from Illumina. Prior to sequencing on the machine, the library was checked for quality. The instrument used for quality inspection was the Agilent Bioanalyzer and the reagent used was the Agilent High Sensitivity DNA Kit. The concentration of a qualified library should be greater than 2 nM, so the Quant-iT PicoGreen dsDNA Assay Kit reagents also should be used for quantitative analysis of the library on the Promega QuantiFluor dsDNA System.

The qualified library obtained after screening was diluted and then mixed according to the desired sequencing amount ratio. After denaturation to single strands by NaOH, 2×300 bp double-ended sequencing was performed on the MiSeq using the MiSeq Reagent Kit v3 reagent.

All the raw sequences generated in this study had been submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP181532.

3. Results

3.1. Cultivation of aerobic granular sludge

According to the operation of the reactor and the shape of the sludge, the whole cultivation process can be divided into three stages: the granular sludge formation stage, growth stage, and mature stage.

Stage I (0–45 d): When the reactor started to run, the inoculated sludge was loose and the sedimentation performance was poor (Fig. 2a). To avoid the loss of biomass with the water, the sedimentation time was set to 25 min. At the beginning of the granular sludge formation stage, it was found that the amount of sludge did not change much, but the color of the sludge gradually changed from dark brown to light brown. After the reactor had been operated for about 10 d, the sludge had adapted to the new environment, and the automotive coating wastewater was gradually added to the influent water (Table S1). At about 45 d, finer particles appeared in the reactor. At this time, the sludge sedimentation time in the reactor was 3 min, the sludge concentration was 4.3 g/L, the sludge volume index(SVI) was 33 mL/g, and the particle size was measured to be 0.25–0.68 mm. The color of the sludge changed from brown to pale yellow. During this stage, the sludge stratified when it settled. The upper layer was loose flocculent sludge and the lower layer granular sludge.

Stage II (45–90 d): In this stage, the granulation phenomenon of the sludge became obvious, the particle size increased significantly, and the proportion of particles in the reactor also increased (Fig. 2b). With the gradual compaction of the granular structure, the resistance to external impacts also increased, and the proportion of automotive coating wastewater in the influent water increased. At the same time, in the sludge sedimentation process, it can be
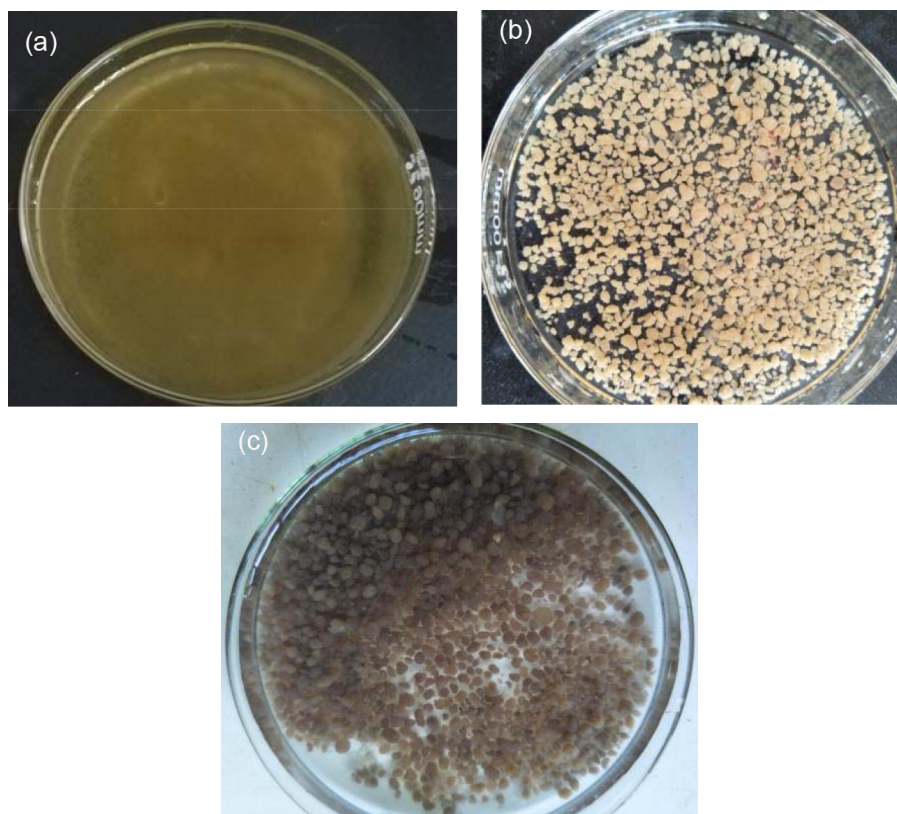

Fig. 2. Images of sludge morphology during different periods: (a) seed sludge, (b) granular sludge at 60 d of operation, and (c) granular sludge at 90 d of operation.

clearly seen that the particles settled quickly, and the flocculation sedimentation performance was not ideal with the discharge of water. There was a clear interface between the sewage and the sludge. The average concentration of sludge in this stage was 4.54 g/L, the SVI was 36 mL/g, and the average size of the particles was 0.75 mm.

Stage III (90–110 d): The morphology of the particles stabilized after the reactor was operated for about 90 d. The particles were spherical and their structure dense (Fig. 2c). The average particle size of the sludge in this stage was 1.05 mm, the average concentration of sludge was 5 g/L, the SVI was 35 mL/g, and the sedimentation performance was good. The reactor was mainly composed of aerobic granular sludge. At this time, all the influent water was automotive paint wastewater.

At the beginning of Stage I, the initial activity of the inoculated sludge was low, the effluent COD value was high, and the COD removal rate was low, only about 70% (Fig. 3a). After several days of operation, the effluent COD gradually decreased and the removal rate increased. At 10 d, automotive coating wastewater was gradually added to the influent water, and the COD effluent concentration and removal rate showed little fluctuation. However, because the amount of sludge added at the beginning was small, the impact on the sludge also was small. Fine granular sludge appeared in Stage II. After that, the COD removal rate generally stabilized at about 85%, and the effluent value also

generally stabilized at 100 mg/L. It was indicated that, with the progress of the reaction, the sludge had adapted to the water quality of the automobile coating wastewater, and the state of the aerobic granular sludge tended to be stable. At the beginning of Stage III, all the influent water in the reactor was automobile coating wastewater, and the COD removal rate had small fluctuations, but the overall effect was a relatively stable environment that was not adversely affected.

The average removal rate of NH_4^+-N was about 90%. This showed that the environment of the reaction system and the regulation of the operation parameters were more suitable for the growth of nitrosating bacteria and nitrifying bacteria (Fig. 3b). It can be seen from Fig. 3b that the removal efficiency of ammonia nitrogen gradually improved after a short period of nonadaptation in Stage I, and reached about 95% later in Stage II. Compared with the earlier stage sludge, the aerobic granular sludge after maturation had a better removal effect on NH_4^+-N. The removal rate of NH_4^+-N remained high when all the influent was automobile coating wastewater, and the concentration of NH_4^+-N in the effluent was below 5 mg/L. This shows that the number of nitrifying bacteria in the reactor was adequate to remove NH_4^+-N from the sewage.

It can be seen from Fig. 3c that the removal efficiency of PO_4^{3-}-P was relatively weak, with the average removal rate only 70.71%, and the effluent concentration was high,
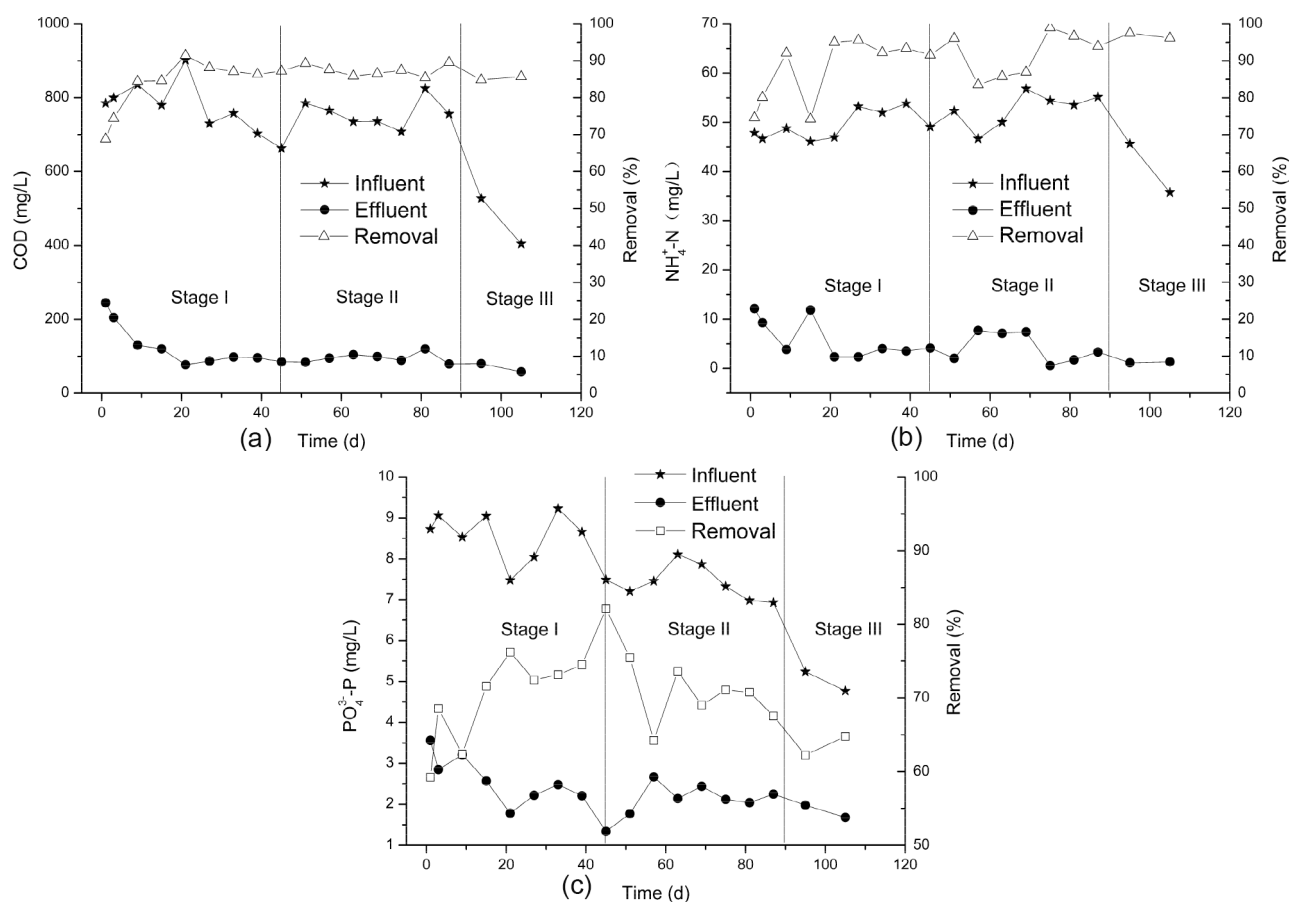

Fig. 3. Reactor performance during its operation: (a) COD removal, (b) ammonia nitrogen removal, and (c) TP removal.

at not less than 1 mg/L. Moreover, the concentration of and removal rate from the effluent fluctuated greatly in the first stage. By about 70 d, the removal efficiency of PO_4^{3-}-P gradually stabilized with the maturity of the granular sludge, and the effluent value fluctuated between 1.5 and 2.5 mg/L. The mechanism of biological phosphorus removal is that phosphorus is removed from wastewater by aerobic phosphorus uptake and anaerobic phosphorus release by polyphosphate-accumulating organisms (PAOs). The SBR reactor used in this experiment was in the aeration stage for a long time, and there was no alternating between anaerobic and aerobic conditions, which was unfavorable to the growth of PAOs. A lower PAO content in the system will lead to a low removal efficiency of PO_4^{3-}-P [11].

3.2. Bacterial abundance and diversity spatial features of the granular sludge

Sludge samples were taken from the SBR reactor at the start (S0) and then every 10 days (S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, and S11) thereafter.

After high-throughput sequencing, 655,367 primitive sequences were generated from the 12 samples collected, and the effective sequence number was 435,536 after removing the questionable sequences such as chimeras. The acceptable sequences were merged based on 97% sequence similarity, and 4,061 OTUs were obtained. The OTUs of the 12 samples were 1,058 (S0), 1,011 (S1), 1,122 (S2), 822 (S3), 814 (S4), 1,062 (S5), 883 (S6), 1,038 (S7), 1,183 (S8), 1,173 (S9), 1,146 (S10), and 905 (S11). From these data, it can be seen that, in the initial stage of aerobic granular sludge culturing, the diversity of the bacterial species increased slightly. When the flocs gradually changed into fine particles, the diversity of the bacterial species decreased. With the increase of aerobic granular sludge particle size, the diversity of the bacterial species increased.

As could be seen in Fig. 4, these five sludge samples had more unique OTUs. In addition, they shared a relatively small number of OTUs, accounting for only 3.03% of the total, indicating that the diversity of microorganisms in the process of sludge granulation was relatively high. The

Fig. 4. Venn diagram of samples S1, S3, S4, S5, and S7.

OTUs common among S1 and four other samples (S3, S4, S5, and S7) accounted for 8.03%, 7.21%, 8.08%, and 6.55% of the total, respectively, showing a decreasing trend with time. From this, we can see that although the bacterial community composition changed greatly during the whole process, some bacteria were retained. According to the unique OTUs of each sludge sample, the proportions of S1 (473), S3 (209), S4 (129), S5 (247), and S7 (387) were 11.65%, 5.15%, 3.18%, 6.08%, and 9.53%, respectively. The shortening of sedimentation time had a certain effect on the diversity spatial features of granular sludge, but the community structure restored after the granule was stabilized.

Microbial diversity can be characterized by alpha diversity indices, which reflect the diversity and richness of species within a single sample. In 16S rRNA sequencing data analysis, community richness (Chao1) and community diversity (Shannon and Simpson) indices generally are used to express the diversity (Table 1).

The Chao1 values fluctuated throughout the stage I, first decreasing and then rising, and finally declining to 906.17 after reaching its maximum value of 1374.55 on 80 d. Shannon values also showed the same pattern, from 7.93 on day 0 to 5.69 on 30 d, then gradually rising to 7.06 at 80 d, and then dropping to 6.43. The reason for this may be that the automobile coating wastewater was added to the sludge at around 10 d, and the sludge was impacted by the coating wastewater. The bacteria that could not adapt to the environment in time were eliminated. At the same time, the sedimentation time also was decreasing and some bacteria were discharged from the reactor in a short time, while the bacteria with good flocculation and sedimentation performance were retained, resulting in a significant change in the bacterial community composition of the activated sludge. With the increase of the amount of automobile coating wastewater in the reactor, the microorganisms were gradually domesticated and gradually adapted to the environment in the reactor. The bacterial community changed again, and the evenness and richness increased. At this time, the sludge in the reactor changed from the original flocculent to small particles. From the data in Table 1, it can be seen that the change in bacterial community composition during the growing stage was smaller than that during the aerobic granular sludge formation process.
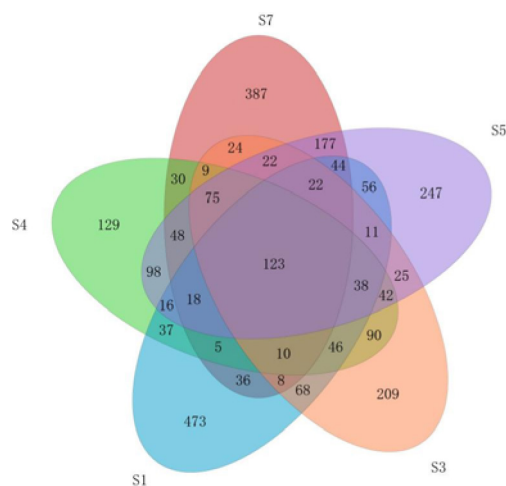
3.3. Bacterial community structure characteristics of the granular sludge

At the phylum level, 37 phyla were found in the 12 sludge samples. Microorganisms with abundance less than 1% in all samples were compiled in the Others category (Fig. 5). As can be seen from Fig. 5, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* accounted for a considerable proportion of the whole, with average abundances of 56.07%, 29.38%, and 6.23%, respectively, which was a similar result to that in a previous study on granular sludge using MiSeq pyrosequencing analysis [12]. As *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* are ubiquitous in soil, this indicated that the character of the sludge was similar to that of soil [13].

Proteobacteria had the absolute advantage, and its abundance was not less than 43% in all stages of the sludge samples, with its highest abundance reaching

Table 1
Sequences from the twelve samples

Sludge sample	Primitive sequences	Effective sequences	OTUs	Chao1	Shannon	Simpson
S0 (0 d)	55533	34681	1058	1058.00	7.93	0.986195
S1 (10 d)	52585	35803	1011	1011.03	6.62	0.955049
S2 (20 d)	50596	35656	1122	1122.30	7.15	0.972800
S3 (30 d)	51970	38087	822	879.69	5.69	0.877697
S4 (40 d)	54433	33929	814	814.00	6.49	0.966936
S5 (50 d)	56556	36524	1062	1066.37	6.97	0.973914
S6 (60 d)	51551	36209	883	891.26	6.27	0.938720
S7 (70 d)	48251	32914	1038	1038.00	6.97	0.973494
S8 (80 d)	60830	40488	1183	1374.55	7.06	0.972357
S9 (90 d)	59021	38855	1173	1289.35	6.75	0.951946
S10 (100 d)	56777	36406	1146	1148.73	6.85	0.957996
S11 (110 d)	57264	35984	905	906.17	6.43	0.956102

64.81%. The percentage of *Proteobacteria* increased gradually from 43.18% of the initial inoculated sludge to 66.74% at 60 d, and gradually stabilized at about 60%. The average abundance of *Proteobacteria* in Stage II was higher than that in the first stage. The phyla *Bacteroidetes* experienced a brief rise and fall in Stage I and early Stage II, and then the abundances of the samples were more similar in the later stages and lacked significant fluctuations. The abundance of the phyla *Firmicutes* was lower than that of the two phyla just mentioned. It reached a maximum value of 13% at 30 d, and gradually decreased when particles appeared and then increased in size.

In addition to the above-mentioned phyla, several other phyla, including *Actinobacteria*, *Spirochaetes*, *Planctomycetes*, *Chloroflexi*, *Verrucomicrobia*, *Acidobacteria*, *Nitrospirae*, *Chlorobi*, and *Fusobacteria*, were the major (abundance > 1%) phyla in 1 of the 12 samples. These bacteria interact with each other to ensure the degradation of organic compounds, while maintaining the stability of the reaction system.
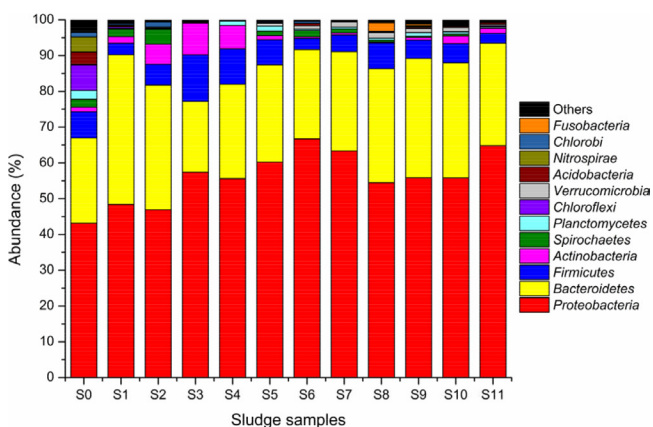
In the 12 sludge samples, 89 microbial classes were detected, among which 16 were found in all 12 samples. About half of 89 of the classes were found in inoculated sludge samples with low abundance. In the same way, 22 of the 89 classes were the dominating ones (>1% in at least one sample; Fig. 6).

The average abundance of *Betaproteobacteria* was the highest (31.19%), followed by that of the *Alphaproteobacteria* (16.88%). *Betaproteobacteria* increased slightly during the initial stage of the reaction. After it had been running for about 40 d, the shortening of the sedimentation time resulted in greater sludge loss and the decrease in *Betaproteobacteria* abundance. Later, with the formation and growth of granular sludge, the abundance of *Betaproteobacteria* increased to a certain extent and then became stable. *Alphaproteobacteria* increased from 5.32% at the beginning to 28.67% at its peak, and then remained at about 20%. In addition, *Clostridia*, *Cytophagia*, *Gammaproteobacteria*, *Saprospirae*, *Actinobacteria*, *Epsilonproteobacteria*, and *Sphingobacteriia* were the most active classes in the sludge. There

Fig. 5. Bacterial community compositions at phylum level as revealed by Illumina sequencing.
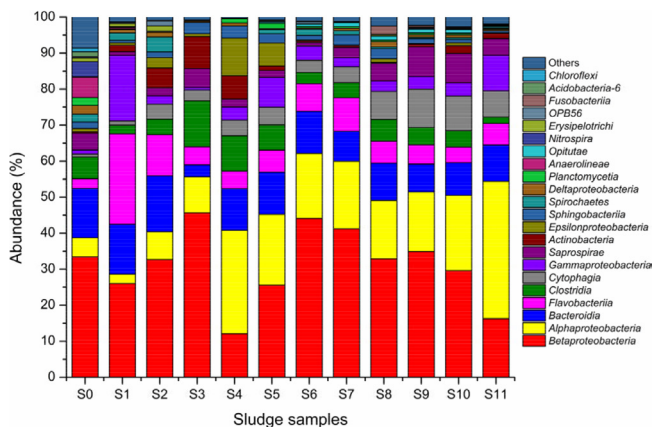

Fig. 6. Bacterial community compositions at class level as revealed by Illumina sequencing.

were also a few *Spirochaetia*, *Deltaproteobacteria*, and *Planctomycetia*.

4. Discussion

4.1 Spatial difference in microbial community structure of the granular sludge at different stages

The 2-D principal component analysis (PCA) analysis of each sludge sample was based on all 16S rRNA V4–V5 sequences detected (Fig. 7). PCA shows the similarity between samples. The closer the distance between samples, the higher the similarity in microbial community structure between them and the smaller the difference. Therefore, the farther the distance is, the more dissimilar the community is. Through PCA, the similarity between different stages of the sludge community during the process of sludge granulation as the proportion of automobile coating wastewater increases can be obtained. The maximum ratios of original data difference were 46.32% (PC1) and 19.57% (PC2), according to Fig. 7. The samples with high similarity can be divided into three categories: granule formation stage, growth stage, and stable stage, which correspond to the three stages of the granular sludge culture process. However, samples from 0 d and 40 d, 20 d and 50 d were obviously closer, respectively. It demonstrated that the granular stage and the growth stage shared similar bacterial communities. The distance between the sludge samples and the other samples was larger when the sludge samples had been running for 10 d, which indicated that the system environment was disturbed and the microbial community structure had changed significantly. Sludge after 30–70 d of operation belongs to the stage of particle formation and growth. As can be seen from Fig. 7, the distance between these samples gradually decreased, and the sludge was much more stable than that from the start-up stage. In addition, it is evident that the four sludge samples from 80–110 d were very close together. Because the sludge in this stage gradually matures into a stable state and the influent is entirely automobile coating wastewater, the microorganisms have adapted to the reaction system and the dynamic change is small, which
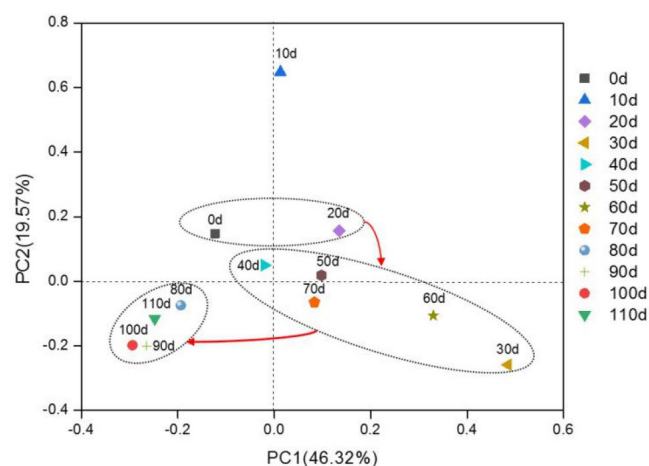
also shows that the environment of the whole system was more stable at that time.

For comparing the bacterial communities across samples, UPGMA (Unweighted pair-group method with arithmetic means) clustering analysis were performed on the Weight Unifrac distance matrix, and the similarity between samples is displayed in the form of a phylogenetic tree (Fig. S1). The length of the branches can reflect the degree of similarity between samples. As the branching lengths of S2 and S3, S6 and S7, and S9 and S10 are equal, this indicates that the similarities between S2 and S3, S6 and S7, and S9 and S10 are higher. The branching lengths of S0, S1, and S4 are relatively long, and thus those samples are different from the other sludge samples. S2, S3, S5, S6, and S7 are aggregated together as are S8, S9, and S10. It is obvious that there are significant differences in microbial community structure at the different stages of sludge granulation, but the overall structure became more and more similar and gradually stabilized.

4.2. Differences in the dominant microorganisms for different operational stages and the causes for such differences

Among the bacteria, *Proteobacteria* was the most dominant phylum in all the sludge samples, and this result is consistent with those in previous studies [14]. In previous studies, denitrification by *Proteobacteria* was detected [15,16].

Bacteroidetes (19.78–34.87%, averaging 29.38%) suddenly increased in abundance in Stage I and decreased early in Stage II. Compared with the previous COD removal curves, the removal rate of COD also increased rapidly from 0 d to 10 d, decreased after 10 d, and remained stable in the later period. It can be deduced that *Bacteroidetes* may be related to the degradation of organic matter, which was in accordance with the results of previous research [17].

The abundance of *Firmicutes* was not as high as that of the two phyla mentioned above, and a significant decrease was observed during particle formation, from 13.00% on 30 d to 3.13% on 60 d. This phenomenon may be related to hydraulic shear force in the process of particle formation. The microcolonies of *Firmicutes* (mainly *Clostridia*) were vulnerable to hydraulic shear force and could have easily been washed out during the aerobic granulation process [18,19]. In addition, the phyla of *Firmicutes* played key roles in NO_2^--N, NO_3^--N, and NH_4^+-N consumption [20].

At the class level, *Betaproteobacteria* was the dominant group in S0, S1, S2, S3, S5, S6, S7, S8, S9, and S10, as shown in Fig. 6. *Alphaproteobacteria* in S4 and S11 was the dominant group at the class level. It has been reported that several groups of nitrification, denitrification, and other nitrogen-cycle-related microorganisms were involved in the *Betaproteobacteria* class, and most hydrogenotrophic denitrifiers belonged to *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* [21,22].

According to previous studies, *Betaproteobacteria* can resist shear stress and contribute to the formation of aerobic granular sludge [23]. Therefore, the difference between the dominant bacteria in S4 and those in other samples may be from the sludge loss in the system caused by the shortening of the sedimentation time and the decreasing abundance of *Betaproteobacteria*. In S11, the dominant bacteria changed to

Fig. 7. The 2-D principal component analysis (PCA) analysis of each sludge sample.

Alphaproteobacteria because of the stable particle morphology, the steady hydraulic shear force, and the decreased dominance of *Betaproteobacteria*. In addition, by comparing the abundance of *Gammaproteobacteria* with the removal rate of P in Fig. 3, we can find that the two have similar changing trends, and thus can speculate that *Gammaproteobacteria* plays an important role in the phosphorus removal process. Yoshie et al. [24] and Bond et al. [25] found that *Gammaproteobacteria* played a significant part in denitrification and phosphorus removal reactors.

Comparing the results of this test with the previous conclusions, no major differences were found at the phylum and class levels. However, at the genus level, the results of this experiment differ somewhat from those of other people's research.

A total of 403 genera were detected, of which 52 belonged to all 12 samples. To understand more deeply the bacterial community structure characteristics of each stage, a heat map combined with phylogenetic trees comprising the 50 top most abundant genera was constructed (Fig. S2). The genera with average abundances of more than 5% were identified as *Unclassified Comamonadaceae*, *Zoogloea*, and *Flavobacterium*. Among them, *Unclassified Comamonadaceae* was most prominent, with an average abundance of 14.00%. Its abundance rose rapidly after the addition of the automobile coating wastewater and remained at a high level, until it decreased and stabilized at the end. It is known that *Comamonadaceae* was associated with the removal of aniline [26], and aniline had been confirmed to exist in automobile coating wastewater. *Zoogloea* existed in all stages, with an average abundance of 6.98%. Its abundance in the first and second stages was relatively low and gradually increased from the beginning of the third stage. Members of *Zoogloea*, such as *Zoogloea ramigera*, had long been considered the typical activated sludge bacteria responsible for the formation of activated sludge flocs and for improving the purification process [27]. The average abundance of *Flavobacterium* (6.52%) was similar to that of *Zoogloea*. However, the abundance of *Flavobacterium* in different stages was similar, and no big fluctuations occurred. We can also see from the heat map that the abundances of *Dechloromonas*, *Nitrospira*, *Dok59*, and *Azospira* in the inoculated sludge (S0) were much higher than those in other stages were. The abundances of *Sulfurospirillum*, *Microbacterium*, *Paracoccus*, *Treponema*, *Clostridium*, *Dyadobacter*, *Simplicispira*, *Pelosinus*, and *Janthinobacterium* in the first and early second stages were significantly higher than those in the latter stages. On the contrary, the abundances of *Amaricoccus*, *Aquincola*, *Runella*, *Acidaminobacter*, and *Pseudoxanthomonas* were higher in the later period than in the earlier period. *Amaricoccus* was not found in the inoculated sludge (S0), but the abundance of *Amaricoccus* increased gradually in the later time and remained at a high level. Therefore, this group may be related to the removal of specific unknown pollutants in automobile coating wastewater.

In this study, the treatment by aerobic granular sludge of automobile coating wastewater was excellent. As the proportion of automobile coating wastewater and the growth of granular sludge expanded, the removal efficiency of the main organic matter was significantly improved. High-throughput sequencing based on Illumina MiSeq technology showed that microbial abundance varied at different stages of sludge granulation and remained at a higher level. The structure of the microbial community differed in different stages and gradually stabilized after the aerobic granular sludge reached a stable state. The presence of the dominant groups promoted granular formation, COD removal, nitrification, denitrification, and phosphorus removal. In addition, research on the removal of specific organic pollutants will be carried out in subsequent studies.

Acknowledgements

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Supplementary Information

Table S1
Distribution ratio of water intake for different operational stages

Time(d)	Ratio of synthetic wastewater to automobile coating wastewater	Time(d)	Ratio of synthetic wastewater to automobile coating wastewater
1–10	100 : 0	50–54	70 : 30
11–15	99 : 1	55–59	65 : 35
16–20	97 : 3	60–64	60 : 40
21–23	95 : 5	65–69	50 : 50
24–26	93 : 7	70–74	40 : 60
27–29	91 : 9	75–79	30 : 70
30–34	89 : 11	80–84	20 : 80
35–39	85 : 15	85–89	10 : 90
40–44	80 : 20	90–110	0 : 100
45–49	75 : 25		

Fig.S2. Heat map combined with phylogenetic trees of the top 50 genera.

Fig. S1. Hierarchical cluster analysis of 12 sludge samples.