Bioresource Technology 273 (2019) 462–467

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Rapid reformation of larger aerobic granular sludge in an internal-circulation membrane bioreactor after long-term operation: Effect of short-time aeration

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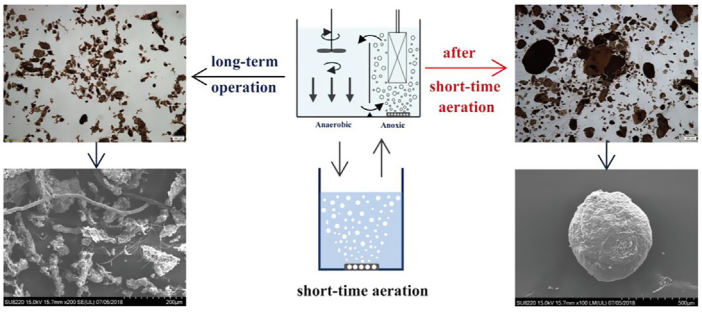
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Aerobic granular sludge
Filamentous bacteria
Membrane bioreactor
Microbial community
Hydraulic conditions

ABSTRACT

The investigation aimed at revealing the influence of an external disturbance on the rapid reformation of larger aerobic granular sludge (AGS) in an internal-circulation membrane bioreactor (IC-MBR) after long-term operation. The used IC-MBR was continuously operated well for more than one year, in which, the biomass was still in the state of AGS with a balanced average size at around 200 μm and an even size distribution. By providing short-time aeration to the biomass within this bioreactor, the characteristics of biomass were totally changed in a very short time, including the surface hydrophilicity, physic-chemical properties, and the structure of microbial community, which created suitable conditions for the growth of filamentous bacteria (*Saccharibacteria*). Such a variation was very beneficial to the reformation of larger AGS, which resulted in the average size of AGS increased to nearly 400 μm with a compact structure and clear edge in no more than one month.

1. Introduction

Aerobic granular sludge (AGS), containing aerobic surface and anaerobic interior simultaneously, is a large microbial aggregate with compact structure that allows simultaneous removal of carbon, nitrogen and phosphorus (Zhang et al., 2016). In the late of last century, AGS was observed and successfully cultivated in sequence bioreactors (SBRs) (Beun et al., 1999; Morgenroth et al., 1997), which aroused a

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https://doi.org/10.1016/j.biortech.2018.11.029
Received 26 September 2018; Received in revised form 6 November 2018; Accepted 8 November 2018
Available online 09 November 2018
0960-8524/

great interest in using AGS as an alternative process of the conventional activated sludge (CAS) to treat both municipal and industrial wastewater. Up to date, an SBR is verified as the most effective way to cultivate AGS, and even a full-scale wastewater treatment plant (WWTP) with this approach has been built and operated successfully (Pronk et al., 2015). The merits of AGS, including rich biodiversity, high settling velocity, enhanced microbial functions, resilience to toxicity and good performance in simultaneous removing multiple contaminants, have been verified by different studies, which showed a promising aspect to replace CAS as the next generation technique for treating wastewater (van Loosdrecht and Brdjanovic, 2014).

SBR, though so far is regarded as the most commonly-used way to cultivate AGS, actually need very complex conditions to obtain mature AGS, such as a suitable applied organic loading rate, the presence of a feast-famine regime. Based on these basic conditions, the hydrodynamic selection of an upward flow subsequently washes the light floc sludge out, and keeps the dense mature AGS remaining and accumulating within the reactors. In this meaning, the successful cultivation of AGS in an SBR mainly relies on two essential factors, one is the occurrence of primary core granules, and the other is the hydrodynamic selection to wash out the light floc sludge from the reactor. Additionally, in SBRs, Verawaty et al. (2012) also found that the crushed granules acted as nuclei for floc sludge to attach, which accelerated the granule formation, and Long et al. (2014) rapidly cultured irregular and pale yellow granular sludge by inoculating a certain ratio of mature granules (about 25%) in the start-up stage. However, in SBRs, when filamentous bacteria overgrow, the settleability of biomass becomes worse, which causes most of sludge being washed out and the mature AGS disappearing, thus, the overgrowth of filamentous bacteria must be inhibited in an SBR (Liu and Liu, 2006), and the operation under an alkaline condition is an effective way to restrict the overgrowth of filamentous bacteria. Even though most reports about mature AGS were from SBRs (Nancharaiah and Kiran Kumar Reddy, 2018), the application in large-scale WWTPs was still too difficult due to the height of SBRs was a crucially restrictive factor (Corsino et al., 2016), therefore, conceiving a continuous-flow reactor might be a promising solution (Juang et al., 2010) to solve this problem.

In a previous investigation, an internal-circulation membrane bioreactor (IC-MBR) with continuous-flow was successfully built, and in which, the well-defined AGS was found to be self-cultivated directly (Chen et al., 2017). In this bioreactor, three factors, including the total retention of sludge particles by membrane modules, the occurrence of filamentous bacteria and internal circulation, were verified to be very essential in forming AGS. Such a finding was very interesting, and showed a novel cultivation mechanism that never reported before, in which, filamentous bacteria exhibited a special role in forming mature AGS, and was quite different with that in SBRs. This bioreactor was continuously operated for more than one year, and showed excellent performance in removing organic pollutants, total nitrogen (TN) and total phosphorous (TP). All the previously reported mechanisms about forming AGS almost focused on the initial stage of forming AGS from floc sludge (Chen et al., 2017). However, a practical bioreactor is generally operated for a long time, and the mature AGS within it may experience a series of continuous processes, including formation, growth, maturity, aging and breaking up. Actually, such a phenomenon was observed in a previous report (Wu et al., 2018), which indicated that, after long-term operation, the characteristics of biomass in an AGS bioreactor were quite different from that at the start-up stage. In such a situation, how does a sudden external disturbance influence on the performance of a long-term operating MBR? This issue is very essential to keep the stability of the whole system. Therefore, in the present investigation, an experiment was designed to explore the effect of short-time aeration on the reformation of larger AGS with the purpose to provide useful references for the practical application in the future.

2. Materials and methods

2.1. Experimental procedure

A previously reported IC-MBR (Wu et al., 2018) was used in the present investigation after adjusting the composition of influent water. Prior to the investigation, it has been continuously operated for 383 days, which showed excellent performance in producing high-quality effluent. This work continued the operation of this bioreactor, and the observation and data collection started from the 384th day (the first day of the present investigation). The whole experiment period lasted for another 8 weeks, and was divided into two phases: (1) Phase I lasted for 4 weeks, all the operational parameters kept unchanged and were same with the previous experiment; (2) Phase II started from the 28th day, on which, all the biomass was transferred into an aeration tank, and continuously aerated for 3 h (11:00–14:00) at an aeration rate of 7.33 L/min. After that, all the biomass were returned back quickly to the IC-MBR, and operated under the same conditions with that of Phase I. All of the experimental process and apparatuses were the same with the previous report unless other stated. The bioreactor was divided into two chambers with a total effective working volume of 36 L. The aeration area was equipped with an aerator and membrane module (effective area: 0.5 m^2, pore dimension: 0.2 μm; MOF-1d, Tianjin Motianmo Membrane Technology Co., LTD, Tianjin, China). A stirring paddle was mounted in the middle of the mixing area. Synthetic wastewater was input into the mixing zone with a flow rate of 4.5 L/h. The daily DO concentration and water temperature in the bioreactor was the average value of the data recorded on the same day (0:00–24:00). During the whole experimental period, the hydraulic retention time (HRT), aeration rate and rotation speed were kept stably at 8 h, 7.33 L/min and 110 rpm, respectively. The membrane module was not taken out for cleaning till the TMP reached at around 30 kPa. The used synthetic wastewater was prepared by mixing Dextrose Monohydrate (412.84 mg/L) and other nutrients, including NH_4Cl (76.43 mg/L), KH_2PO_4 (17.56 mg/L), $NaHCO_3$ (160 mg/L), $MgSO_4$ (40 mg/L), $MnSO_4$ (12 mg/L), $CaCl_2$ (8 mg/L), and $FeSO_4$ (0.6 mg/L), in tap water. The ratio of carbon (as chemical oxygen demand, COD), nitrogen and phosphorus (COD:N:P) in the influent was set at 100:5:1 during the whole operation.

2.2. Analysis of water quality and sludge property

The performance of the bioreactor was evaluated in terms of the removal of organic pollutants, nitrogen- and phosphorus-containing substances. The regular water quality index, including COD_{Cr}, TN, NH_3-N, NO_2^--N, NO_3^--N and TP was chosen as reference parameters to measure their value in the effluent. All the indexes were measured according to the standard methods (APHA et al., 2005).

A laser diffraction particle size analyzer (Mastersizer 3000, Malvern, United Kingdom) was used to measure the size distribution of sludge samples. The morphology of sludge was observed with a digital optical microscope (CX41, Olympus, Japan) and a scanning electron microscope (SEM) (SU8220, Hitach, Japan). Before SEM observation, the sludge sample was pretreated by referencing the method of Cetin et al. (2017).

2.3. Extraction and analysis of extracellular polymeric substances (EPS)

The sludge samples were collected directly from both the aeration and mixing zone of the bioreactor, then sieved with a 0.2-mm-sieve, the residual sludge on the sieve was AGS (larger than 0.2 mm). A heat extraction method was adopted to extract the loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Li and Yang, 2007). The polysaccharides (PS) content in EPS was measured by using the anthrone method with glucose as the standard (Frølund et al., 1996). The protein (PN) content in EPS was measured by using a modified Lowry

colorimetric method, in which the bovine serum albumin was used as the standards (Frølund et al., 1995).

2.4. Microbial community

To determine the microbial community of the bioreactor, and subsequently evaluate the distribution of filamentous bacteria and their variation during the whole period, 10 mL of sludge was collected from the bioreactor (5 mL sludge from the aeration and mixing zone, respectively) every 10 days and then stored at −80 °C. The first sludge sample (original sample) was collected on 4 days before this observation as a comparison, then 10 days later, the second sludge sample (S6) was collected at the same position of the bioreactor, and other samples were collected subsequently in the same way and time interval. All the sludge samples for measuring their microbial community were named by the time sequence of sampling date, namely, original (4 days before the experiment), S6 (day 6), S16 (day 16), S26 (day 26), S36 (day 36), S46 (day 46), S56 (day 56).

Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) was used for quantifying DNA samples, and 30–50 ng of DNA was adopted to generate amp icons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). A panel of proprietary primers was designed to anneal the relatively conserved regions bordering at the V3 and V4 hypervariable regions. The V3 and V4 regions were amplified using forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC". Indexed adapters were added to the ends of the 16SrDNA amplicons to generate indexed libraries (Kong et al., 2018).

DNA libraries were validated by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA) was used for quantifying the DNA libraries. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 300 paired-end configuration; image analysis and base calling was conducted by the MiSeq Control Software (McSwain et al.) on the MiSeq instrument (Fu et al., 2016). The sequences were processed and analyzed by Majorbio Bio-Pharm Biotechnology Co., Ltd. (Shanghai, China). All of the sequences were clustered into Operational Taxonomic Units (OTUs) based on a 97% identity threshold by Silva128 16S as reference databases (Chen et al., 2018b; Quast et al., 2013).

3. Results and discussion

3.1. Variation of DO value and the growth of filamentous bacteria

The DO value at both zones and the temperature of the bioreactor was automatically measured by the installed DO probes, and the results are shown in Fig. 1.

As shown in Fig. 1, the temperature gradually decreased from 28.8 to 16.6 °C, then increased to 23.1 °C, which indicated it ranged in a normal fluctuating scope. The DO value in the mixing zone was very stable to be kept constantly at 0 mg/L, but on day 28, short-time aeration was applied on the biomass, which caused the DO value in this zone quickly increased to 1.84 mg/L and then decreased to 0.2 mg/L in one day. While, the DO concentration of the aeration zone ranged from 1.49 mg/L to 5.04 mg/L (average 2.63 ± 0.72 mg/L), indicating a stable aerobic status formed in this zone.

During the experiment, a small amount of sludge sample was taken out from the bioreactor and observed their morphology every day with the representative images shown in the Supporting Information (SI). After long-term operation under constant operating conditions, the characteristics of the biomass in a bioreactor tended to be stable, especially the size distribution of AGS, which might achieve a balance in its size development (Dahalan et al., 2015). As shown in SI, the

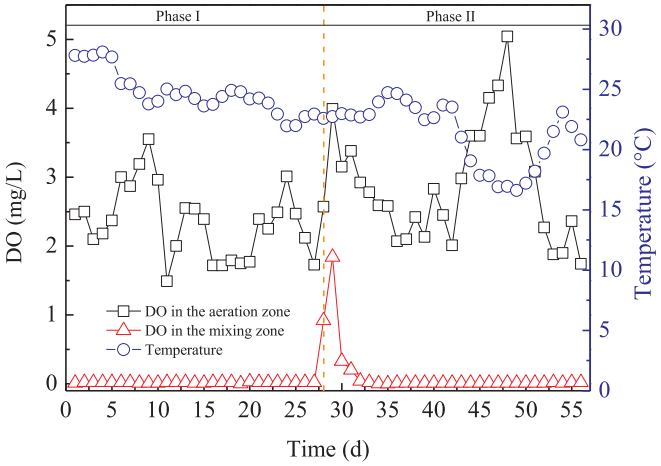

Fig. 1. Variation of DO and temperature.

images exhibited the morphology of sludge on different dates before day 28 was very similar, and the sludge was all in a granular status (> 200 μm), but with a very narrow size distribution. However, after the aeration, the DO value fluctuated for a very short time, but as a consequence, the growth of filamentous bacteria was promoted greatly, which caused the morphology of sludge changed obviously.

The causes of filamentous bulking are complicated and generally include these factors as low DO concentrations, low organic loading rates, low temperatures and low substrate concentration gradients (Guo et al., 2014). By adjusting these factors, the growth of filamentous bacteria can be stimulated. Tian et al. (2011) and Yang et al. (2013) once induced limited filamentous bulking with low DO. Banti et al. (2017) stimulated the growth of filamentous bacteria by adjusting the ratio of food to microorganism and DO concentration. In general, filamentous bacteria have a higher growing advantage over other microorganisms from the limited substrate in bulk liquid (Martins et al., 2004; Martins et al., 2003), and they also have a larger storage capacity to resist starvation (Beccari et al., 1998). The specific growth rate of filamentous bacteria was higher than that of non-filamentous bacteria at low substrate concentration (Cenens et al., 2000). After aeration, some of the nutrients were consumed, which caused a famine status within the bioreactor, and also led to faster growth of filamentous bacteria. The morphology of sludge on day 36 (only one week after aeration) (SI) was changed, and it showed quite an obvious difference with that before aeration. In the days followed, filamentous bacteria grew continuously, and acted as a bridging effect to bring more tiny granular sludge together, which caused the AGS growing larger quickly (SI).

3.2. Variation of AGS after aeration

For giving a quantitative description to the growth of AGS, the size and its distribution of the biomass within the bioreactor was measured every 4 days after the beginning of this experiment. The results are shown in Fig. 2.

A parameter – median size (D50), which meant the average size of a group of granules, was used in this section to describe the continuous variation of the size of sludge samples. Fig. 2 indicated the average size of sludge was about 200 μm and it kept stable before day 28. Such a result verified that the AGS had attained a balanced size distribution after long-term operation and was very similar to a reported work (Dahalan et al., 2015). However, after a sudden external disturbance, namely, short-time aeration, the average size changed greatly, which grew from about 200 μm to nearly 400 μm in no more than one month (4 weeks). The sizes of AGS in this study were very close to that cultivated in SBRs. Chen et al. (2018a) cultivated compacted granules with

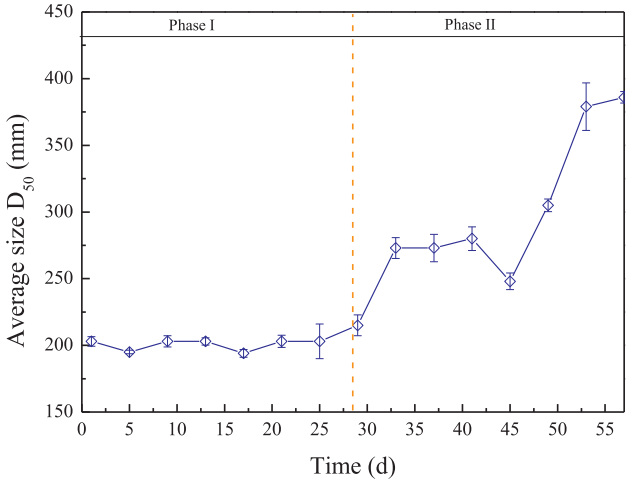

Fig. 2. Variation of the sludge size over time.

the size ranging from 0.46 to 0.9 mm in an SBR. Caluwe et al. (2017) used two different SBRs to culture AGS with median granule size at 264.7 μm and 307.4 μm respectively. The obvious variation in the size of AGS would influence on the performance of the bioreactor and result in the succession of its microbial community, which will be further investigated in the following sections.

3.3. Performance of the bioreactor

Performance of the bioreactor is the most concerned issue in the present investigation. For making a comparison, during Phase I and II, the water samples in the bioreactor and the effluents were collected and measured their regular water quality indexes, including COD_{Cr}, TN and TP, to evaluate the performance of the bioreactor, and NH_3-N, NO_2^--N, NO_3^--N was measured to detect their variation in the bioreactor. All the indexes were measured every day according to the standard method with the results shown in Fig. 3.

As shown in Fig. 3(a), the COD value in the effluent fluctuated for a very short time at the beginning of this experiment, but it stably kept at lower than 10 mg/L subsequently even after the biomass being aerated for 3 h. The COD removal was stably kept at a high value (higher than 98.48%) during the whole experimental period, which indicated the used bioreactor performed very well in removing organic pollutants even encountering a sudden external disturbance.

During Phase I and II (Fig. 3(b)), the concentrations of NH_3-N and NO_2^--N were very low, which implied quite excellent nitrification of the bioreactor. The NO_3^--N value fluctuated from 0 to 3 mg/L, indicating the denitrification was not very stable. On day 28, all the biomass was aerated for 3 h, which created a total aerobic state within the bioreactor and worsened the denitrification of the bioreactor, therefore, the NO_3^--N value increased sharply after that day and kept at a relatively high level for about 5 days, then it gradually decreased to a low value and kept stable in the following weeks. In general, during these two phases, it obtained a good result in total removing TN (Fig. 3(c)) except the period from day 28 to 33, during which, the denitrification conditions were destroyed by the total aeration of biomass. However, after 5 days, the performance of total removal of TN recovered completely and the TN value kept at a lower value than that in Phase I.

Total removal of TP also needs an alternant aerobic-anaerobic condition. The continuously recorded data in Fig. 1 indicated that an obvious DO gradient actually formed within the bioreactor, and in which, the aeration and mixing zone maintained at an aerobic and anaerobic state (the DO value nearly closed to 0 at the mixing zone), respectively. Therefore, in Phase I, an excellent result in removing TP was successfully achieved. After aeration on day 28, the removal of TP deteriorated for a short time (only one day), and then recovered very quickly. Comparing with these two phases, a higher efficiency in removing TP seemed obtaining at Phase II than that at Phase I, which implied more suitable conditions were obtained during Phase II.

In summary, the sudden external disturbance (short-time aeration) resulted in a great change in the size distribution of AGS, and created a combining aerobic-anaerobic condition, which was beneficial for the removal of TN and TP, while, such a variation had no obvious influence on the removal of COD.

3.4. Variation of extracellular polymeric substances (EPS)

In AGS, microorganisms are encapsulated in EPS matrix and compose an integral microbial community. In this meaning, the composition of EPS plays an important role in forming AGS and keeping its structure stable (Nancharaiah and Kiran Kumar Reddy, 2018). In this section, each sludge sample was divided into two sub-samples with a 0.2 mm-sieve. According to a general meaning, the sub-sample with the size larger than 0.2 mm was regarded as AGS, and those less than 0.2 mm, regarded as floc sludge (FS). The results are shown in Fig. 4.

In general, the mixed microbial populations imbed in the three-dimensional glue of bound EPS (Verawaty et al., 2013), and the bound EPS can be roughly classified as TB-EPS and LB-EPS. TB-EPS mainly attaches on the cell surface peripheral capsules with a certain shape, and LB-EPS sheds into the surrounding environment without a clear edge (Sheng et al., 2010). Fig. 4 shows the variation of EPS and PN/PS in AGS and FS, respectively, which indicates that both LB-EPS and TB-EPS in AGS are higher than that in FS. The average value of LB-EPS and TB-EPS in AGS was 4.64 mg/gVSS and 28.01 mg/gVSS, respectively, but in FS, the average LB-EPS and TB-EPS was 2.14 mg/gVSS and 22.99 mg/gVSS. No matter in AGS or FS, the content of TB-EPS was far higher than that of LB-EPS, which indicated that TB-EPS played a main role in keeping sludge stable.

PN and PS are the main components in EPS (Zhu et al., 2018), and the ratio of PN/PS stands for the surface properties of biomass. Higher value of PN/PS generally means a more hydrophobic surface. Comparing with the values of PN/PS in Fig. 4(a) and (b), the value of AGS was obviously higher than that of FS, which implied that AGS was more hydrophobic than FS, and might be a major reason leading to granulation. After the aeration on day 28, the value PN/PS showed an increasing tendency in AGS, but for FS, it declined slowly. High PN/PS value would reduce the surface negative charge of AGS, thus, the electrostatic repulsion between AGS decreased and the hydrophobicity enhanced, which promoted the twining of small AGS by filamentous bacteria to reform larger AGS (McSwain et al., 2005).

3.5. Analysis of bacterial communities

High-throughput sequencing (HTS) is a useful tool to reflect the composition and variation of microbial communities. The AGS within the bioreactor was collected at a regular time interval (10 days) to detect its microbial community with the results shown in Fig. 5.

Under constant operating parameters, the operation of a bioreactor gradually becomes stable, and the microbial community within the bioreactor experiences a continuous succession with a gradual and slow change in its microbial structure (Tang et al., 2014). Fig. 5 reveals the relative abundance of various microorganisms at the family level during these two phases. At Phase I, the microbial community kept relatively stable, the ratio of the dominant microorganisms gradually changed along with time. However, at Phase II, a great change occurred in the composition of microbial community after the short-time aeration. *norank_p_Saccharibacteria*, a kind of filamentous bacteria (Hugenholtz et al., 2001), showed the most dominant variation among all the microorganisms, it accounted for a very small proportion before day 26 (original: 3.14%, S6: 3.12%, S16: 3.40%, S26:14.57%), but after aeration, it increased to a very high ratio on day 36 (S36: 64.21%). In the

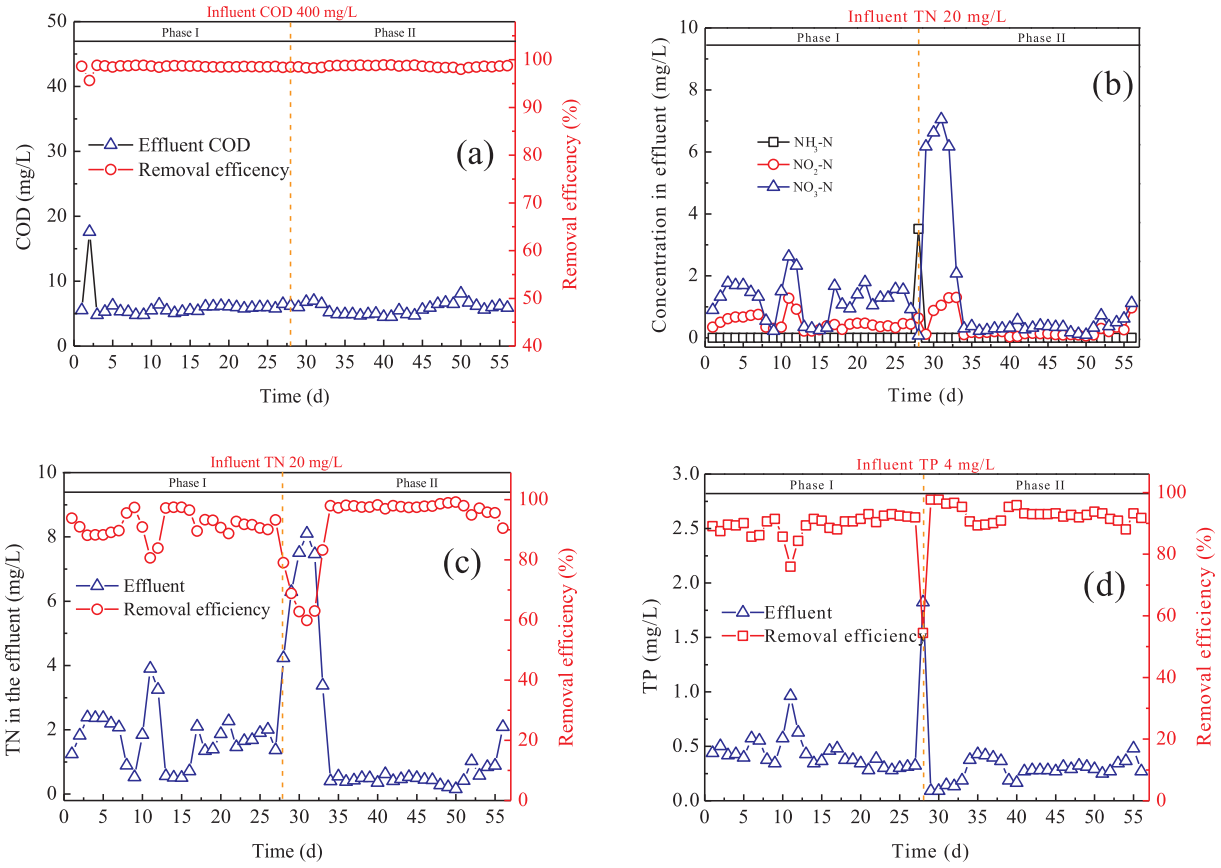

Fig. 3. Performance of the bioreactor: (a) COD_{Cr} removal; (b) variation of NH_3-N, NO_2^--N and NO_3^--N within the bioreactor; (c) TN removal; (d) TP removal.

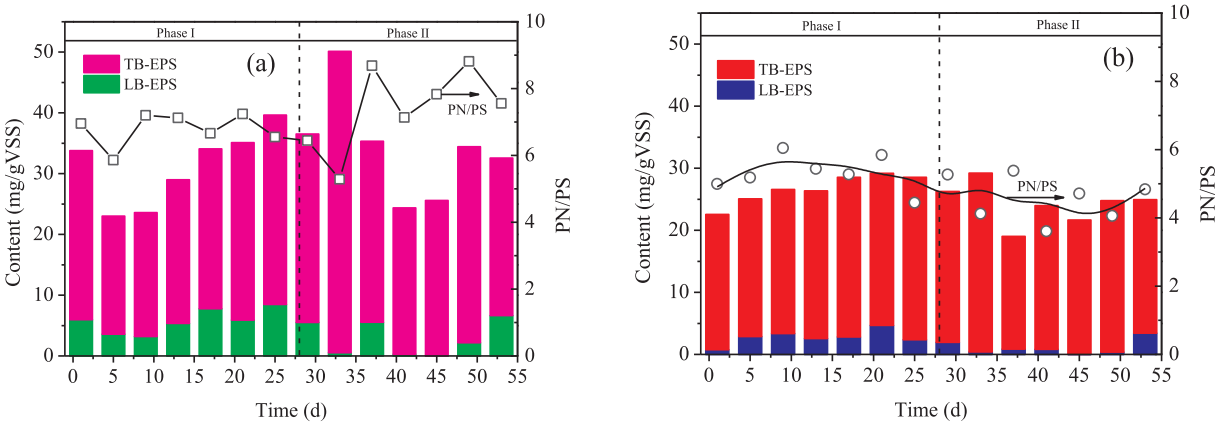

Fig. 4. Variation of EPS along with time: (a) EPS in AGS; (b) EPS in FS.

following days, its ratio gradually decreased, but still was the most dominant microbial species (S46: 53.89% and S56: 48.77%). *Rhodocyclaceae* was the most dominant family before the short-time aeration, which occupied the highest ratio among all the families before day 28 (original: 13.63%, S6: 15.21%, S16: 20.06%, S26: 11.96%). Being a kind of denitrifying rod-shaped bacteria (Zhao et al., 2015), their growth was greatly inhibited by the aeration, thus, its ratio in the microbial community sharply decreased after the aeration (S36: 2.75%, S46: 2.54% and S56: 3.58%). *Thiotrichaceae* is a common kind of filamentous bacteria, and was one of the dominant species at the beginning of this experiment, but was gradually replaced by other species (original: 21.21%, S6: 15.18%, S16: 7.59%, S26: 0.64%, S36: 0.60%, S46: 1.69%, S56: 1.62%). Both *Thiotrichaceae* and *Saccharibacteria* are different kinds of filamentous bacteria, they compete for the same food and other sources for surviving. The results of competition led to their changes in the composition of microbial community and caused the succession of the relevant community even before aeration. *Myxococcales*, known as “slime bacteria”, generally secrete slime to adhere to the interface between the cell body and substratum (Wang and Zheng, 2017), its increased gradually after filamentous bacteria overgrowth (original: 0%, S6: 0.14%, S16: 0.08%, S26: 0.37%, S36: 0.38%, S46: 0.71% and S56: 0.10%). Overall, the structure of the microbial community within the bioreactor was greatly changed by the short-time aeration, and the growth of some of filamentous bacteria (such as *Saccharibacteria*) was also promoted obviously. Such a result was in accordance with the above SEM results and the microscopic observation shown in SI, which might be an important reason leading to the reformation of larger AGS.

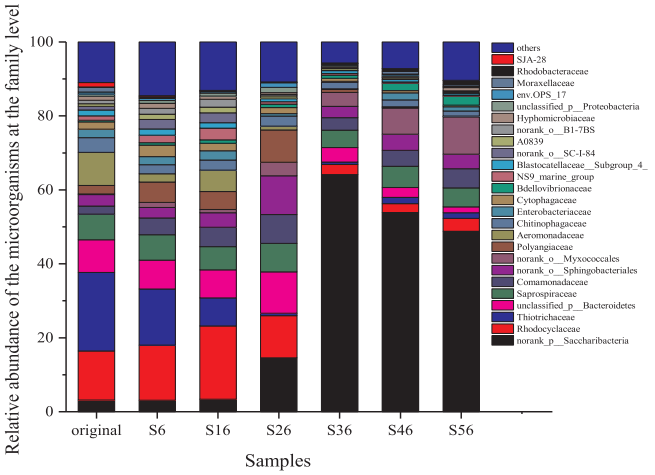

Fig. 5. Relative abundance of the microorganisms at the family level.

4. Conclusions

The microbial community in an IC-MBR experienced a continuous succession with a gradual and slow change in its structure after long-term operation, and the average size of AGS within the bioreactor reached a balance at around 200 μm with an even distribution. Interestingly, after short-time aeration to the biomass, the growth of some filamentous bacteria (*Saccharibacteria*) was greatly promoted, and the surface of AGS tended to be more hydrophobic, which obviously changed the microbial community in this bioreactor in a very short-time, and quickly caused the reformation of larger AGS (nearly 400 μm) with a compact structure and clear edge.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China [No. 21476050].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.11.029.

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