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Gradient reduced aeration in an enhanced aerobic granular sludge process optimizes the dominant microbial community and its function†

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The laboratory-scale aerobic sludge process was used to treat municipal sewage using a novel energy-saving aeration strategy. For comparison, two strategies of regulation (reducing the aeration directly and reducing the aeration gradually) were operated in parallel. Results showed that using an operation strategy with reduced aeration during the famine period led to a significant reduction of energy consumption ($p < 0.01$). Much of the aerobic sludge was washed out and granular sludge disintegrated irreversibly when the direct reduction of aeration was applied; the MLSS dropped gradually from 6.33 g L^{-1} to 1.94 g L^{-1} and the SVI reached 325 mL g^{-1}. At the same time, the zeta potential and sludge yield rose from −17.3 mV and 1.13 kg MLSS per kg COD to −20.7 mV and 1.96 kg MLSS per kg COD, respectively. The removal efficiency of NH_4^+-N, TN and COD decreased to 61%, 48% and 82%, respectively. *Leadbetterella* spp., which belongs to the phylum *Sphingobacteria*, became the dominant microorganism in the bioreactor. By contrast, when the gradient reduced aeration strategy was applied, Shannon diversity indices increased significantly ($p < 0.01$) and the aerobic granular structure remained stable, revealing that the richness and diversity of the aerobic sludge were retained. FISH results revealed that the enrichment of functional microorganisms such as *Zoogloea* spp. for EPS secretion enriched in the inner part of the granular sludge. Results of 2-DE demonstrated that four extracellular proteins PPIase, pyrophosphate phosphohydrolase, OprF and OMP, which can secrete EPS, were enriched in the stable granules under the condition of gradient reduced aeration. Therefore, a scientific method to regulate the structural stability of the aerobic granular sludge was achieved in this study.

Water impact

In recent years, aerobic sludge granulation has played an important role in upgrading the technology for wastewater treatment because of its excellent settling ability and efficient pollutant removal. According to the key limitations of granular stability, an innovative and energy-saving aeration strategy is developed to achieve stable aerobic granules and improve their performance.

1. Introduction

Aerobic granular sludge (AGS) technology has received increasing attention for biological wastewater treatment in recent years.[1–5] As a special form of biofilm, AGS has overcome some inherent problems such as poor settling ability and easy sludge expansion in the conventional activated sludge process and has the potential to be developed as a next-generation biological wastewater treatment technology. Currently, aerobic granular sludge is applied to slaughterhouse wastewater,[6] toxic organic wastewater, high concentration organic wastewater,[7] dairy wastewater,[8] phenolic wastewater[9,10] and dye wastewater treatment, resulting in significant achievements.

However, there are limitations to aerobic granule sludge technology, such as long start-up time, high energy consumption and insufficient denitrification ability. These problems have restricted its engineering application in wastewater treatment.[11–13] Studies on substrate composition as well as dissolved oxygen (DO),[14] pH and temperature fluctuations[15] have greatly improved start-up times, and an aerobic granular

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sludge bioreactor can now be started up within four weeks.[16,17] However, the instability of AGS remains unresolved and often occurs during the long-term operation of the process.[18] Studies have shown that the overgrowth of filamentous microorganisms can lead to reactor failure when the DO is low.[19] At an organic loading rate (OLR) of 6.0 kg COD m^3 d^{-1}, the compact aerobic granules grew in size, but gradually lost their stability due to filamentous growth.[20] The presence of proteolytic bacteria belonging to the genera *Pseudomonas* spp., *Raoultella* spp., *Acinetobacter* spp., *Pandoraea* spp., *Klebsiella* spp. and *Bacillus* spp. inside the aerobic granules has been confirmed to be responsible for inner core deterioration, leading to granular disintegration.[21] Another study reported that a non-cellular protein core in aerobic granules provided the granular mechanical stability.[22]

Many studies have analyzed aerobic granular sludge granulation and its stable operation from the perspective of microbial communities. It has been reported that the dominant sludge microbial community can be divided into six classes: *Paracoccus marcusii*, *Nitrosomonas* spp., *Thauera* spp., *Rhodocyclaceae* spp., *Zoogloea ramigera* and *Proteobacterium*. Among these, *Paracoccus marcusii* is a facultative anaerobic bacterium that possesses nitrification and denitrification abilities, whereas *Nitrosomonas* spp. was previously reported to be a dominant ammonia oxidizing bacterium (AOB) and plays an important role in ammonia oxidation.[23] *Thauera* spp. is a kind of denitrifying bacterium that can grow under both anaerobic and aerobic conditions.[24] *Rhodocyclaceae* spp. and *Proteobacterium* are important phosphorus removal microbes. A sliced sample study revealed that mature granules have a spherical core with anaerobic *Rhodocyclaceae* spp. covered by an outer spherical shell with both aerobic and anaerobic strains;[25] *Zoogloea ramigera*, which produces a slime matrix, contributes to the sludge granulation. A "concentration-to-extinction" approach was adopted to isolate the functional microbes from aerobic granules cultivated at an OLR of up to 21.3 kg m^3 d^{-1}.[26] The functional microbes were identified to be *Zoogloea resiniphila* and two uncultured strains that might include α-*Proteobacteria* (*i.e.*, *Meganema perideroedes*), β-*Proteobacteria* (*i.e.*, *Aquaspirillum* spp.), γ-*Proteobacteria* (*i.e.*, *Thiothrix* spp.) and *Bacteroidetes*, which enrich in the activated sludge and possess the ability to produce extracellular polymeric substances (EPS). Researchers have suggested that these bacteria may play an important role in reinforcing the filamentous sheath, strengthening the floc and enhancing the surface hydrophobicity.[26]

Currently, research examining the influence of the relevant functional microbial community on aerobic granular sludge is limited. Lots of studies showed that EPS played a key role in sludge granulation and stability. Thereinto, extracellular protein (PN) is the main component of EPS, and could enhance the hydrophobicity of the cellular surface.[22] Lots of PN can bind with multivalent cations such as Ca^{2+}, Mg^{2+} and Fe^{3+}.[27] On the other hand, some PN, as extracellular enzymes, can hydrolyse EPS or other macromolecule substances to contribute to the granule stability.[28] All of these properties favor aerobic sludge granulation, and different PN contents have varied functions in aerobic granular sludge. Nevertheless, some PN haven't been analysed for their specific structure and function, as experimental methods were limited. Therefore, the use of 2-dimensional gel electrophoresis (2-DE) technology to explore the main PN components represents a pioneering approach. Based on the analysis of PN using 2-DE technology, we can illustrate the function of the main PN component and its role in granular stability. Therefore, it is of great significance to expound the mechanism of aerobic sludge granulation fully and enhance the application of this technology.

This study used a laboratory-scale bioreactor treating municipal sewage to evaluate an innovative aeration approach that enhances the granular stability, and to elucidate the relationship between the sludge microbial community and its function in an enhanced aerobic granular sludge process. The experiment focused primarily on the process of sludge granulation, disintegration (directly reducing the aeration) and the restoration of stability (gradient reduction of aeration). This provided a scientific method to regulate the structural stability of aerobic granular sludge. Genomic and proteomic analyses were also used to characterize the microbial community and identify the relevant proteins associated with the enhanced granular sludge process.

2. Materials and methods

2.1. Reactor and operation

A sequencing batch reactor (SBR) with a working volume of 6 L was operated in 3 h cycles. The detailed conditions for the experimental operation of the reactor are provided in Table S1.† The treatment object was simulated municipal sewage. Air bubbles were supplied through dispensers at the reactor's bottom at a superficial gas velocity of 2.5 cm s^{-1}. The effluent was discharged at a volumetric exchange ratio of 70%. Mature aerobic granules were cultivated in the reactor with a mixed liquor suspended solid (MLSS) value of approximately 4.0–6.0 g L^{-1} and a sludge volume index (SVI_{30}) of approximately 40–60 mL g^{-1}.

Using the successful start-up aerobic granular sludge reactor as a platform, step aeration methods were used to enhance the operation performance of the aerobic granular reactor as follows (Fig. 1). Directly decreasing the aeration intensity of the aerobic starvation stage: the high aeration intensity of the feast stage was maintained with a 2.5 cm s^{-1} superficial gas velocity for 1 h. The aeration intensity of the aerobic starvation stage directly decreased to a 1.0 cm s^{-1} superficial gas velocity and was maintained at this level up to the end of the cycle. Gradient reduction of the aeration intensity of the aerobic starvation stage: over a 10 d period, the aeration intensity of the aerobic starvation stage was decreased in gradients from a 2.5 cm s^{-1} superficial gas velocity to a 1.0 cm s^{-1} superficial gas velocity, and then stable operation was maintained.

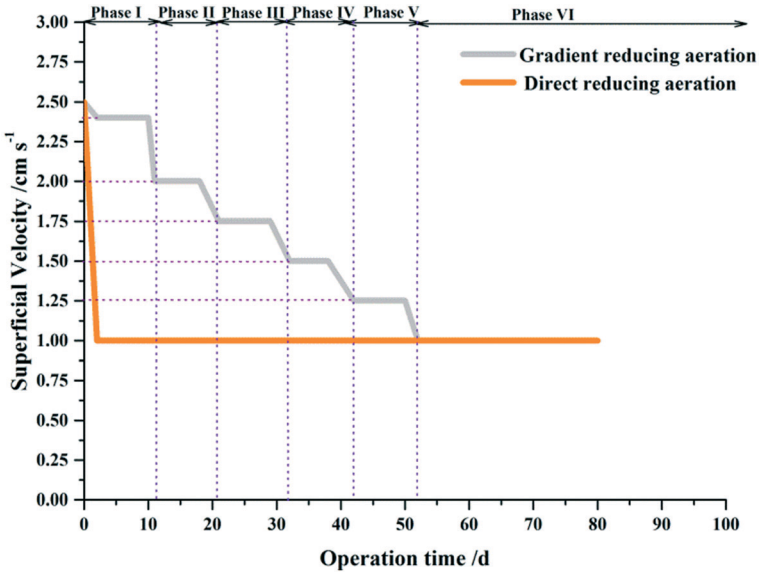

Fig. 1 Variations in aeration intensity during the aerobic starvation stage.

2.2. Synthetic wastewater and inoculated sludge

Both reactors were inoculated with seeding sludge taken from an aerobic tank at the Qige Municipal Wastewater Treatment Plant in Hangzhou, China. The SVI_{30} of the seed sludge was 80 mL g^{-1}. Synthetic wastewater was prepared by diluting the synthetic stock solution (Table S2†) with tap water to set the influent COD concentration at about 500 mg L^{-1} during the reactor operation.

2.3. Analytical methods

2.3.1. Analysis of sludge property and water quality

2.3.1.1 Particle size distribution of the granules. The granular size distribution was analyzed using a laser particle size analysis system (QICPIC, Sympatec, Germany) following a previous study,[29] and the proportion of the granular size over 200 μm was measured to assess the degree of sludge granulation as described by Verawaty *et al.*[30]

2.3.1.2 Scanning electron microscopy (SEM) analysis. The external structure of the sludge samples was analysed by scanning electron microscopy (SEM) as follows: the aerobic granular sludge samples from reactors with a sludge size of over 200 μm were sieved and first fixed with 2.5% glutaraldehyde in a phosphate buffer (pH 7.0) for more than 4 hours, washed three times in the phosphate buffer, then post-fixed with 1% OsO_4 in the phosphate buffer for 1 hour before washing three times again in the phosphate buffer. For dehydration, the sludge samples were first dehydrated with a graded series of ethanol (50%, 70%, 80%, 95%, and 100%) for approximately 15 to 20 minutes at each step; the samples were then transferred into a mixture of alcohol and isoamyl acetate (v : v = 1 : 1) for approximately 30 minutes and then into pure isoamyl acetate for approximately 1 h. Finally, the sludge samples were dehydrated in a critical point dryer with liquid CO_2 (Hitachi, Model HCP-2, Japan). The dehydrated sludge samples were coated with gold–palladium and observed by SEM (Hitachi, Model TM-1000, Japan).

2.3.1.3 Contact angle of granules. The contact angle was applied to assess the hydrophobicity of aerobic granular sludge and was analysed by static drop technology. Sludge samples were washed three times with PBS and filtered in a cellulose acetate membrane (0.45 μm). The membrane with evenly distributed sludge was then dried for 10 min. The contact angle was measured using a contact angle meter (Dataphysics, OCA20, Germany).[31]

2.3.1.4 Porosity analysis. Granules with a diameter of >200 μm were screened out for analysis of the surface area and porosity. Then, the samples were prepared according to the preparation method for SEM. Dry granules were analyzed with an automatic instrument (Micromeritics, TRISTAR 3020, USA) using the corresponding nitrogen adsorption–desorption isotherms at 77 K. The P/P_0 ranged from 10^{-5} to 0.99. The Brunauer–Emmett–Teller (BET) method was used to calculate the specific surface area.[32]

2.3.1.5 Zeta potential analysis. The zeta potential of granular sludge was analyzed with a zeta potential measurement analyzer (Malvern, Zetasizer nano zs90, UK). Samples were first washed three times with deionized water, and then centrifuged to remove supernatants at 2000 rpm for 5 min. A homogenizer (Allegra, 64R Centrifuge, USA) was used to crush sludge samples. 30 mL of mixed sludge suspension was homogenized at 8000 rpm for 1.5 min (Su *et al.*, 2014), and then filtered through a 200 mesh screen to remove unbroken granules for zeta potential analysis.[33]

2.3.1.6 Other analysis. The biomass concentration including MLSS, mixed liquor volatile suspended solids (MLVSS) and SVI_{30} were analyzed according to standard methods.[34] The water quality including the pH, DO, chemical

oxygen demand (COD), TN, NH_4^+-N, NO_2^--N and NO_3^--N levels was measured regularly according to the APHA standard methods.[34]

2.3.2. Analysis of microbial diversity

2.3.2.1 16S rRNA sequencing. The genomic DNA of the biomass in granule samples was extracted following the protocol of the Power Soil DNA extraction kit (MO BIO Laboratories, Inc.). The total DNA extracted from the samples was used as a template and the V3–V4 region of the bacterial 16S rRNA was amplified with the primer (338F 5′-ACTCCT ACGGGAGGCAGCAG-3′; 806R 5′-GGACTACHVGGGTWTCTA AT-3′). All reactions were carried out in 25 μl (total volume) mixtures containing approximately 25 ng of the genomic DNA extract, 12.5 μl of the PCR Premix, 2.5 μl of each primer, and PCR-grade water to adjust the volume. PCR was performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) set to the following conditions: initial denaturation at 98 °C for 30 seconds; 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 53 °C for 30 seconds, and extension at 72 °C for 45 seconds; and then final extension at 72 °C for 10 minutes. The PCR products of the samples were sent for sequencing using the Illumina MiSeq platform (PE300, CA, USA). The reads were filtered using Quantitative Insights Into Microbial Ecology (QIIME) quality filters. The CD-HIT pipeline was used for picking operational taxonomic units (OTUs) through making the OTU table. Sequences were assigned to OTUs at 97% similarity. Representative sequences were chosen for each OTU, and taxonomic data were then assigned to each representative sequence using the Ribosomal Database Project (RDP) classifier. In order to estimate the Alpha Diversity, the OTU table was rarified at a minimum sequencing depth level of 90%, and four metrics were calculated: the Chao 1 metric to estimate the richness, the observed OTUs metric as the count of unique OTUs found in the sample, the Shannon index and the Simpson index.

2.3.2.2 Fluorescence in situ hybridization (FISH). Fluorescence *in situ* hybridization (FISH) analysis was used to identify the bacterial populations. As described by Winkler *et al.*,[35] a total of 0.3 mL of the sample in 1× PBS was added to the fixative solution (0.9 mL 4% formaldehyde in 1× PBS) and incubated on ice for 2 h. The samples were centrifuged at 13 000 rpm for 5 min. Subsequently, the supernatants were removed, and the pellet was resuspended in 1 volume of 1× PBS; this step was repeated once. Next, the pellet was resuspended in 1 volume of ethanol (−20 °C), and the fixed samples were stored at −20 °C.

For the hybridization, 10 μL of the paraformaldehyde fixed sample was placed on a slide. The samples were dehydrated using an increasing ethanol series (3 min each in 50, 80, and 100% ethanol) and then dried. BET420a (β-*Proteobacteria*) and ZRA23a (*Zoogloea* lineage, not *Z. resiniphila*) probes were used for FISH analysis.[36] A total of 10 μL of the hybridization buffer was added to each well with 1 μL of each probe. The remaining hybridization buffer was poured onto the tissue, and the slide was immediately incubated at 46 °C for 1.5 h. The slides were quickly rinsed with a small portion of washing buffer and incubated in the remaining washing buffer for 15 min in a preheated water bath (48 °C). The slides were rinsed carefully with distilled water, dried and stored in the dark. The cells were observed with a Zeiss microscope.

2.3.3. Analysis of the EPS content and component

2.3.3.1 EPS content analysis. EPS was extracted from a sludge sample using a heating method. The polysaccharide (PS) content in EPS was then quantified using the phenol–sulfuric acid method with glucose as the standard. The protein (PN) content in EPS was further determined by a modified Lowry colorimetric method with bovine serum albumin as the standard.

2.3.3.2 Two-dimensional gel electrophoresis (2-DE) analysis. 2-DE was carried out using the Bio-Rad 2-DE system. Briefly, 150 μg of protein samples was applied for isoelectric focusing (IEF) using the ReadyStrip immobilized pH gradient (IPG) strips, 17 cm, pH 4–7. The strips were placed into a Protean IEF cell and rehydrated at 50 V for 12 h. The proteins were separated based on their isoelectric point according to the following protocol: 250 V with a linear climb for 30 min, 1000 V with a rapid climb for 60 min, 10 000 V with a linear climb for 5 h and 10 000 V with a rapid climb until 60 000 V was reached. After the completion of focusing, the strips were equilibrated by two steps, first in a buffer containing 50 mM Tris-HCl, pH 8.8, 30% glycerol; 7 M urea, 2% sodium dodecyl sulfate and 1% dithiothreitol, followed by further treatment in a similar buffer (but containing 4% iodoacetamide instead of dithiothreitol). Each step lasted for 15 min. After equilibration, the IPG strips were applied to 12% homogeneous sodium dodecyl sulfate-polyacrylamide electrophoresis gels for electrophoresis using a PROTEAN II xi Cell system. The system was run at 10 mA per gel for 30 min, followed by 30 mA per gel for 5–6 h until the bromophenol blue line reached the bottom of the gels. The gels were then silver stained using Silver Stain Plus kit reagents according to the manufacturer's instructions. The silver-stained gels were scanned using a Densitometer GS-800 and then analyzed using PD-Quest software. The individual protein spot quantity was normalized as follows: the raw quantity of each spot in a member gel was divided by the total quantity of the valid spots in the gel, and normalized spot intensities were expressed in ppm. Quantitative analysis was performed using a Student's *t*-test between protein gels from sham-operation and the IR group. The significantly differentially expressed protein spots ($P < 0.05$) with a 1.5 fold or more increased or decreased intensity between sham-operation and the IR group were selected and subjected to further identification by MALDI-TOF MS/MS. The spot intensities of these spots (possible IR-related proteins) in other groups (IR + SA, IR + NG, IR + CSN) were also examined.

2.3.4. Statistical analysis. Statistical analysis between all the variables was conducted by a *t*-test using SPSS (SPSS 17.0). $p < 0.05$ was considered to be statistically different, and $p < 0.01$ was considered to be significantly different.

3. Results and discussion

3.1. Reactor performance

The aeration intensity was directly reduced when the reactor was in the aerobic starvation stage. The DO of the SBR decreased from 6.37 mg L^{-1} in the feast stage to 3.72 mg L^{-1} in the aerobic starvation stage, resulting in irreversible disintegration of granular sludge that was washed out of the reactor after 30 days of operation. In addition to the disintegration, there was an obvious decrease in the ability to remove pollutants. The removal efficiency of TN decreased from 68% to 48% (Fig. S1†), and decreased from 95% to 82% for COD. MLSS gradually dropped from 6.33–1.94 g L^{-1} (Fig. S2†), whereas the SVI reached 325 mL g^{-1}. Reducing the aeration intensity directly led to a sharp drop in granular sludge hydrophobicity which was indicated by a decrease in contact angle from 82.4° to 69.6°. The zeta potential increased from −17.3 mV in the stable aerobic granular sludge to −20.7 mV in the disintegrated sludge. The corresponding increase in the sludge yield was from 1.13 kg MLSS per kg COD to 1.96 kg MLSS per kg COD.

Gradient reduction of the aeration intensity during the aerobic starvation stage was applied to the SBR following the decline of the granule performance. DO was reduced slowly and maintained at 4.5–5.0 mg L^{-1}. In addition to the regulation, there was a slight fluctuation in the removal efficiencies of NH_4^+-N and COD. The removal efficiency of TN showed a downward trend during the initial regulation period and increased soon thereafter with the enhancement of denitrification. By the end of the aeration regulation process, the reactor maintained an efficient and stable operation. The removal efficiency of NH_4^+-N and COD remained above 90% and 95%, respectively, whereas the removal efficiency of TN reached approximately 85% (Fig. S3†). Moreover, the reactor showed enhanced sludge properties after the gradient reduction of aeration. MLSS was maintained at 4.5 g L^{-1} (Fig. S4†), whereas the SVI was reduced to 25 mL g^{-1}. The SRT of the aerobic granules was controlled in the range of 10.32–11.25 d and the average granule size stayed above 200 μm (data not shown). Granule hydrophobicity significantly improved, with the water contact angle increasing from 81.9° to 102.4° and the zeta potential decreasing from −17.1 mV to −13.7 mV, respectively. These results indicated that the gradient reduction of the aeration intensity during the aerobic starvation stage improved the AGS performance. SEM observations of the sludge morphology after directly reducing the aeration showed that the granular structure was fluffy and loose (Fig. 2). By contrast, the sludge of steady state granules after the gradient reduction of aeration showed that the pore size was reduced from 412.8 to 296.5 nm, but the porosity and compactness were increased. Therefore, the granules may change the pore structure and substrate metabolism to adapt to the DO variation.[2]

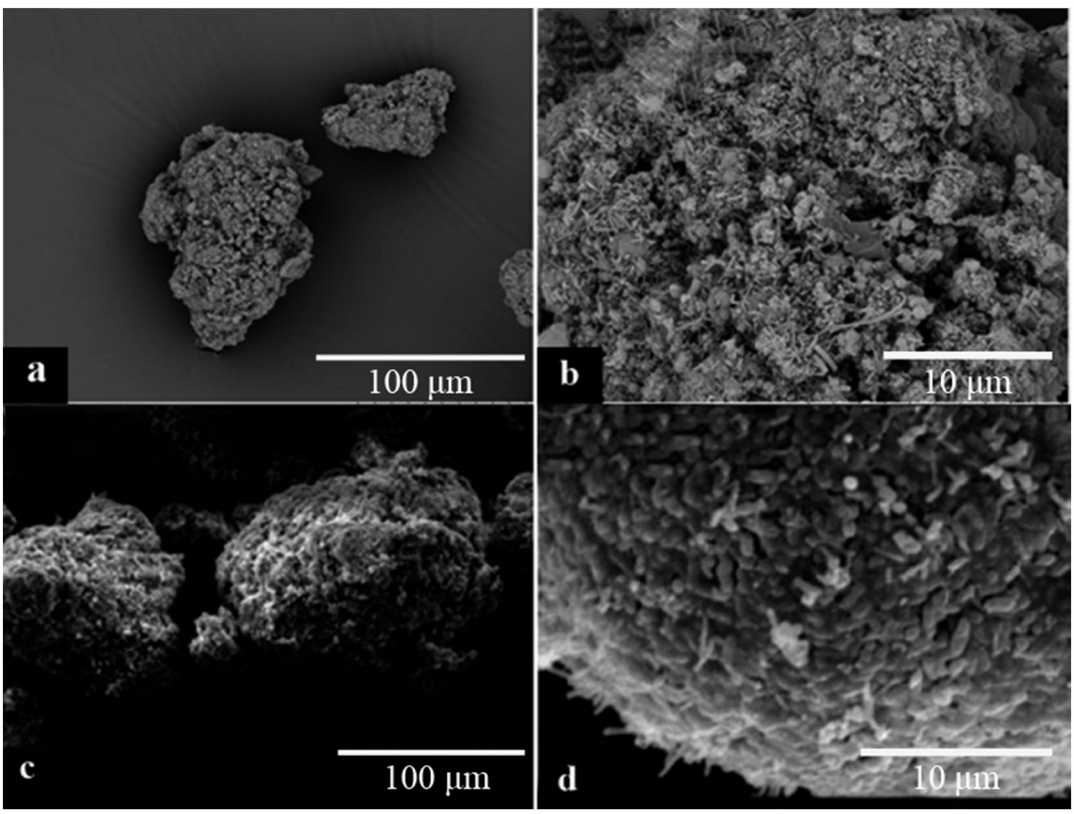

Fig. 2 SEM images of the granular sludge: a and b: directly reducing the aeration (500× and 4000×); c and d: gradient reduction of the aeration (600× and 3000×).

3.2. Variation of the microbial community under different aeration strategies

Comparing the microbial communities of AGS samples before regulation (sample A) and the disintegrated sludge sample after directly reducing the aeration intensity during the aerobic starvation stage (sample B) showed that the Shannon index decreased from 4.76 in sample A to 3.92 in sample B. The data indicated that mass of microbes were washed out of the reactor within the irreversibly disintegrating sludge, resulting in a significant reduction ($p < 0.01$) in species abundance. However, community diversity was recovered in the aerobic granular sludge sample collected after gradient reduction of the aeration intensity during the aerobic starvation stage (sample C). Compared with sample B (the Shannon index was 3.92), the Shannon index of the granular sludge reached a peak value of 5.79, and results showed that the aerobic granular sludge formed after the gradient reduction of aeration achieved a higher richness and a stronger impact resistance performance.

Microbial diversity analysis of aerobic granules before directly reducing the aeration and after directly reducing the aeration is shown in Fig. 3. The graph shows that the dominant microbe at the phylum level in aerobic granules before directly reducing the aeration was *Proteobacteria* (45% of the total bacteria), followed by *Bacteroidetes* (31% of the total bacteria). By contrast, the proportion of *Bacteroidetes* in the aerobic granules after directly reducing the aeration rapidly rose to 63%, whereas the proportion of *Proteobacteria* decreased to 23% (Fig. 3b). Thereinto, the species distribution at the genus level demonstrated that *Leadbetterella* spp. appeared as the dominant microbe (45% of the total bacteria) in the aerobic granules after directly reducing the aeration. Regulation of the aeration intensity by direct reduction led to a rapid drop in the reactor DO during the aerobic starvation stage. The PN content was significantly reduced ($p < 0.01$), and the original dominant bacterial community change was caused by the lower hydraulic shear force.[7] Cell autoinducers were stimulated, thereby promoting the growth of *Leadbetterella* spp. belonging to *Sphingobacteria*, which can secrete EPS.[37] However, the enrichment of *Leadbetterella* spp. might not provide the reactor with a stable EPS composition, and the reactor eventually failed to recover a stable microbial population. As a result, the aerobic granular sludge was irreversibly disintegrated and the mass of the sludge was washed out of the reactor.

By contrast, the succession of the microbial community following the gradient reduction in aeration showed obvious shifts in the microbial community (Fig. 4). *Proteobacteria* was highly enriched (74% of the total bacteria) in the aerobic granules after the reinforcement. The proportion of *Zoogloea* spp. in the total bacteria increased to 45%. Additionally, *Microlunatus* spp., *Thauera* spp., and *Rhodobacter* spp. were found at proportions of 17%, 10% and 2%, respectively. All of these bacteria are commonly found in stable aerobic granular sludge.[38,39] Our experiments confirmed that the specific enrichment of functional bacteria occurred during the gradient aeration reduction process. Thereinto, *Zoogloea* spp. was responsible for the production of EPS, and *Thauera* spp. and *Rhodobacter* spp. were responsible for nitrogen removal. All the bacteria mentioned above contributed to the efficient and stable operation of the aerobic granular sludge.

FISH was performed to identify the dominant bacteria and analyze the aerobic granular sludge following the direct reduction of the aeration intensity and the gradient reduction of the aeration intensity from the perspective of the microbial community. Both β-*Proteobacteria* and the main EPS-secreting bacteria (*Zoogloea* spp.) were identified in the aerobic granular sludge after the gradient reduction of aeration. As shown in Fig. 5, β-*Proteobacteria* was found in the whole granule, whereas *Zoogloea* spp. were primarily located in the outer layer of the granules in the same approximate location

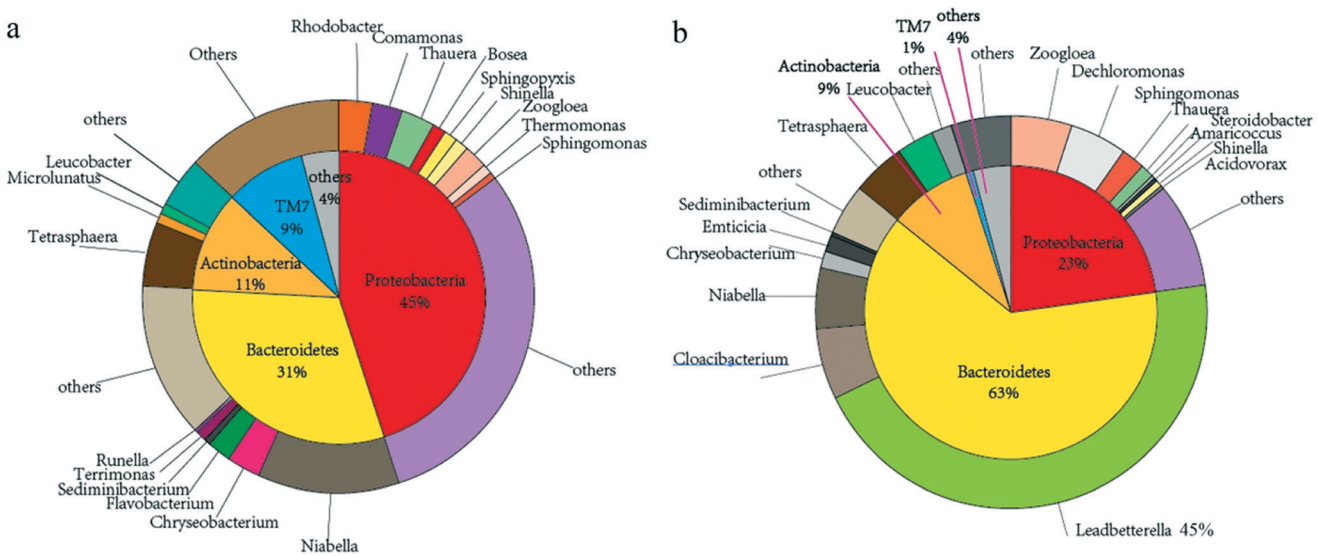

Fig. 3 Microbial distribution of aerobic granules before directly reducing the aeration (a) and after directly reducing the aeration (b).

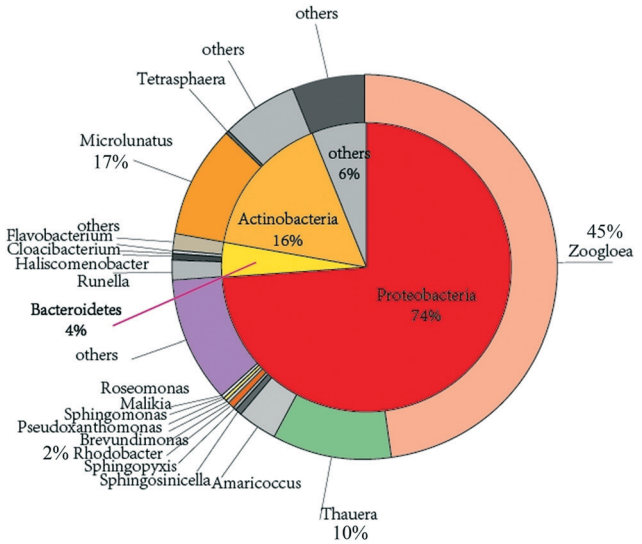

Fig. 4 Microbial distribution in stable aerobic granules after the gradient reduction of aeration.

as the EPS as described by recent studies.[3,40] This result confirmed that *Zoogloea* spp. are important for the structure stabilization of aerobic granular sludge.

3.3. Functional analysis of intracellular proteins in the aerobic granular sludge

As shown in Fig. S5,† the EPS content and PN/PS decreased significantly ($p < 0.01$) under the direct decrease of aeration intensity while the EPS content and PN/PS increased during operation under the gradient decrease of aeration intensity. It was speculated that the gradient reduction of aeration intensity strategy increased the PES content, especially the PN content to enhance the aerobic granule stability.

To examine the expression and functions of intracellular proteins during granulation, a proteomic analysis was performed using 2-DE/MS on floc sludge and stable AGS samples (Fig. 6). More than 30 spots were selected after comparing the gel images for the proteins from the floc sludge and stable AGS. The results showed that the proteins with increased expression levels within the aerobic granular sludge were mostly acidic proteins. Four types of intracellular proteins were identified as key enzymes involved in EPS secretion: periplasmic peptidyl-prolyl isomerase (PPIase), pyrophosphate phosphohydrolase, outer membrane protein F (OprF) and outer membrane protein (OMP).[41] Among them, PPIase is a multifunctional protein that has a highly conserved structure and has been widely identified in prokaryotic and eukaryotic organisms. It can accelerate cell protein secretion and reconstruction by catalyzing proline's *cis–trans* isomerization. In its role as a type of molecular chaperone, PPIase possesses several other functions, such as introducing new peptide folds, transforming proteins into active proteins, and transmembrane positioning. A previous study showed that it acts as a protein secretion catalyst and helps with the secretion of extracellular PN to strengthen agglomeration and the physical strength of granules.[42] Therefore, to some extent, the existence of PPIase speeds up the granulation process and makes the aerobic granule sludge different from the flocs. Pyrophosphate phosphohydrolase is a common enzyme in cells and has been demonstrated to be abundantly enriched in stable-state granules. It promotes cell proliferation and the secretion of extracellular materials; moreover, pyrophosphate phosphohydrolase helps hydrolyze high levels of ADP-RNA in the cell, which inactivate protein functions.[43] Previous studies have speculated that the existence of pyrophosphate phosphohydrolase in granules helps maintain the structural stability and efficient reactor operation by improving the stability of the extracellular proteins. OprF is the main specific channel protein and is fixed in the inner surface of phospholipid acetamide and the surface of lipopolysaccharide on chitosan. It plays a role in maintaining the cell morphology, bacterial growth in low osmotic pressure environments and is the main oligomer that participates in transmembrane transport.

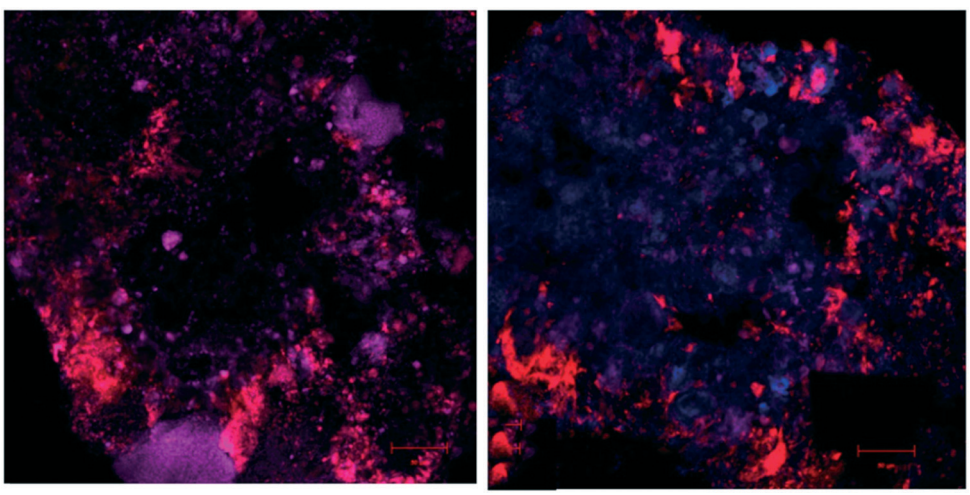

Fig. 5 FISH of a stable aerobic granule under the condition of gradient reduction of aeration (red: *Zoogloea* spp.; purple: β-*Proteobacteria*; blue: DAPI).

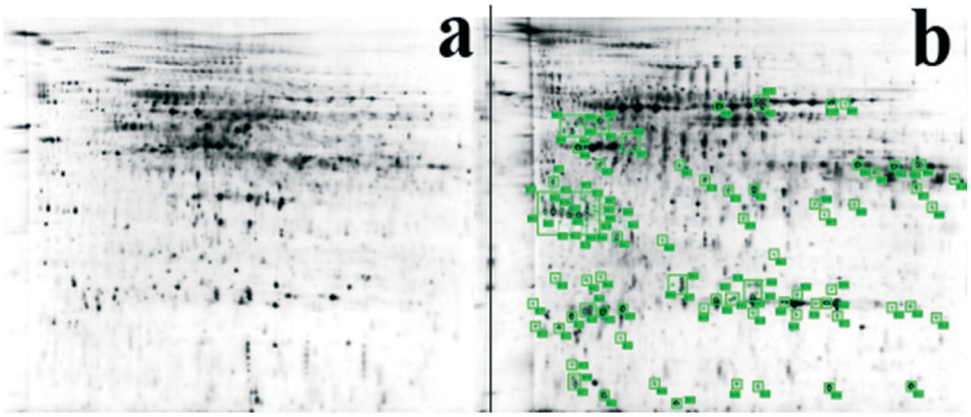

Fig. 6 2-DE analysis of intracellular proteins in flocculent sludge (a) and granular sludge (b).

The above studies have confirmed that it is related to adherence between bacteria.[44,45] Thus, we can speculate that OprF increases the reunion of aerobic granular sludge and plays a role in the process of nutrient transport, metabolic waste discharge, and oxygen mass transfer. OMP is embedded in the outer membrane layer of Gram-negative bacteria. It plays an important role in maintaining the microbial membrane structure and morphology, regulating metabolism, and ensuring material transport and nutrient intake. Previous studies have confirmed that it can promote polymer transfer and transmembrane transport of extracellular materials, thereby accelerating the granulation process. In contrast to the sludge floc, the abundance of intracellular proteins that increase the secretion of EPS may be related to the main EPS secretion bacteria *Zoogloea* spp. However, the relationship between them remains unclear at present, and further research needs to be conducted.

4. Conclusions

Using an operation strategy with reduced aeration during the famine period led to a significant reduction ($p < 0.01$) of energy consumption as the air flow rate decreased by 60%, but much of the sludge was washed out and the granules disintegrated irreversibly with the decrease of the PN/PS ratio when the direct reduction of aeration was applied. When the process was switched to a gradient reduction mode in aeration, the richness and diversity of the aerobic sludge were retained and the structure of the granular sludge remained stable. The functional microorganisms such as *Zoogloea* spp. for EPS secretion were enriched in the inner part of the granular sludge, and four types of proteins, namely PPIase, pyrophosphate phosphohydrolase, OprF and OMP, were identified, which are the key enzymes involved in EPS secretion and microbial aggregation.

Conflicts of interest

There are no conflicts to declare.

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