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RESEARCH ARTICLE

Response of aerobic granular sludge to organic loading rate under micro-electric stimulation environment

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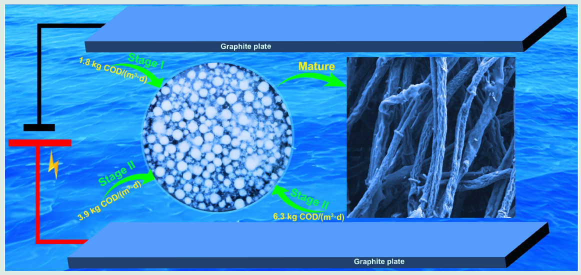
HIGHLIGHTS

- The role of OLR on AGS under a complicated environment is rarely established.
- The dynamic OLR affected the behaviors of AGS and reactor performance.
- The variation of EPS content and constitutions was revealed.
- The main function microbes under the OLR-varied condition were identified.



ABSTRACT: Aerobic granular sludge (AGS) is a neoteric wastewater treatment technology. The organic loading rate (OLR) exhibits a critical effect on the AGS formation process. The special role of OLR on AGS is rarely established, especially in a complicated environment. This work explored the influence of OLR on the AGS system under a micro-electric stimulation environment. The dynamic OLR affected the behaviors of AGS and reactor performance. AGS cultured under a dynamic OLR environment showed a more compact structure and the AGS system displayed an excellent capacity in removing pollutants. The stable texture of AGS is related to the extracellular polymeric substance (EPS). The main constitutions of EPS include tryptophan protein, tyrosine protein, humic acid-like substance, and fulvic acid-like substance. The OLR-varied environment may provide a selective condition, impacting the microbial population. The prevail bacteria were *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (21.98%), *Lactococcus* (23.93%), and *Chryseobacterium* (5.58%) in OLR-varied AGS system. The evolution of the microbial population induced the change in bacterial community functions, such as carbohydrate metabolism, replication and repair, and membrane transport functions. This work provides valuable insights into the OLR on AGS processes, helping to the stability of AGS-based systems.

KEYWORDS: Biological treatment technology, Aerobic granular sludge, Organic loading rate, Microbial community structure, Metabolism function

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1 Introduction

The extensive usage of chemicals and rapid development of industrial areas have caused many environmental risks, for example, the increase of contaminants (e.g., antibiotics and perfluorinated compounds) and the prevalence of viral surrogates in water bodies (Barrios-Hernández et al., 2021; Liu et al., 2023a; Wang et al., 2024). These environmental risks have challenged the capacity of traditional wastewater treatment processes. The aerobic granular sludge (AGS), featuring high biomass, more pyknotic texture, large size, and excellent settleability compared with floc sludge (Kedves et al., 2019), is regarded as an innovative sewage treatment technology (He et al., 2023). The special structure of AGS impedes the oxygen permeation and penetration of substances from water to the core of AGS, thereby establishing a concentration gradient. From dissolved oxygen concentration, the external aerobic, intermediate anoxic, as well as internal anaerobic microzones are distributed in AGS (Li et al., 2024). Consequently, compared with activated sludge, AGS exhibits excellent performance for simultaneously removing organics and nutrients (i.e., NH_4^+-N and TP) (Yuan et al., 2020; Chen et al., 2023). Yang et al. (2023) developed a two-stage AGS system to eliminate phosphorus and nitrogen pollution from municipal wastewater and found that the system was highly efficient for the elimination of phosphorus (91%) and nitrogen (81%).

In addition, granular sludge systems cover less surface area and lower capital costs compared with the flocculent activated sludge biosystem (He et al., 2020a; Mills et al., 2024). Therefore, the AGS-based process is very promising in wastewater treatment and has garnered wide attention from industrial and academic areas. Currently, there are four mechanisms for explaining the formation of AGS, including the microbial auto-coagulation mechanism, selective pressure-driven mechanism, extracellular polymer mechanism, and quorum sensing mechanism (Lv et al., 2014; Nancharaiah and Reddy, 2018; Zhang et al., 2019a). However, some bottlenecks still limit the development of the AGS process toward practical application, e.g., the interminable granulation stage and the instability of the AGS system (Cao et al., 2024). In recent years, many innovative strategies have been utilized to shorten the sludge granulation period, e.g., micro-electric fields and magnetic fields (Zhu et al., 2021), introducing algae (Liu et al., 2023b; 2024a) or exogenous substances (e.g., signal molecules, inert materials, and metal ions) (Pishgar et al., 2020; Shuai et al., 2021; Nancharaiah et al., 2023). In our previous work (Li et al., 2024), we applied micro-electric stimulation to enhance the formation of AGS and found that introducing micro-electric stimulation facilitated the start-up stage of the AGS system (25 d).

The organic loading rate (OLR) is vital during the aerobic sludge granulation process (Liu et al., 2023c; 2024b). This is because OLR affects the physical characteristics of the sludge granulates (e.g., shape, granulate size, and settling velocity) and wastewater treatment efficiency. For instance, AGS formed at a low OLR environment often display a smaller and denser structure, however, an extended granulation period is required (Peyong et al., 2012). The sludge granulates shape and grain size impact the diversity and abundance of functional microorganisms. The wastewater treatment efficiency of the AGS process differs from the composition of wastewater (He et al., 2020b). Urban domestic sewage typically exhibits a low OLR, whereas industrial sewage has a higher OLR (> 3.5 kg COD/(m^3·d)). These variations of OLR have challenged the stability of the AGS-based biological processes. The efficiency of the AGS-based processes still depends on the organics load of the influent. Therefore, AGS cultured at a constant OLR strategy lacks the alleviating ability for the wastewater with various OLR.

The special role of OLR on AGS under a complicated environment (e.g., electric stimulation) is rarely established. Based on our reported study (Li et al., 2024), the purpose of the present work is to investigate the impact of dynamic OLR on AGS system under a complicated environment (i.e., micro-electric field environment). (i) The dynamic behaviors of granular sludge were revealed at different OLR. (ii) The performance of the AGS system at various OLR stresses was investigated. (iii) The effects of OLR stress on AGS stability and the changes in EPS concentration as well as composition were carefully examined. (iv) The dynamic evolution of microbial community structure over various OLR was analyzed. The present work provides valuable insight into the OLR stress on AGS processes and a feasible strategy to reinforce the stability of AGS under a complicated environment.

2 Materials and methods

2.1 Operation of AGS bioreactors

Two cylindrical reactors (R_1 and R_2) with a diameter of 80 mm, height of 900 mm, and volume of 3.2 L were employed as AGS bioreactors (Fig. S1). The reactors were equipped with a pair of graphite electrode plates, which had a plate area of 800 mm^2 and connected with a GPD-4303S direct-current power (3.0 V). The electric field was used to promote the process of sludge granulation (Guo et al., 2019). R_2 was carried out at different OLR conditions to investigate the influence of OLR on AGS. The OLR was set as 1.8 kg COD/(m^3·d) (1st–12th day), 3.9 kg COD/m^3·d (13th–24th day), and 6.3 kg COD/(m^3·d) (after 25th day). The corresponding COD values of the influent were 600, 1100, and 2300 mg/L (Table S1). R_1 bioreactor, as a control reactor, was carried out at a constant OLR condition. Aerobic flocculent sludge was sampled from the aeration tank in the Yanchangbu wastewater treatment plant in Lanzhou, China, and then inoculated in the bioreactors to cultivate aerobic sludge granulates. For a reactor, a complete operating cycle (144 min) included water injection, aeration reaction, sedimentation, and drainage, which were respectively set as 8, 130, 2, and 4 min. The influent volume was set as 1.6 L during an operating cycle. R_1 and R_2 were carried out under the different OLR conditions. The feeding water was simulated with $C_6H_{12}O_6$, CH_3COONa, NH_4Cl, KH_2PO_4, K_2HPO_4, $CaCl_2$, $MgSO_4$, $FeCl_3$.

2.2 Analytic methods

The residual chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), and total phosphorus (TP) in wastewater were analyzed according to the reported methods (Liu et al., 2023d). The integrity coefficient (IC) of AGS and specific oxygen utilizing rate (SOUR) were determined according to the reports (Zhang et al., 2020; de Sousa Rollemberg et al., 2018). The microstructure characteristics of AGS were investigated by a Sigma 300 scanning electron microscopy (SEM, ZEISS, Germany). The wet screening method was employed to analyze the distribution of the size of granular sludge (Ren et al., 2018).

The means of thermal extraction (Han et al., 2021) was employed to extract extracellular polymer substance (EPS) of granular sludge samples, which included the extraction of tightly bound EPS (TB-EPS), loosely bound EPS (LB-EPS), and soluble EPS (S-EPS) in different granular layers. The polysaccharides (PS) concentration and protein (PN) content were tested according to the anthrone-sulfate method (Zhang et al., 2020) and the Lowry method (Frølund et al., 1995), respectively. Besides, the fluorescence spectra of TB-EPS, LB-EPS, and S-EPS were further analyzed using a Hitachi F-7000 fluorescence spectrophotometer. The emission wavelength (λ_{em}) scanned from 220 to 550 nm with a step size of 5 nm during a scanning process. The excitation wavelength (λ_{ex}) was increased from 220 to 450 nm with an increasing interval of 10 nm. The excitation/emission slit width was fixed at 5 nm.

2.3 Analysis of 16S rDNA

16S rDNA was analyzed to explore the microorganism's community composition of granular sludge. The mature sludge granulates were sampled from two bioreactors of R_1 and R_2. The bacterial DNA was extracted, among which the V3–V4 region was further amplified through the specific primers of 341F and 806R. Subsequently, the sequencing procedure was carried out (Shanghai Omicsmart Company, China). The obtained OTU data were studied against the SILVA database (version 138.1) with a confidence threshold of 0.8–1.0. The PICRUSt2 program was used to predict functional information of the bacterial community.

3 Results and discussion

3.1 Behaviors of AGS at different OLR

3.1.1 Granulate size distribution

Figure 1 shows the size variation of granular sludge in different stages. At stage I (12 d) (Fig. 1(a)), the particle size was mainly centered in 1–3 mm in the R_1, accounting for 56.4%. In the experimental reactor (R_2),

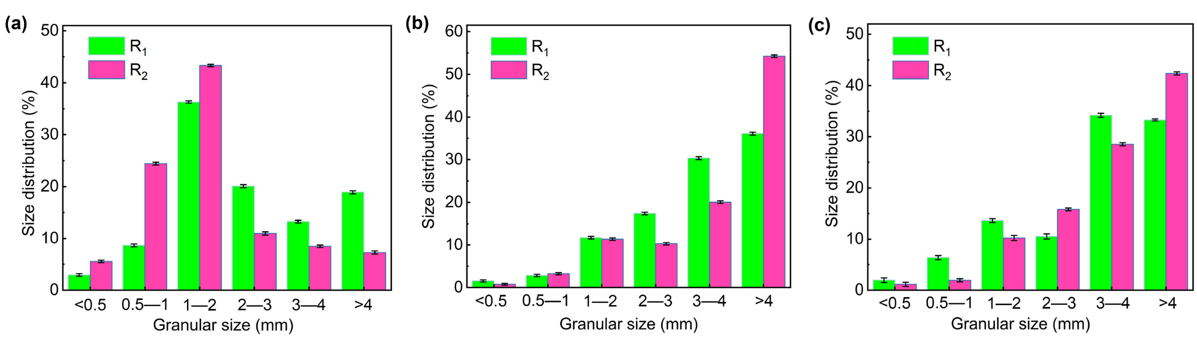

Fig. 1 Distribution of AGS size at (a) stage I, (b) stage II, and (c) stage III.

67.7% of the AGS was concentrated in the range of 0.5–2 mm. This difference is probably caused by the difference in OLR (3.9 kg COD/(m^3·d) for R_1 *vs* 1.8 kg COD/(m^3·d) for R_2). A lower OLR is more advantageous for the formation of the small size granular (Ni et al., 2009). At stage II (24 d) (Fig. 1(b)), the size of granular sludge increased in both reactors. It is also found that AGS in R_2 exhibited a larger size compared with that of R_1. AGS with a partial size of 3–4 mm accounted for 30.4% in R_1 and 20.6% in R_2. 54.3% of AGS has a larger size (> 4 mm) in R_2, which is higher than that of the control (36.7% in R_1). With the OLR elevated to 6.3 kg COD/(m^3·d) (stage III, 70 d) (Fig. 1(c)), the granular sludge was gradually mature. The proportion of AGS with a partial size greater than 3 mm in the R_2 bioreactor (74.3%) was higher than that in the R_1 bioreactor (66.4%), demonstrating that the particle size of AGS in R_2 was larger compared with R_1.

3.1.2 Morphology of AGS

The morphology of AGS was observed for further analyzing the response of OLR. After cultivation for 74 d, the morphology of AGS cultivated at variational OLR environments (Figs. 2(a)–2(c)) was different compared with the control (Figs. 2(d)–2(e)). In the variational OLR reactor, AGS showed a pale yellow and dense granule with an average size of *ca*. 2.9 mm (Fig. 2(a)). However, the white granular sludge can be observed in the constant OLR reactor (Fig. 2(d)), displaying a loose texture. The size of AGS in R_1 (2.2 mm) was smaller than that in R_2 (2.9 mm), which is consistent with the results of size distribution. These results indicate the variational OLR strategy is favorable to forming dense granular sludge, which is beneficial for the steadiness of the AGS system during the contaminate removal process.

Further, the microstructure character of AGS was analyzed by SEM. At the low magnification, there was no observed difference from AGS in R_1 (Fig. 2(b)) and R_2 **(**Fig. 2(e)**)**. A large number of filamentous bacteria entwined with each other, forming the major structure of AGS. This intertwined state of filamentous bacteria endowed the AGS with ample void structure, which is conducive to transferring oxygen and nutrients and fostering microbes (Lyu et al., 2021). The filamentous bacteria are commonly regarded as a substrate for microbes growing (Chen et al., 2022). There were obvious differences on the AGS surface at the high magnification. (i) Compared with the control (R_2, Fig. 2(f)), a greater number of microbes can be observed in the R_1 reactor (Fig. 2(c)) including coryneform bacteria (*ca*. 2.7 μm in length) and cocci with a diameter *ca*. 0.8 μm. (ii) The coryneform bacteria dominated the AGS system in R_1. (iii) The diameter of filamentous bacteria in R_1 (2.4 μm) was larger than that in R_2 (1.8 μm), which is benefit for the attachment and reproduction of bacteria. These results indicate that OLR affect the appearance, morphology, and bacterial

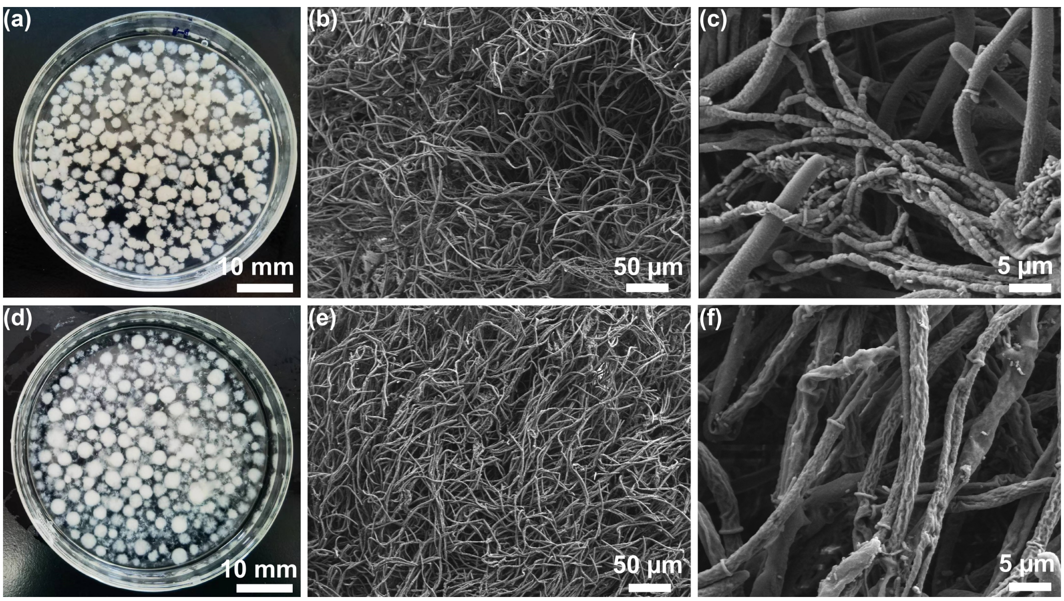

Fig. 2 Photographs and SEM images of mature AGS cultured under (a, b, c) the constant OLR condition in R_1 and (d, e, f) the OLR-varied condition in R_2.

population of granular sludge.

3.1.3 Characteristics of biomass and microbial activity

The dynamic variations of the AGS system during granular sludge formation process were characterized by the indices of MLSS concentration, MLVSS/MLSS ratio, SV_{30}, and SOUR (Fig. 3). The sampled inoculation sludge had a MLSS concentration of 3350 mg/L with MLVSS/MLSS ratio of 0.41 and SOUR of 35.67 mg O_2/(g MLVSS·h). MLSS and MLVSS decreased significantly in both reactors at the beginning of operation. This may be because of the poor sedimentation property of the sludge, which led to biomass loss with the discharging water. With the adaptation of the sludge to the culture conditions, the biomass gradually accumulated, and the MLSS of the R_1 reactor maintained at about 2300 mg/L in the mature stage (Fig. 3(a)). However, in the R_2 reactor, MLSS showed a concentration of 2950 mg/L at the stage I (0–12 d) (Fig. 3(b)). On the 13th day, MLSS concentration decreased to 2100 mg/L with the OLR increasing from 1.8 to 3.9 kg COD/m^3·d. This may be because the increase of OLR impacted the settling property, leading to the decrease in the MLSS concentration. This can be demonstrated by the increase of SV_{30}. In stage III (25th–75th day), OLR was further elevated to 6.3 kg COD/(m^3·d). MLSS concentration was decreased at the beginning of this stage, which was consistence with the beginning of stage II. After that, MLSS gradually increased and eventually stabilized at 2600 mg/L, which was higher than that in R_1. These analyses demonstrate the significant changes in sludge properties under the various OLR conditions.

During the sludge granulation process, the MLVSS/MLSS ratio displayed a significant increase, implying the enhancement of microbial activity, which is consistent with the previous report (Cheng et al., 2023). The microbial activity was further evaluated by SOUR (Figs. 3(c) and 3(d)). During start-up, the SOUR in both reactors was approximately 50 mg O_2/(g MLVSS·h). As the culture time prolonged, the SOUR gradually increased and finally reached a stable state. This is accordant with the variation in the MLVSS/MLSS ratio.

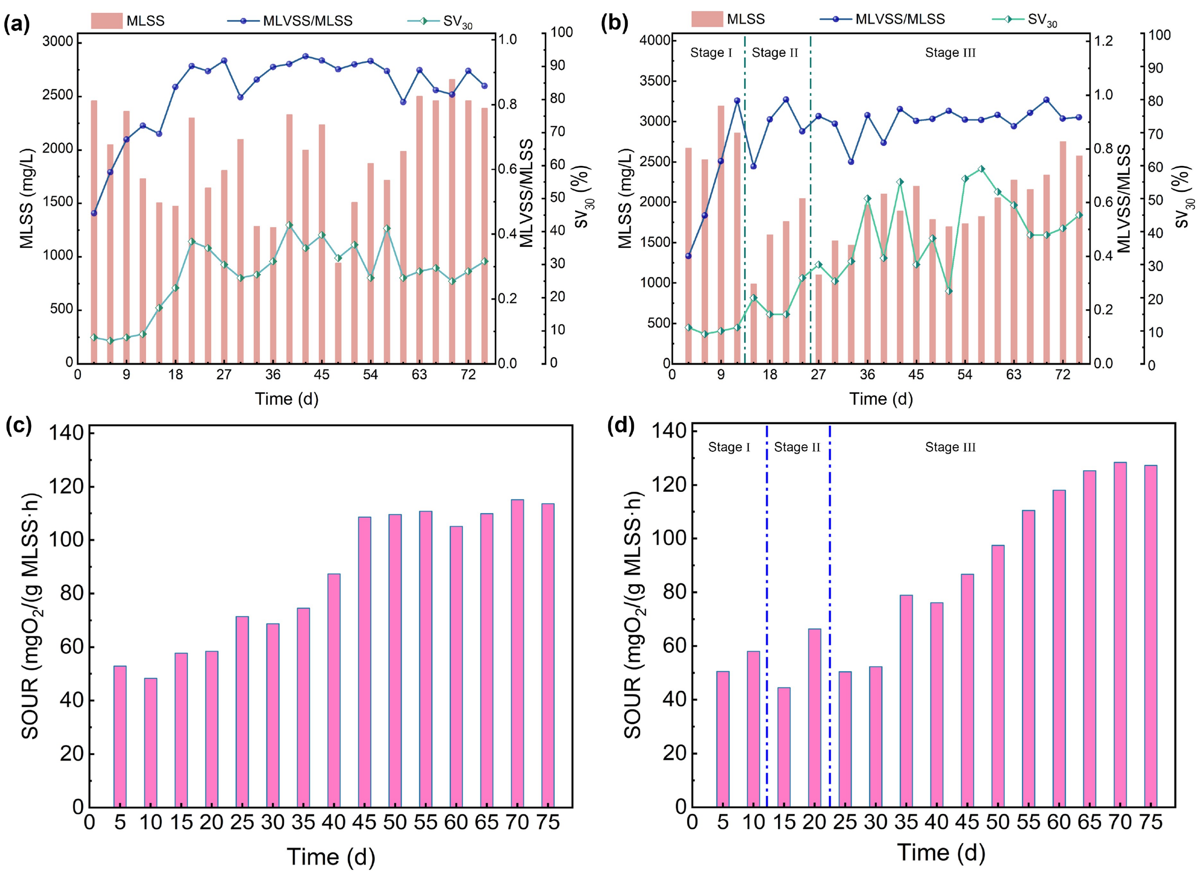

Fig. 3 Changes of MLSS, MLVSS/MLSS, SV_{30}, and SOUR during the sludge granulation process in (a, c) R_1 and (b, d) R_2.

However, there was a decline in SOUR on the 15th day and 25th day in R_2 (Fig. 3(d)), which may be induced by the change in OLR. In the stable phase, SOUR was 110 mg O_2/(g MLVSS·h) in R_1 (Fig. 3(c)) and 123 mg O_2/(g MLVSS·h) in R_2 (Fig. 3(d)). The higher SOUR value in R_2 indicates that the OLR-varied strategy is conducive to enrichment of AGS biomass and improvement of microbial activity.

3.2 Performance of AGS at various OLR stress

The removal performance of nutrients by AGS bioreactor under various OLR stresses was investigated by monitoring COD, TP, and NH_4^+-N concentrations (Fig. 4). As shown in Fig. 4(a), there was a fluctuation in COD removal in stage I. This may be because of the loss of microorganisms with the effluent. In stage II, with the adaption of sludge to the increased OLR, COD removal showed a significant improvement with a removal efficiency of 90%. At the beginning of stage III, the high OLR value of 6.3 kg COD/(m^3·d) impacted the COD removal performance of the AGS system. However, with the formation of sludge granulate and the adaption of AGS to the high OLR ambiance, COD removal showed an increased trend and remained at about 90%, which was higher than that under a constant OLR condition (Fig. S2).

The removal of TP under the constant OLR (R_1 reactor) fluctuates in the range of 60%–90% (Fig. S3). This is because the AGS system was not stable due to the disintegration and reformation of AGS (Hamza et al., 2018), accompanying the change in the activity of the phosphor-accumulating bacteria. For the OLR various conditions (R_2 reactor, Fig. 4(b)), the removal of TP showed a low level at the initial phase. As OLR increased, the removal of TP exhibited a relaxed increase with an average removal rate of 76.2%, which is higher than that of the control (68.4%). This is probably because OLR influenced the microstructure (aerobic, anoxic, and anaerobic zones) of AGS, which is crucial for TP removal by phosphorus-accumulating organisms. Besides, the high OLR provides a sufficient

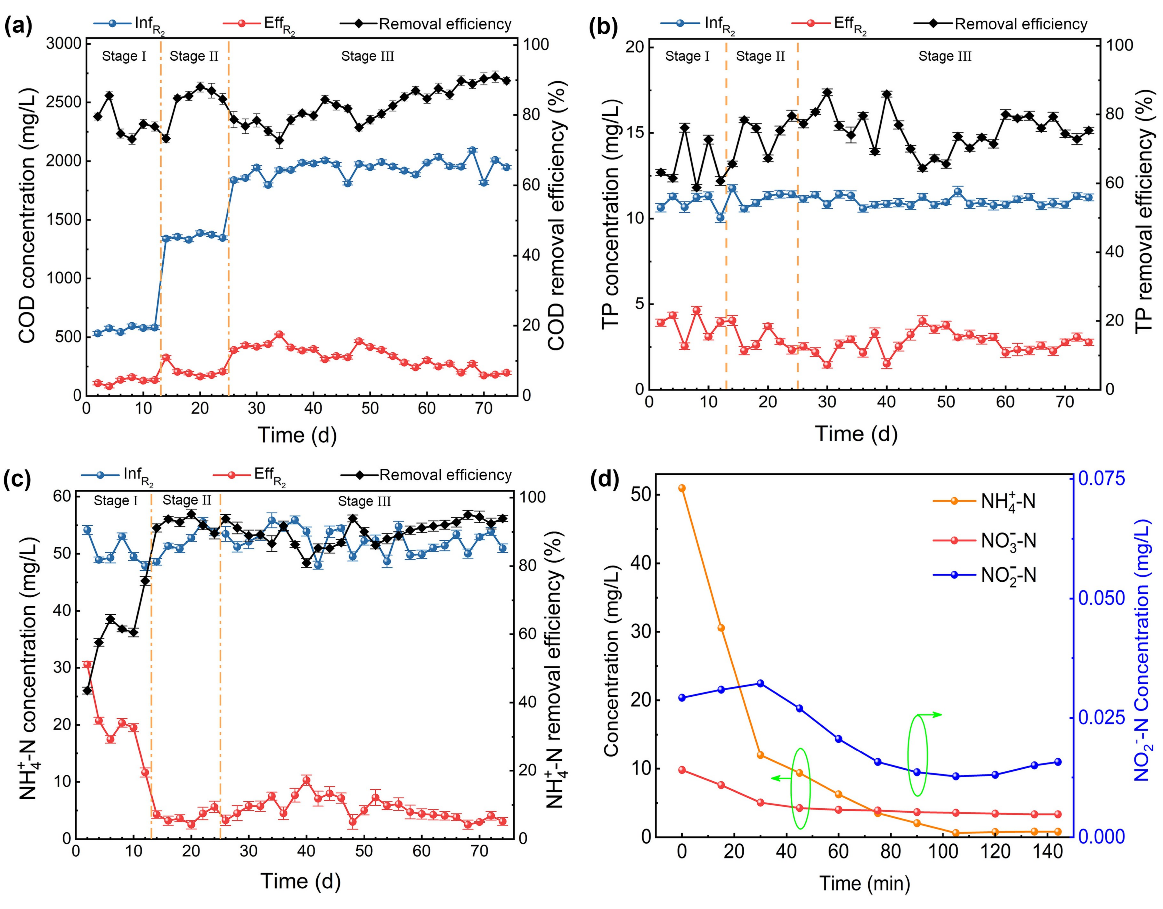

Fig. 4 Removal performance of (a) COD, (b) NH_4^+-N, (c) TP by AGS system under the OLR-varied condition (R_2 reactor), (d) The transformation of nitrogen during the representative cycle.

carbon source for phosphor-accumulating bacteria to uptake phosphorus at aerobic ambient.

As the granular sludge formed, the abatement of NH_4^+-N gradually increased. The residual of NH_4^+-N concentration in the effluent remained below 4 mg/L at the stable state (Fig. 4(c)). From the NH_4^+-N residual in the effluent, the change of OLR has little effect on NH_4^+-N removal. In stage III, although the OLR was 3.5 folds compared with stage I and 1.6 folds compared with control, the elimination of NH_4^+-N was still higher than 90%. In this stage, the mature AGS was formed. Compared with the small-sized granular sludge of stages I and II, the mature AGS has a large granulate size, which creates a more favorable anoxic environment for the propagation of nitrifying and denitrifying microorganisms. Therefore, the removal of NH_4^+-N eventually remained at 98%, which is higher than that under the constant OLR condition (R_1, 92%).

Further, time profiles of NH_4^+-N, NO_3^--N, and NO_2^--N were determined during a typical period on the 75th day to analyze the nitrogen conversion. As displayed in Fig. 4(d), the NH_4^+-N concentration rapidly declined within 30 min and then gradually decreased to 0.81 mg/L, which was less than R_1 (1.19 mg/L, Fig. S5). NO_2^--N concentration gradually increased in the first 30 min, which may be related to the transformation of NH_4^+-N. After that, NO_2^--N content gradually declined and the residual in the effluent was 0.02 mg/L, while that was 0.07 mg/L in R_1 (Fig. S5). Overall, NO_3^--N content displayed a decreasing tendency with a residual concentration of 3.3 mg/L, while a higher residual concentration was observed in R_1 (3.8 mg/L, Fig. S5). These results confirm that the OLR-varied AGS system displayed a high capacity for removing NH_4^+-N.

3.3 Analysis of stability and EPS

3.3.1 Stability of AGS

The integrity coefficient was adopted to evaluate the structural stability of AGS. A lower integrity coefficient signifies a more robust structure of AGS, resisting mechanical shock. As the culturing period prolonged, the integrity coefficient of AGS gradually decreased (Fig. 5(a)**)**, suggesting the mature sludge granulates have a more stable structure. AGS in R_2 displayed a lower integrity coefficient than that in R_1 during the whole period. This result indicates the stability of the AGS structure can be enhanced by the OLR-varied strategy. It is also found that AGS formed under the constant OLR easily disintegrated, which is not conducive to the durability of the AGS reactor. The reason for this phenomenon is probably because OLR influenced the aggregation of sludge cells, which is a major factor affecting the granular process.

3.3.2 Concentration of EPS

EPS is critical for facilitating the formation of AGS and maintaining granulate stability (Wu et al., 2024). Figure 5(b) shows the changes in EPS concentration in the AGS system. Under the OLR-varied condition, the secreted EPS content was gradually increased to 324.8 mg/g SS (on the 9th day), which exceeded the control (R_1, 214.5 mg/g SS). When the OLR value elevated from 1.8 to 3.9 kg COD/(m^3·d), EPS content declined to the amount of the control. When the OLR value was further elevated to 6.3 kg COD/(m^3·d), EPS content further declined to 55.4 kg COD/(m^3·d) (27th day). This may be because the high OLR impacted or even suppressed the growth of microorganisms. Therefore, a lower EPS content was observed compared with R_1 (69.6 mg/g SS, 27th day). In the high OLR environment, microorganisms secreted more EPS to help themselves survive in the harsh environment. This may be the explanation for the increase in EPS content on the 45th day. After that, with the adaption of microorganisms to this OLR environment, EPS content was consistent with the control.

Figure 5(c) shows the effect of OLR on the PN/PS ratio. The ratio of PN/PS was below 1, implying that PN content was less than that of PS during the whole process. As we all know, PS is viscous and hydrophilic. Therefore, PS is vital for promoting microorganisms' cell aggregation and AGS formation. At the OLR of 1.8 kg COD/(m^3·d) condition, the PN/PS ratio was increased and then decreased. This is because of the increase in PS concentration. With the elevation of OLR value from 1.8 to 3.9 kg COD/(m^3·d), the PN/PS ratio maintained a relatively stable state and then increased. The increase of PN concentration and PN/PS ratio on the 24th day indicates that the PN is vital for forming sludge granulation. The boost of OLR from 3.9 to 6.3 kg COD/(m^3·d) led to an obvious decrease in PN/PS ratio. This change reduced cell hydrophobicity, impacted the stability of AGS, and decreased the activity of microorganisms. As sludge granulation progressed, PN concentration and PN/PS ratio gradually increased and ultimately maintained a stable state. This is because PN plays a critical role in regulating the hydrophobicity. Therefore, sludge granulates became dense and mature enough to resist OLR stress, enhancing the structural stability of aerobic sludge granulates as well as the AGS system. These results are consistence with the results of stability of

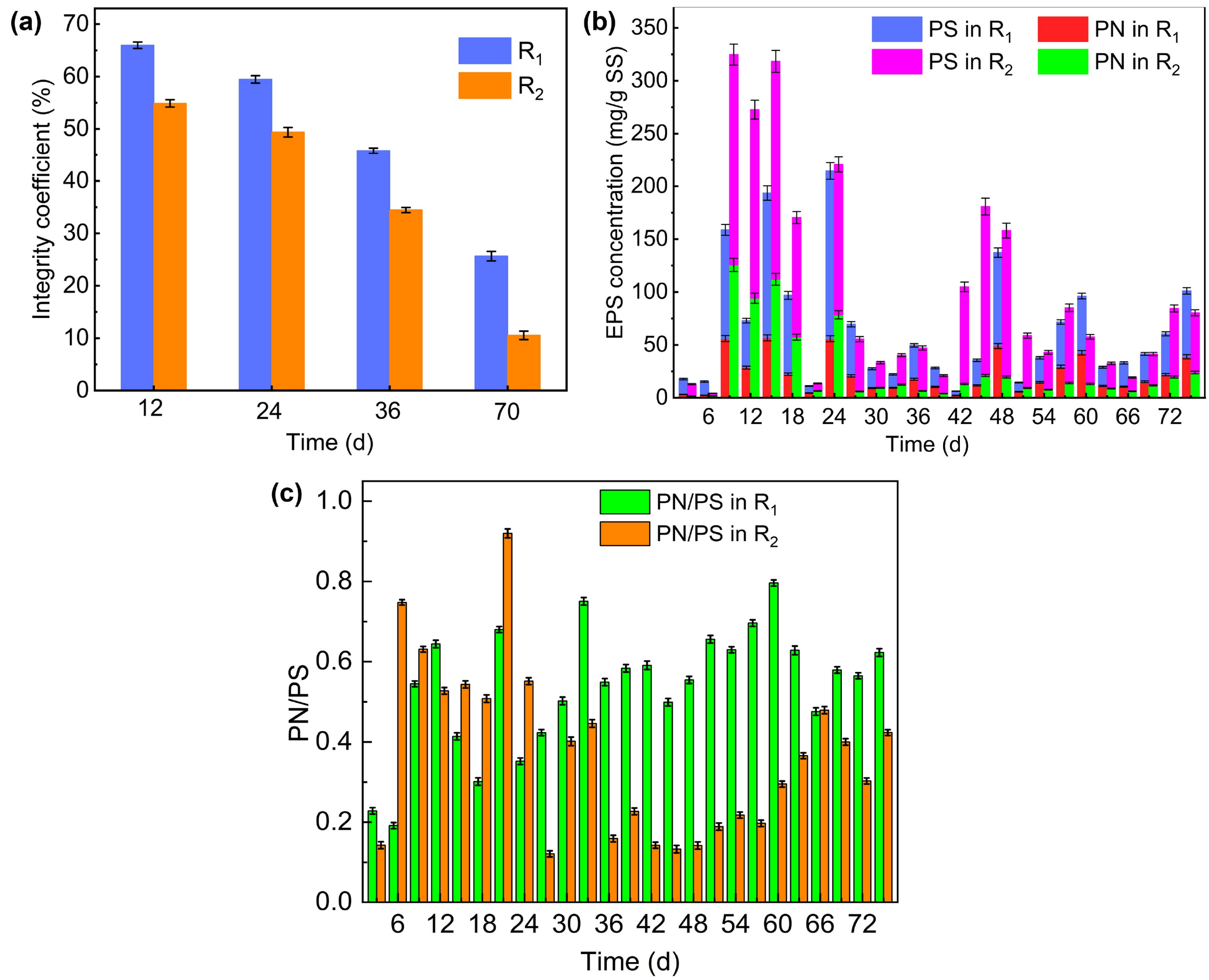

Fig. 5 (a) Integrity coefficient of granular sludge, (b) EPS content and (c) PN/PS ratio under different operating conditions.

AGS.

3.3.3 Composition of EPS

The 3D-EMM was utilized to further analyze the constituents of EPS in mature AGS (70th day). Table 1 presents detailed fluorescence information on EPS, including the peak location and the corresponding composition. There were four main peaks observed in the fluorescence spectra. The peak A ($\lambda_{ex/em}$ = 200–300/300–350 nm), peak B ($\lambda_{ex/em}$ = 280–300/340–360 nm), peak C ($\lambda_{ex/em}$ = 310–460/455–540 nm), and peak D ($\lambda_{ex/em}$ = 220–270/380–500 nm) was represents tyrosine protein, tryptophan protein, humic acid-like substances, and fulvic acid-like substance (Li et al., 2021; 2022; Xu et al., 2022), respectively.

Table 1 Fluorescence spectral characteristics of the EPS samples

Peak	$\lambda_{ex/em}$ (nm)	Composition
A	200–300/300–350	Tyrosine protein
B	280–300/340–360	Tryptophan protein
C	310–460/455–540	Humic substances
D	220–270/380–500	Fulvic acids

The appearance of peak A and peak B in all AGS samples from R_1 (Figs. 6(a)–6(c) and R_2 (Figs. 6(d)–6(f)) indicates that tyrosine proteins and tryptophan proteins were the main constituents of SMP-EPS, TB-EPS, and LB-EPS. Compared with S-EPS and LB-EPS, peak A and peak B exhibit the highest intensity in TB-EPS, indicating that TB-EPS contains more tyrosine protein as well as tryptophan protein. Their concerted action is of significance for the stability of AGS. From the spectra of S-EPS of AGS samples in the R_2 (Fig. 6(d)), the appearance of peak C and peak D indicate that humic substances and fulvic acids existed in the S-EPS. These compounds, possessing abundant carbonyl and carboxyl groups, play a significant role in metabolic processes, coordinating enzymatic reactions,

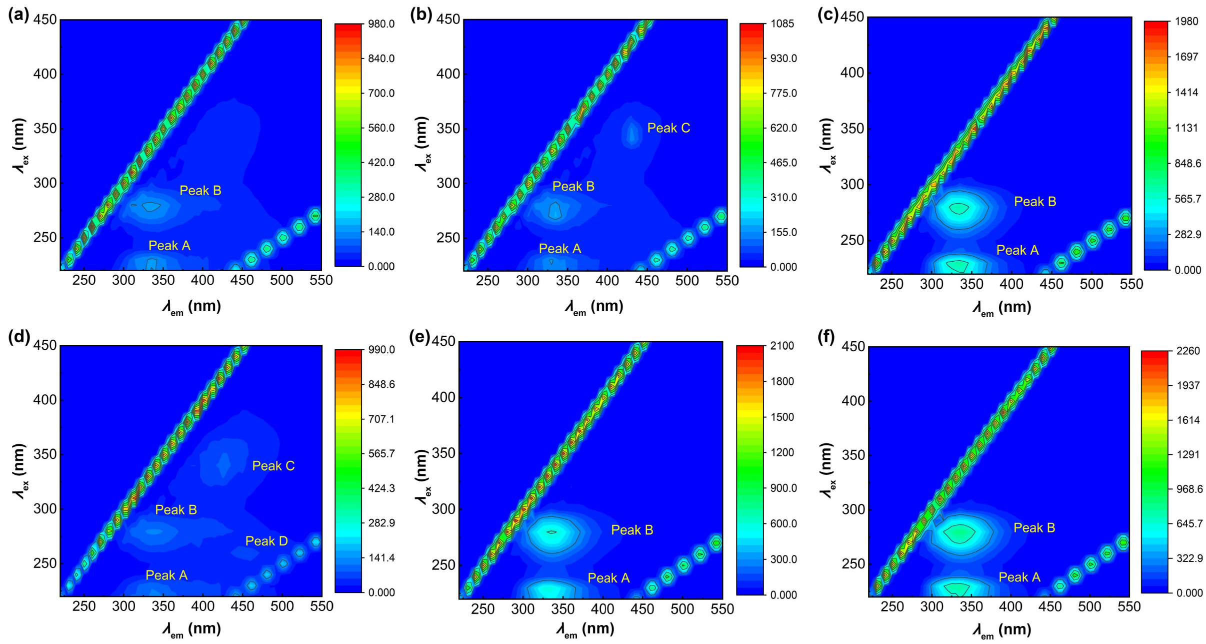

Fig. 6 3D fluorescence excitation-emission matrices spectra of EPS. (a) S-EPS, (b) LB-EPS, and (c) TB-EPS in R_1, (d) S-EPS, (e) LB-EPS, and (f) TB-EPS in R_2.

and adapting environment. From the LB-EPS spectra, the intensity of peak A and peak B in R_2 (Fig. 6(e)) was higher than that in R_1 (Fig. 6(b)). Besides, peak C disappeared in R_2 (Fig. 6(e)). These results indicate that the OLR-varied strategy is positive to secret tyrosine protein and tryptophan protein and negative for humic substances secretion. The spectrum intensity of peak A and peak B in TB-EPS samples revealed that the OLR-varied strategy has little effect on the composition of TB-EPS (Figs. 6(c) and 6(f)).

Overall, as the OLR gradually increased, more EPS was generated to maintain the stability of the sludge granulates. The components of EPS, containing tryptophan protein, tyrosine protein, and humic acid, may contribute to resisting environmental stress and maintaining structural stability (Wei et al., 2016, Zhang et al., 2018).

3.4 Structural characterization of microbial community

3.4.1 Diversity analysis

The richness and diversity of microorganisms were investigated. From Table 2, the coverage indices of AGS samples were higher than 0.99, demonstrating that the obtained data can be utilized to analyze the microbial structure information of granular sludge samples. The Chao and ACE indices serve as indicators of microbial community richness. A higher Chao or ACE index value indicates a higher diversity of microbial species. The ACE index in AGS-R2 (313.7582) declined compared with that of AGS-R1 (315.1608), revealing that OLR-varied strategy impacted the richness of the microbial structure. This result can be further demonstrated by the changes in OTU values (273 in AGS-R_2 *vs* 290 in AGS-R_1, Fig. S6).

The microbial species' evenness can be reflected by the Shannon index and Simpson index. It can be seen that the AGS-R_2 sample shows a lower Shannon index (3.575912) and Simpson index (0.843848) compared with the AGS-R_1 sample. This result indicates that increasing the OLR of the influent would lead to a reduction in the microorganisms' evenness in AGS, which can be further confirmed by the unique OTU values of AGS-R_2 (91) and AGS-R_1 (108). The impact of OLR on microorganisms' evenness may be because of the activity suppression or even the death of partial bacteria at a higher OLR condition. From the other perspective, the OLR-varied strategy may provide a selective environment, where the more active bacteria

Table 2 Richness and diversity characterization indices of the microbial community

Sample	Sobs	Shannon	Simpson	ACE	Coverage
AGS-R_1	290	3.978136	0.886448	315.1608	0.999
AGS-R_2	273	3.575912	0.843848	313.7582	0.999

survive for treating this wastewater.

3.4.2 Evolution of microbial population

The bacteria population structure was analyzed at phylum, class, and genus levels to insight into the dominant microbes. From the results of phylum level (Fig. 7(a)), *Proteobacteria*, *Bacteroidota*, and *Firmicutes* were the predominant microorganisms in the AGS-R_1 sample with an abundance of 61.46%, 20.73%, and 14.57% respectively. However, in the AGS-R_2 sample, *Proteobacteria*, *Bacteroidota*, and *Firmicutes* accounted for 51.23%, 10.28%, and 35.96%, respectively. The decreasing abundance of *Proteobacteria* and *Bacteroidota* may be due to the suppression of the high OLR stress. This is consistent with the decrease in Shannon and Simpson indices. *Proteobacteria* play a significant role in transforming organics into non-toxic substances (Jiang et al., 2023). *Bacteroidetes* belong to heterotrophic microbes and are commonly found in sludge, exhibiting high proficiency in EPS secretion (Liu et al., 2023c). This also responds to the decrease in EPS production at high OLR conditions.

From the class level (Fig. 7(b)), *Alphaproteobacteria*, *Bacilli*, *Gammaproteobacteria*, and *Bacteroidia* prevailed in AGS-R1 and AGS-R2 samples. Compared with AGS-R1, the abundance of *Alphaproteobacteria* (23.16%) and *Bacilli* (35.24%) was increased by 2.43% and 20.98%, respectively. *Bacilli* is a chemoheterotrophic microbe with aerobic denitrification capacity (Zhang et al., 2024). From the aspect of nutrient removal, *Alphaproteobacteria* are active in removing nitrogen and phosphorus (Xia et al., 2024), responding to the changes in the NH_4^+-N and TP removal profiles. The abundance of *Gammaproteobacteria* (28.04%) and *Bacteroidia* (10.28%) declined by 12.38% and 10.45%, respectively. The *Gammaproteobacteria* are important bacteria in organic matter degradation. Especially,

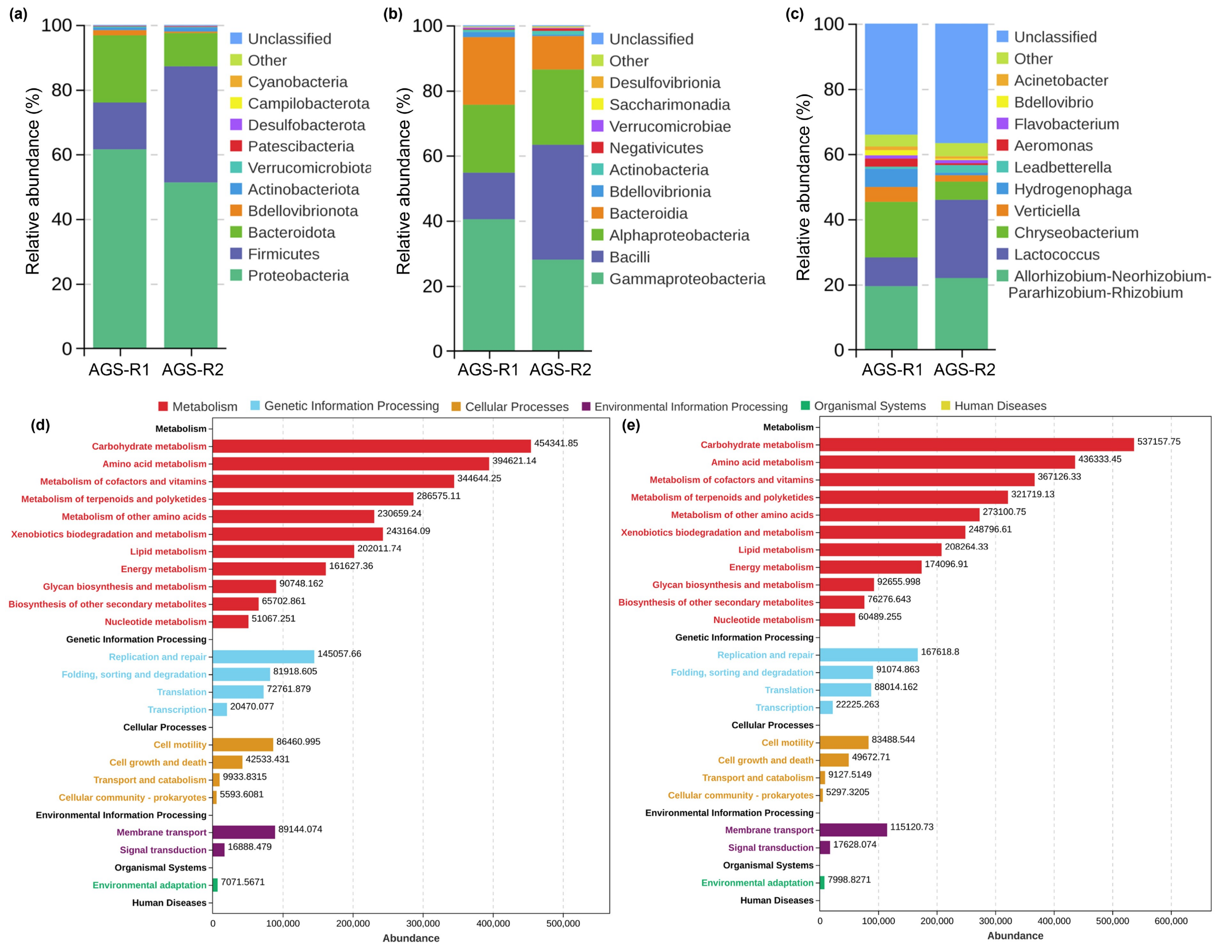

Fig. 7 Microbial community structure at (a) phylum level, (b) class level, and (c) genus level. PICRUSt2 function analysis of AGS in (d) R_1 and (e) R_2.

when glucose was utilized as a carbon source and ammonia nitrogen as a nitrogen source, Gammaproteobacteria would exhibit denitrification capacity. Besides, Gammaproteobacteria efficiently metabolizes glucose to produce acid (Pan et al., 2023).

Figure 7(c) shows the evolution of microbial population structure at the genus level. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Lactococcus*, and *Chryseobacterium* show an abundance of 19.45%, 8.81%, and 17.02% in AGS-R_1 respectively. However, their abundances changed to 21.98%, 23.93%, and 5.58% in ASG-R_2 respectively. It can be seen that the prevailing bacteria in both sludge samples are *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Lactococcus*, and *Chryseobacterium*, which have a critical role during heterotrophic nitrification process and aerobic denitrification process (Xi et al., 2022). The changes in abundance show that these three bacteria were sensitive to external environmental OLR stress. Due to the high OLR in R_2, the abundance of *Chryseobacterium* in AGS-R_2 declined by 11.44% compared with that in AGS-R_1. In AGS-R2, the abundance of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Lactococcus* increased by 2.53% and 15.12%, respectively. The microorganism of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* belongs nitrifying bacterial group and mainly utilizes ammonia nitrogen as a nitrogen source (Guo et al., 2024), which further supports the superior nitrification performance of R_2.

3.4.3 Functions analysis

The functions of the microorganism population were analyzed using the PICRUSt2 program. The results reveal that both samples were associated with metabolism, genetic information processing, cellular processes, environmental information processing, and organismal systems (Figs. 7(d) and 7(e)). Two AGS systems show a robust metabolism function, which displays a vital role in the biological metabolic process. Carbohydrate metabolism displayed the highest abundance in bacterial community function. AGS-R_2 showed a superior metabolism function compared with AGS-R_1, further demonstrating that the OLR-enhanced strategy can improve the pollutant metabolism capacity of the system. AGS-R_2 is better than AGS-R_1 in processing genetic information, which can be confirmed by the higher abundance of replication and repair (167618.8), folding, sorting and degradation (91074.863), translation (88014.162), and transcription (22225.263) functions of AGS-R_2. This is probably because the OLR-enhanced strategy promotes the gene materials and expression in microorganisms' colonization.

AGS-R_2 showed a weaker cellular processes function, especially the abundance of the cell growth and death function decreased by 7229.28, which is consistence with the microbial community diversity results. However, the higher environmental information processing function and environmental adaptation function (7998.8271) were observed in AGS-R_2. This result suggests that the OLR-enhanced strategy is conducive to the AGS system for adapting and resisting the external environment stress. This is because high OLR stress provides a selective environment for the enrichment of functional microbes, donating a more robust ability to adapt to the changes of the environment. Therefore, a group of highly active bacteria survived to maintain the stability of the AGS system.

4 Conclusions

In conclusion, this work explored the influence of OLR on the AGS system under a micro-electric environment. The dynamic change of influent OLR affected the sludge granulates size distribution, morphology, granule strength, biomass, and settleability. The aerobic sludge granulates cultured under an OLR-varied condition showed a compact texture, high microbial activity, and stability. The AGS system showed excellent removal for COD (90%), NH_4^+-N (95%), and TP (76.2%). In addition, the OLR-varied strategy affected the secretion of EPS, including polysaccharides, protein (i.e., tyrosine protein and tryptophan protein), humic acid-like substances, and fulvic acid-like substances. The OLR-varied condition provides a selective environment, where Proteobacteria (51.23%), Bacteroidota (10.28%), and Firmicutes (35.96%) phyla were enriched and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (21.98%), *Lactococcus* (23.93%), and *Chryseobacterium* (5.58%) genera prevailed. The functions of the microorganisms' community were also changed with the variation of OLR to well adapt to the environment, such as carbohydrate metabolism, replication and repair, and membrane transport functions. This work provides valuable insights into the OLR on AGS processes and a strategy for enhancing the stability of AGS systems.

CRediT Authorship Contribution Statement

Yabin Li: Conceptualization, Methodology, Data curation, Funding acquisition, Writing - original draft-editing. **Lanlan Qin**: Software, Validation, Formal analysis. **Xiran Li:** Formal analysis, Investigation,

Data curation, Supervision. **Xiaolong Tang:** Methodology, Investigation. **Xia Zhao:** Conceptualization, Resource, Funding acquisition, Project administration, Writing-review and editing. **Xiaoning Jia:** Methodology, Software. **Xiuqin Kong:** Methodology, Validation.

Conflict of Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this work.

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