

Efficacy of aerobic granular sludge treatment of piggery wastewater under length regulation of truncated modified basalt fibers

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ARTICLE INFO

Editor: Choe Earn Choong

Keywords:

Aerobic granular sludge

Piggery wastewater

Truncated modified basalt Fiber

Stability

Granulation

ABSTRACT

Aerobic granular sludge (AGS) technology offers several advantages over traditional activated sludge systems; however, its performance can be unstable when treating wastewater with high organic and ammonia loads. Truncated modified basalt fibers (TMBF) have been demonstrated to enhance the stability of AGS systems when treating piggery wastewater (PWW), but little is understood about their impact. In this study, the addition of TMBF with lengths ranging from 0.1 to 0.4 mm significantly accelerated sludge granulation, achieving complete granulation in 10 days compared to 17 days in the control reactor. The enhancement was attributed to increased secretion of β -polysaccharides, resulting in smaller cavities within the granules for the 0.1 mm TMBF. In contrast, 0.2 mm TMBF promoted the secretion of proteins, hindering substrate transfer and reducing ammonia oxidation efficiency. The addition of 0.1 mm TMBF resulted in chemical oxygen demand (COD) and ammonia removals of 90.7 % and 96.8 %, respectively. Furthermore, the addition of TMBF enriched the sludge with a substantial number of functional bacteria, *Thauera* and *Chryseolinea*, which were the most abundant genus, contributing to its stable pollutant removal efficiency. These findings provide key insights on potential strategies for optimizing AGS technology to treat industrial wastewater that has high ammonia and COD loads, not usually suited for intensified AGS systems.

1. Introduction

The rapid expansion of large-scale pig farming has resulted in the centralized discharge of large volumes of piggery wastewater (PWW), which primarily consists of urine, manure, residual feed, and barn wash water [1,2]. With high discharge volume and elevated concentrations of carbon, nitrogen, phosphorus, and other pollutants, untreated PWW poses the risk of contaminating the atmosphere, water bodies, and soil [3]. Therefore, efficient treatment of PWW is essential to prevent pollution and mitigate its ecological impact [4].

Aerobic granular sludge (AGS) is a compact and regularly shaped biological aggregate formed through microbial self-aggregation [5]. AGS exhibits high biomass density and strong biomass retention, enabling it to withstand fluctuations in influent load, toxic compounds, and osmotic pressure [6,7]. Additionally, it demonstrates excellent settling ability and enables simultaneous nitrification and denitrification, enhancing nitrogen removal efficiency [8]. Its capacity to reduce

operational costs by 20–25 % further highlights its potential for cost-effective treatment of high-strength wastewater such as PWW [9].

Despite its advantages, AGS faces serious challenges when treating PWW over the long term, primarily due to low carbon availability and the presence of toxic compounds, which may result in instability or even system collapse [10]. Both filamentous and non-filamentous bulking can occur during AGS treatment of PWW. The low COD-to-TN ratio in PWW promotes excessive growth of filamentous bacteria, which, although beneficial for initial granulation, may compromise structural integrity when overgrown [11]. In parallel, The high toxicity of PWW promotes *Zoogloea* overgrowth, leading to non-filamentous bulking [12]. A previous study on AGS treatment of real piggery wastewater reported that *Zoogloea* overgrowth increased polysaccharide secretion, leading to poor sludge settleability, reduced effluent clarity, and lower treatment efficiency [12]. Additionally, fluctuations in influent substrate concentration further hinder AGS cultivation and long-term stability under real-world conditions. These combined factors pose major challenges to

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<https://doi.org/10.1016/j.jwpe.2025.108244>

Received 5 March 2025; Received in revised form 15 June 2025; Accepted 29 June 2025

Available online 4 July 2025

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maintaining AGS stability during PWW treatment.

Given these challenges, the instability of AGS under PWW conditions can be attributed to multiple factors, including: filamentous and non-filamentous bulking that compromise granule integrity [12,13]; hydrolysis in the anaerobic core; loss of functional bacteria [14]; and changes in the composition and function of extracellular polymeric substances (EPS) [15]. Consequently, enhancing AGS stability and preventing particle disintegration have become critical research priorities for the practical application of this technology in high-ammonia wastewater treatment [16].

Modified basalt fiber is an environmentally friendly inorganic fiber, primarily composed of SiO_2 , Al_2O_3 , Fe_2O_3 , and CaO , etc. [17]. It is derived from natural basalt ore, which is melted at high temperatures (1450–1500 °C) and extruded into fibers [18]. Due to its natural abundance and sustainable production process, basalt fiber is widely used in industries such as construction and marine industries [19]. Compared to conventional carriers such as carbon fibers or polymer-based biofilm supports, basalt fiber offers advantages in terms of sustainability, mechanical strength, and cost-effectiveness [20]. It is derived from natural basalt without the emission of toxic gases during production, making it a low-cost alternative [21]. These features are particularly beneficial for large-scale applications such as piggery wastewater treatment, where economical and environmentally friendly solutions are urgently needed [22]. Surface modification enhances the roughness and hydrophilicity of basalt fibers, thereby improving microbial adhesion and making them ideal bio-carriers in wastewater treatment systems [23]. Previous studies demonstrated that truncated MBF (TMBF) had excellent hydrophilicity and biocompatibility, facilitating the rapid attachment and growth of microorganisms in the AGS system [24]. TMBF also facilitates the enrichment of functional microbial communities and supports enhanced denitrification during the treatment of industrial wastewater, such as that from pig farms. Most importantly, TMBF acted as a framework within AGS, connecting proteins and lipids, thus improving the structural stability of the granules [24]. Despite these proven benefits, there remains a lack of in-depth studies on how variations in fiber length affect AGS formation and stability, especially under real-world conditions such as piggery wastewater treatment. While previous research has focused on TMBF's material properties and surface modifications, the role of fiber length in shaping AGS formation and system stability has largely been overlooked. This gap is particularly important for large-scale applications, where the successful integration of TMBF into microbial communities within complex wastewater environments is crucial for sustained system performance.

Determining the optimal length of TMBF is essential for practical engineering applications, where standardized carrier dimensions are typically required. Without such guidance, effective full-scale application of TMBF for stable AGS formation and operation remains challenging. Therefore, this study is significant not only in advancing understanding of TMBF's role in AGS formation but also in offering practical insights for optimizing carrier design for large-scale wastewater treatment systems.

In this study, TMBF lengths of 0.1 mm, 0.2 mm, and 0.4 mm were selected as granulation scaffolds to evaluate their effects on accelerating granulation acceleration and AGS system stability, based on both biological and engineering considerations. The upper limit of 0.5 mm was determined based on the typical minimum diameter of mature aerobic granules (generally >0.5 mm [25]), ensuring that remained embedded within the granule matrix rather than protruding externally. Such internal integration is critical for maintaining structural stability and supporting effective microbial colonization. The lower limit of 0.1 mm was selected as further reduction in fiber length would significantly increase processing difficulty and cost. As this study aims to evaluate TMBF as a low-cost, practical carrier for AGS in real piggery wastewater, fiber preparation needed to remain economically feasible. Excessive processing costs would undermine the purpose of developing TMBF as a

scalable, affordable solution for full-scale applications. Therefore, selecting TMBF lengths within 0.1–0.4 mm ensured relatively stable production costs and enabled a systematic evaluation of fiber length effects without economic bias.

This study investigates the effects of different TMBF fiber lengths on AGS morphology, EPS production, and the composition and spatial distribution of EPS. It also compares the pollutant removal performance of AGS systems incorporating different TMBF lengths. At the molecular level, this study examines microbial community succession and spatial distribution within AGS, providing insights into the dynamic population shifts during long-term operation. This study is distinctive in its real-world application, evaluating how fiber length influences microbial colonization, EPS production, and overall AGS system performance under the complex conditions of piggery wastewater treatment.

2. Methods

2.1. Preparation of MBFs and reactor operation

TMBF with a diameter of 13 μm was procured from Jiangsu GMV New Material Science T&D Co., Ltd. (China) in three lengths: 0.1 mm, 0.2 mm, and 0.4 mm. Before adding the truncated fibers into the reactors, the fibers were ultrasonically cleaned three times using acetone and deionized water. The cleaned fibers were then thoroughly dispersed in 50 mL of tap water using ultrasonic treatment. After the reactor startup, the fully dispersed fibers were periodically at a dosage of 50 $\text{mg}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ until granulation was complete. The final concentration of fibers in the reactors was 6.25 $\text{mg}\cdot\text{L}^{-1}\cdot\text{cycle}^{-1}$, a value selected based on previous studies on the application of fibers in wastewater treatment [24,26]. These studies demonstrated that this dosing level effectively enhances the granulation and stability of AGS without causing operational difficulties in reactor systems. Further research is recommended to investigate the scalability of this approach in pilot-scale systems to further validate its feasibility for engineering applications.

Four sequencing batch reactors (SBRs) were configured as follows: R-0 (no fiber), R-0.1 (0.1 mm TMBF), R-0.2 (0.2 mm TMBF), and R-0.4 (0.4 mm TMBF). Each SBRs had an inner diameter of 5 cm, an effective height-to-diameter ratio (H/D) of 11, and a total volume of 1.08 L. Each reactor was operated with eight 3-h cycles per day and a 50 % water exchange per cycle. pH was not controlled and typically ranged from 8 to 9.5 by the end of each cycle. Cycle phases included 3 min feeding, 158–170 min aeration, 3–15 min settling, 4 min effluent withdrawal, and 4 min idle time. During aeration, airflow was maintained at 2 L/min to keep dissolved oxygen (DO) levels between 7.0 and 7.5 mg/L. The temperature was maintained at 25 ± 2 °C throughout the experiment. DO levels remained within 7.0–7.5 mg/L in all reactors.

2.2. Piggery wastewater characteristic and inoculated sludge

The PWW used in this experiment was collected from a pig farm in Zhenjiang, China. It consisted of urine, wash water, blood, and feces. Prior to use, the wastewater was pre-filtered through a 1.0 mm sieve to remove large particles and stored at -10 °C. Detailed characteristics are listed in Table S1. To maintain an ammonia ($\text{NH}_4^+\text{-N}$) concentration of approximately 100 mg/L in the influent, the wastewater was diluted accordingly. Due to the reduced COD following dilution, sucrose was added as a carbon supplement to adjust the COD to 700 mg/L. Although this adjustment increased the C/N ratio compared to typical undiluted piggery wastewater (which often has a relatively low COD/TN ratio) [27], it was necessary to ensure successful AGS formation and stable system operation, which were essential for investigating the mechanistic effects of TMBF on granule development and nitrogen removal. Carbon supplementation, particularly with sucrose, has been shown to promote microbial aggregation and granulation, which are critical for AGS formation and effective nitrogen removal [28]. Therefore, sucrose was added to simulate a favorable and representative condition for piggery

wastewater treatment.

Activated sludge used for inoculation was collected from the aerobic tank of a municipal wastewater treatment plant in Zhenjiang. The sludge was yellow-brown and consisted primarily of fine floc particles, with protozoa observed microscopically. Prior to use, it was screened through a 60-mesh sieve to remove large impurities. The initial mixed liquor suspended solids (MLSS) and sludge volume index (SVI₃₀) were 6.6 g/L and 69.2 mL/g, respectively.

2.3. Analytical methods

Scanning electron microscopy (SEM) images were obtained using a field-emission SEM (JSM-7001F, JEOL, Japan) to observe sludge morphology. pH values were measured using a PHS-3C pH/DO meter. Standard methods [29] were used to analyze MLSS, MLVSS, SVI₃₀, COD, NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, and total inorganic nitrogen (TIN). Due to sample limitations, measurements were taken once per sampling point. Although replicates were not available, operational parameters were carefully controlled to ensure data reliability. EPS was extracted using the heating method [30]. Protein and polysaccharide content were quantified using the modified Lowry and anthrone-sulfuric acid methods [31,32]. Sludge particle size distribution (AGS₀, AGS_{0.1}, AGS_{0.2}, and AGS_{0.4} are the sludge in R-0, R-0.1, R-0.2 and R-0.4) was measured using a laser diffraction analyzer (Mastersizer 3000, Malvern Instruments, UK). Spatial distribution of proteins (FITC), α -polysaccharides (Con A), and β -polysaccharides (Calcofluor White) in AGS samples was visualized using confocal laser scanning microscopy (CLSM; Leica TCS SP5 II, Germany). AGS samples were cryosectioned into 40 μ m slices (Leica CM3050S) and prepared according to Chen et al. [33].

2.4. Statistical analysis

To evaluate whether there were statistically significant differences in pollutant removal performance among the reactors during the stable

stage, Kruskal-Wallis one-way analysis of variance (ANOVA) was performed using Python 3.11. Given the non-parametric nature of the dataset and the absence of replicates, the Kruskal-Wallis test was selected over parametric alternatives. The removal efficiencies of COD, NH₄⁺-N, and TIN were independently assessed. A *p*-value <0.05 was considered statistically significant. The statistical analysis was supplemented by boxplot visualization to illustrate interquartile variation and potential outliers across reactors.

2.5. Microbial and functional gene analysis

Microbial community analysis was conducted on sludge samples from four SBRs at different time points: days 1 (seed), 10, 17, 30, and 50. 16S rRNA gene sequencing (targeting the V3–V4 region with primers 341F/805R) was performed by Sangon Biotech (Shanghai, China). Genomic DNA was extracted using the E.Z.N.ATM Mag-Bind Soil DNA Kit (Omega Bio-tek, USA). Functional gene prediction was performed using PICRUSt2 based on the 16S rRNA data. Functional annotations were assigned using the KEGG database, with a focus on genes involved in ammonia oxidation, nitrate reduction, and denitrification [34].

3. Results and discussion

3.1. Granulation process and stability of aerobic granules

Using these three TMBF lengths to cultivate AGS led to changes in sludge characteristics in each reactor, as shown in Fig. 1. MLSS, SVI₃₀, and VSS/SS were dynamically monitored after reactor startup. The results indicated that R-0.1 and R-0.2 maintained higher MLSS levels and, consequently, were likely to have higher sludge activity. In contrast, R-0 and R-0.4 exhibited lower MLSS values and reduced sludge activity.

During the first 5 days, both MLSS and SVI₃₀ decreased rapidly due to the reduction in settling time from 15 to 3 min, which facilitated the retention of denser AGS and removal of flocculent sludge [35]. By day 5, R-0 had the highest MLSS (4.26 g/L), followed by R-0.2 and R-0.4, while

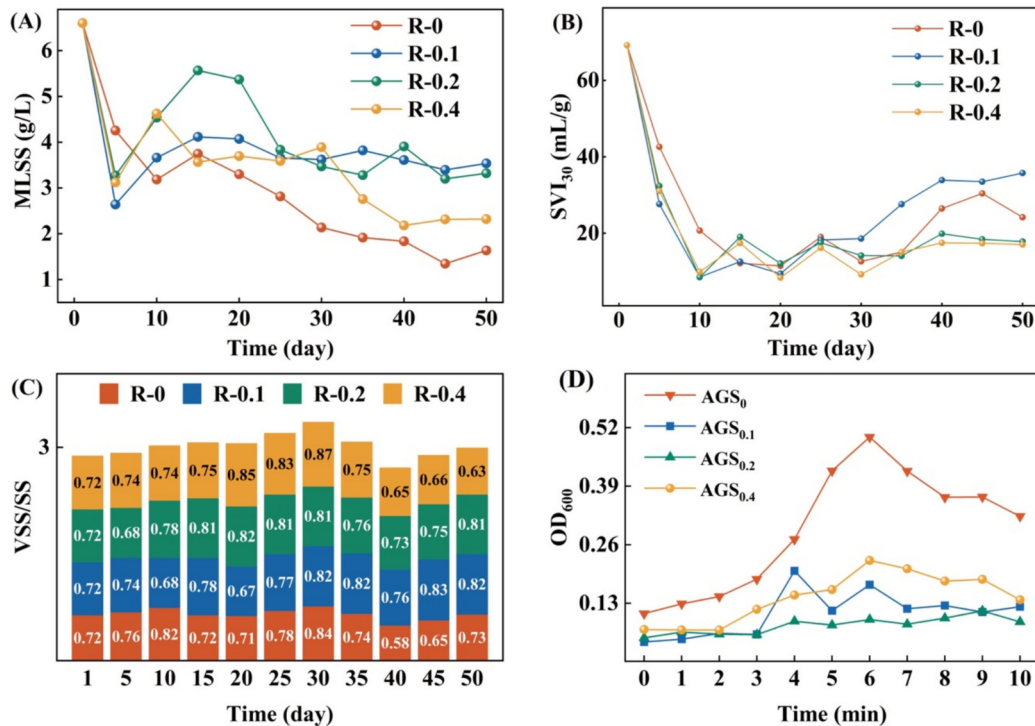


Fig. 1. (A) MLSS, (B) SVI₃₀, (C) VSS/SS and (D) sludge particle size during the operation of different reactors: R-0/AGS₀ (no truncated fiber introduction), R-0.1/AGS_{0.1} (introduction of 0.1 mm TMBF), R-0.2/AGS_{0.2} (introduction of 0.2 mm TMBF), R-0.4/AGS_{0.4} (introduction of 0.4 mm TMBF).

R-0.1 had the lowest (2.64 g/L), likely due to intensified collisions and biomass washout. However, this initial decrease did not affect long-term stability, with R-0.1 maintaining granule stability throughout the experiment.

By day 10, the MLSS values in R-0.1, R-0.2 and R-0.4 were higher than in R-0 (3.2 g/L), with R-0.2 and R-0.4 exceeding 4.5 g/L, and R-0.1 reaching 3.7 g/L (Fig. 1). This indicates that 0.2 mm and 0.4 mm TMBF enhanced sludge retention, while 0.1 mm TMBF caused greater biomass loss due to stronger collisions, leading to weaker early granule retention. Particle size measurements (Fig. 1 D) showed that by day 10, AGS sizes in R-0.1 (212 μm), R-0.2 (246 μm), and R-0.4 (274 μm) exceeded 200 μm , confirming successful granulation [36]. This acceleration in AGS formation is attributed to TMBF's strong microbial affinity, which promotes aggregation and improves sludge retention [18]. From a practical engineering perspective, such early enhancement in AGS formation is highly desirable, especially in treating high-strength piggery wastewater, where rapid formation of compact granules can enhance system stability and biomass retention [37].

SEM image in Fig. 2 (A) showed that a significant sludge adhered to TMBF surfaces, forming smooth AGS granules (Fig. 2 B), with microbial attachment on exposed fibers, confirming TMBF's strong microbial affinity. Although R-0.1 had the lowest MLSS early on, it maintained stability around 3.5 g/L after day 15, while R-0 and R-0.4 declined to 1.64 g/L and 2.32 g/L, respectively. This suggests that despite initial washout, 0.1 mm TMBF enhanced long-term sludge retention and reactor stability. In contrast, larger 0.4 mm fibers caused rapid aggregation but compromised granule integrity over time, leading to internal voids, uneven biomass [26], and reduced nutrient diffusion, as indicated by a lower VSS/SS ratio (0.63 vs. 0.73 in R-0). Smaller fibers (0.1 mm and 0.2 mm) maintained compact structures, improving nutrient and oxygen transfer, and promoting sustained microbial activity, as shown by higher VSS/SS ratios, reflecting better biomass stability.

By day 17, granulation was achieved in R-0 with an average particle diameter of 338 μm (Fig. 1 D), although R-0 still exhibited significant suspended sludge, indicating a delayed onset of AGS formation. By day 20, the particle diameters of AGS_{0.1}, AGS_{0.2} and AGS_{0.4} reached 558 μm , 517 μm and 504 μm , respectively, confirming maturation with granule sizes >500 μm [38]. In contrast, AGS₀ had a diameter of 435 μm , indicating it had not yet matured. This highlights that TMBF promotes both granulation and maturation of AGS. By day 30, AGS_{0.1} showed the largest diameter at 958 μm , while others remained around 750 μm , suggesting 0.1 mm TMBF accelerated particle growth during maturation. Between days 30 and 40, AGS₀ grew to 1630 μm (Fig. 1 D), while AGS_{0.1}, AGS_{0.2}, and AGS_{0.4} remained below 1400 μm , with AGS_{0.2} at 1260 μm . This suggests that TMBF limits granule growth beyond 1000 μm , likely due to its role in promoting microbial attachment, which forms a compact matrix. It is worth noting that when AGS diameter exceeds 1000 μm , oxygen mass transfer to the granule core can become significantly limited, potentially leading to undesired anaerobic conditions or microbial stratification [37]. Therefore, the observed moderation in granule size growth in the presence of TMBF may help mitigate such risks, contributing to more stable reactor performance during long-term operation.

Fig. 2 (C, G and H) shows AGS₀ after freeze-sectioning, revealing a loose sludge distribution with large internal voids. In contrast, AGS_{0.4} has a denser structure with smaller voids, leading to a smaller particle size. AGS_{0.1} and AGS_{0.2}, however, displayed tight sludge distribution with no significant voids. SEM images of AGS_{0.2} (Fig. 2 I and J) show that TMBF was embedded and tightly enveloped by the sludge, creating a more compact structure. While AGS_{0.1} stabilized quickly in size, AGS_{0.2} continued to grow, reaching 1490 μm by days 40–50, suggesting successful granulation but approaching the threshold where mass transfer limitations could affect system performance. Granules over 1000–1500 μm may experience reduced oxygen penetration, causing anaerobic zones that could disrupt nitrification [39,40]. Smaller granules, however, improve oxygen diffusion and nitrification efficiency [41].

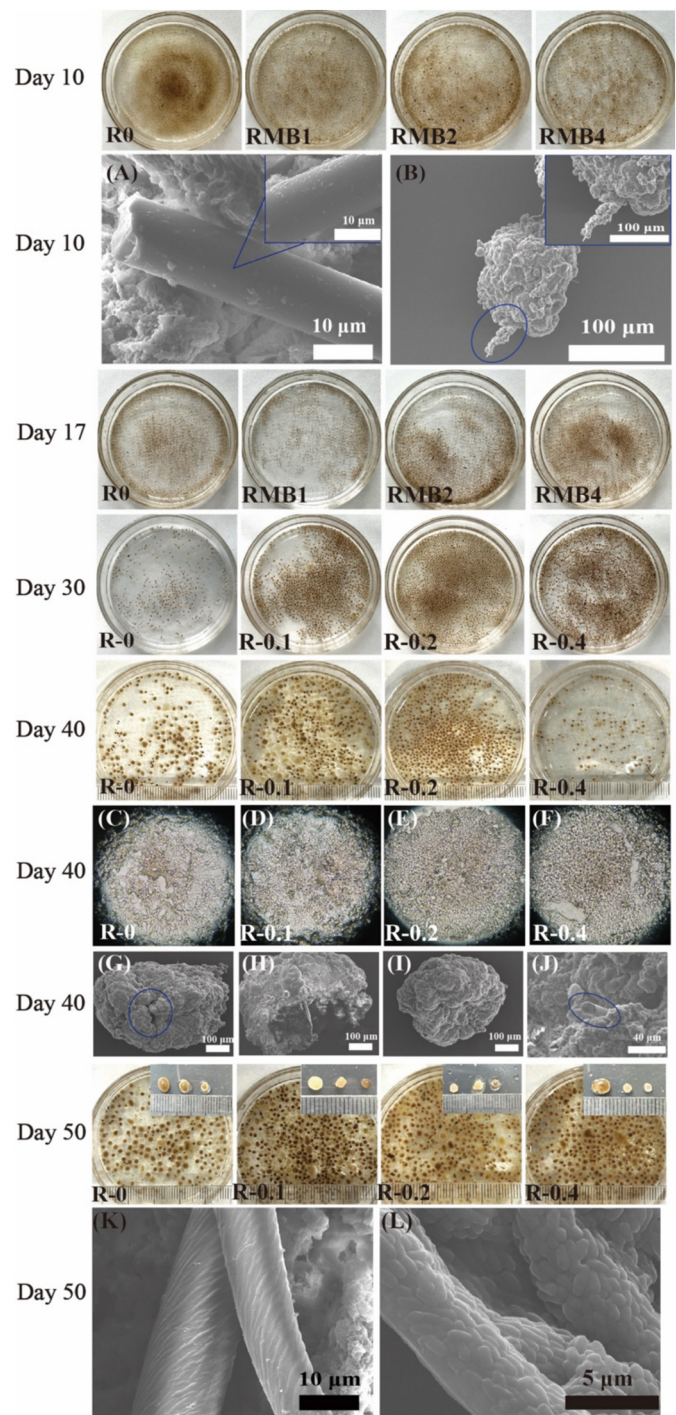


Fig. 2. Photo and SEM images (A, B) of sludge morphology, and the microscopic images of frozen sections (C ~ F); (G) is the SEM image of whole AGS₀, (H) is the SEM image of cut particle of AGS₀, (I) is the SEM image of whole AGS_{0.2}, (J) is the SEM image of cut particle of AGS_{0.2}, (K, L) are the SEM images of adhesion of EPS, bacteria, and fibers in cut granules.

Although no significant disintegration was observed in AGS_{0.2} during the experiment, its growth near 1500 μm indicates potential mass transfer issues in the future. In contrast, AGS₀ decreased from 1630 μm to 1040 μm due to instability from mass transfer limitations, leading to disintegration and reaggregation [42]. SEM images of AGS_{0.1} (Fig. 2 K and L) show that TMBF, covered with EPS and bacteria, helped enhance the granules' structural compactness and stability.

Although TMBF fibers integrate into granules during the initial

granulation phase and are likely retained due to microbial attachment, their long-term behavior in the AGS system remains unverified. Once incorporated, the fibers should not be easily flushed out, even if the granules break down and reassemble, as microorganisms adhere to the fibers. However, further experimental validation is needed, and a pilot-scale study could assess the long-term need for additional TMBF and its effects on residual sludge treatment.

To further evaluate the structural stability of AGS, samples from each reactor on day 50 underwent ultrasonic disruption. The OD_{600} values of the sludge suspensions were measured to assess the degree of granule breakage. The rate of increase in OD_{600} of the supernatant represents the rate of granule disintegration. Fig. S1 showed that AGS₀ exhibited the highest OD_{600} value, indicating the greatest degree of granule disintegration, while AGS_{0.4} showed moderate stability. AGS_{0.2}, however, displayed the most stable structure, as indicated by the lowest OD_{600} increase. This confirms that 0.2 mm TMBF most effectively enhances the structural stability of AGS.

3.2. Extracellular polymeric substances secretion

EPS plays a crucial role in the aggregation of sludge, facilitating the connection of bacteria to each other or to carrier surfaces, thereby promoting the formation of AGS. Proteins (PN) and polysaccharides (PS), as major components of EPS, are vital for maintaining the structural stability of AGS. This study measured and compared the changes in PN and PS contents in different sludge samples across operational stages, as shown in Fig. 3.

Based on the granulation progression and system performance described in 3.1, the operational period was divided into three phases: granulation, maturation, and stable stages. For R-0, the granulation stage was defined as days 1–17, maturation as days 18–30, and the stable stage as days 31–50. For reactors with TMBF (R-0.1, R-0.2, and R-0.4), the granulation stage was days 1–10, maturation was days 11–20,

and the stable stage was considered from day 21 onwards. As granules formed, the EPS content in all reactors gradually increased. By day 10, EPS content in R-0 reached 15.1 mg/g MLSS, while R-0.1, R-0.2, and R-0.4 was 25.7, 27.5, and 25.3 mg/g MLSS, respectively. These results indicate that TMBF stimulated microbial EPS secretion, accelerating granule formation, which is one reason for the shorter granulation time observed in R-0.1, R-0.2, and R-0.4 compared to R-0.

During the maturation stage, R-0.1 exhibited the highest average concentrations of PN and PS, at 21.9 and 15.3 mg/g MLSS, respectively, resulting in a high PN/PS ratio. A higher PN content and PN/PS ratio effectively reduced the surface charge of sludge, facilitating microbial aggregation and granule formation [43]. After 20 days of operation, the EPS content in R-0.1 and R-0.2 continued to increase, reaching maximum values of 73.6 and 65.5 mg/g MLSS, respectively, and then stabilized. In contrast, the content in R-0 and R-0.4 showed greater fluctuations during the stable stage and remained at lower levels.

The average PN concentration in R-0 and R-0.4 was 30 and 28.2 mg/g MLSS, respectively, while R-0.1 and R-0.2 maintained higher PN levels at 43.7 and 52.8 mg/g MLSS. Additionally, the PN/PS ratio in R-0 and R-0.4 gradually increased to 3.1 and 2.6, respectively, before declining to around 0.7. A lower PN/PS ratio was unfavorable for granule aggregation, reduced sludge hydrophobicity, and increased the risk of AGS disintegration [44]. In contrast, the PN/PS ratio in R-0.1 and R-0.2 increased significantly, with R-0.2 stabilizing at 4.0. A higher PN/PS ratio enhances AGS hydrophobicity and promotes microbial aggregation, resulting in more stable AGS structure in AGS_{0.2}.

The experimental results indicate that while 0.4 mm TMBF did stimulate some EPS secretion, its effect was less significant compared to the 0.1 mm and 0.2 mm TMBF. Specifically, 0.4 mm TMBF did not promote as high PN secretion, nor did it sustain as favorable a PN/PS ratio, both of which are critical for enhancing granule stability and aggregation. In contrast, the 0.1 mm and 0.2 mm TMBF consistently supported higher PN concentrations and a more stable PN/PS ratio, leading

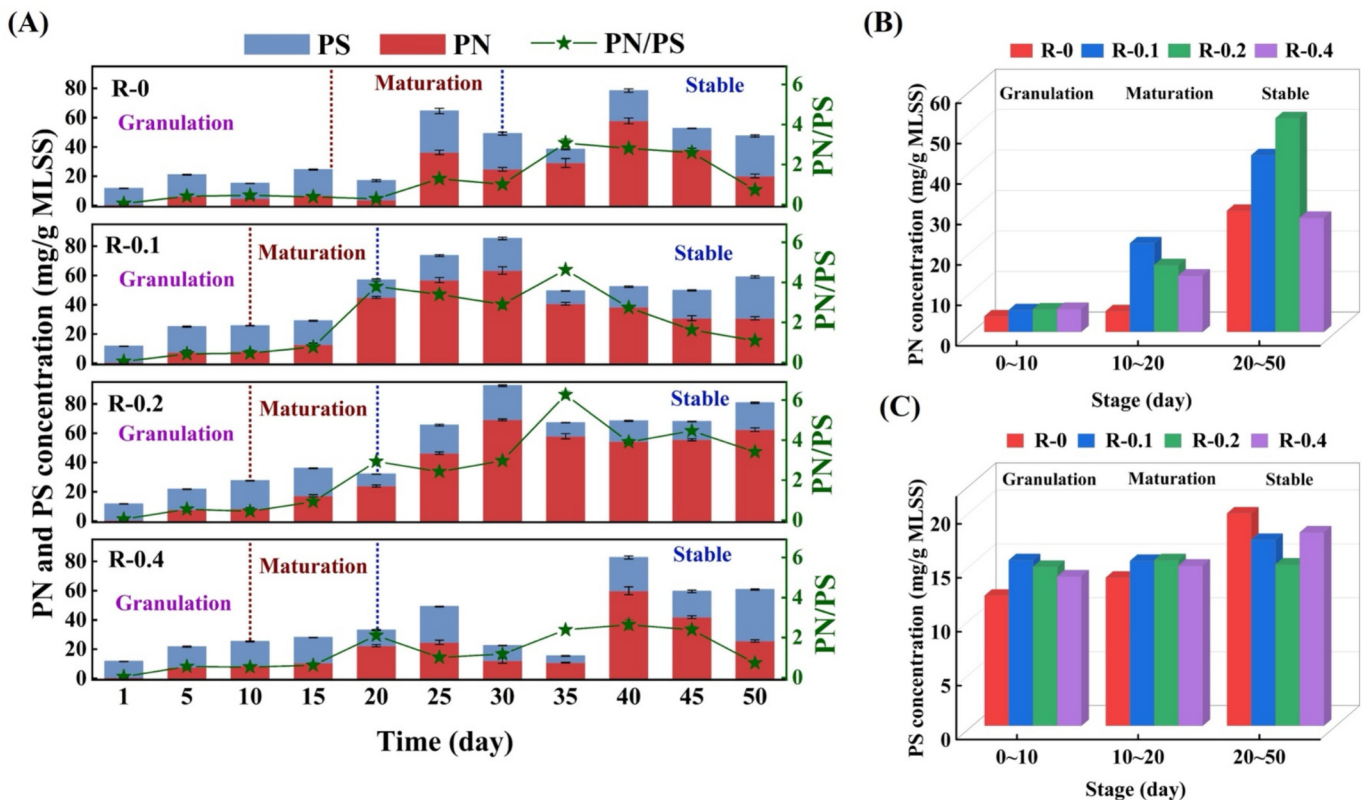


Fig. 3. (A) The variation in PN and PS content, as well as the PN/PS ratio, during the operation of different reactors; (B) and (C) are PN and PS average concentrations in different operational stages.

to enhanced structural stability in AGS.

One limitation of this study is that the EPS analysis did not differentiate between loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS), which are known to play distinct roles in granule development. TB-EPS is typically associated with structural integrity and internal scaffolding of AGS, while LB-EPS contributes more to surface interactions and microbial adhesion [45]. Without distinguishing these two fractions, the specific mechanisms by which TMBF regulates granule formation and stabilization remain partially unclear.

To fully elucidate how TMBF influences the fine-scale organization and functional roles of EPS within AGS, future investigations should focus on the spatial distribution and biochemical characteristics of LB-EPS and TB-EPS. Studies employing EPS fractionation and characterization techniques such as FTIR or EEM are recommended to better understand the regulatory effects of TMBF on EPS behavior and AGS performance [24,46].

3.3. Confocal laser scanning microscopy of EPS

After the experiment concluded, AGS samples were cryosectioned and analyzed using multicolor fluorescence staining and confocal laser scanning microscopy to investigate the impact of TMBF on the spatial distribution of different EPS components. Fig. 4 illustrates the distribution of PN (green fluorescence), α -PS (red fluorescence), and β -PS (blue fluorescence) in AGS₀, AGS_{0.1}, AGS_{0.2}, and AGS_{0.4}. The results revealed that in AGS₀, PN and α -PS were primarily located along the periphery, with a small amount of β -PS present in the core region. This is due to the tendency of EPS to surround microbial cells, leading to cavity formation inside AGS₀, resulting in lower microbial and EPS concentrations in the granule core.

In AGS_{0.1}, although PN and α -PS were also mainly distributed on the periphery, a notably greater amount of β -PS was found in the core compared to AGS₀. β -PS plays a crucial role in granular sludge, forming a

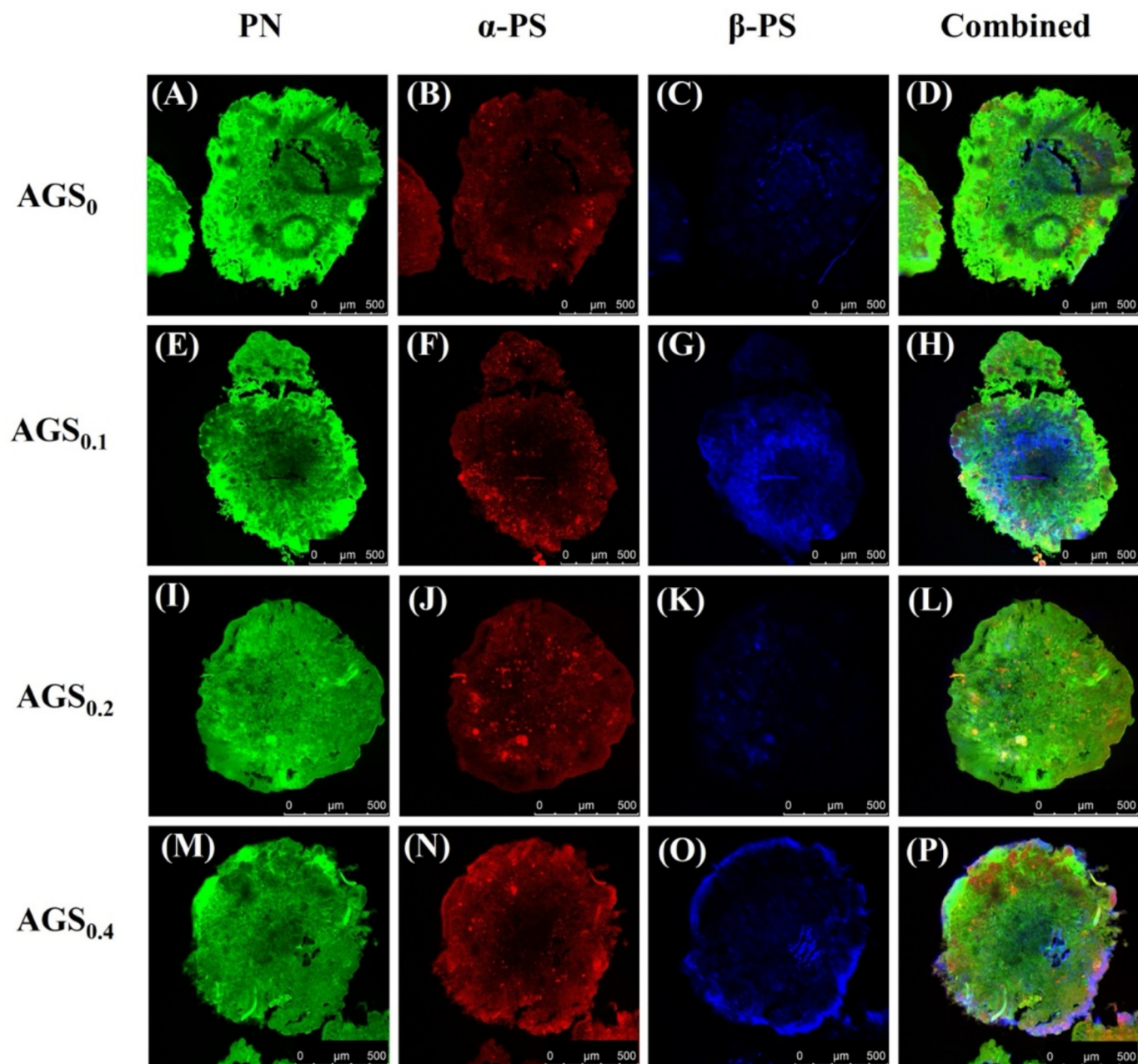


Fig. 4. CLSM images of EPS, protein (FITC, light green, A, E, I and M); α -polysaccharides proteins (Con A, light red, B, F, J and N); β -polysaccharides (calcofluor white, light blue, C, G, K, and O); Combined image (D, H, L and P) from the reactor operation on the 50th day.

mesh-like structure within microcolonies that embeds all cells into the matrix [47]. It acts as a scaffold in the granular core, connecting PN and α -PS and thereby providing mechanical stability to prevent granule disintegration under external forces [48]. This explains the smaller cavities and lower OD₆₀₀ values, indicating enhanced structural stability (Fig. 2 and Fig. S1). PN and α -PS enhance AGS hydrophobicity and integrity [49]. These findings suggest that 0.1 mm TMBF stimulated the secretion of β -PS in the core region, thereby increasing granule stability.

In contrast, in AGS_{0.4}, β -PS was predominantly distributed on the periphery, with lower abundance in the core. This indicates that TMBF of different lengths stimulates the secretion of β -PS in different spatial locations, thereby maintaining the external structural stability of AGS_{0.4}.

In AGS_{0.2}, the distribution of PN and PS was noticeably different. PN and α -PS were relatively uniformly distributed throughout the granule, whereas β -PS was less abundant, corresponding to the higher PN/PS ratio. Studies have shown that insufficient β -PS cannot effectively fix and connect PN and α -PS, potentially leading to AGS disintegration [50]. However, in this study, OD₆₀₀ values indicated that AGS_{0.2} had stronger stability than the other AGS types. Scanning electron microscopy observations revealed that 0.2 mm TMBF was embedded within the AGS, with a clear and compact granular structure. This suggests that 0.2 mm TMBF can partially substitute for β -PS as a structural scaffold, maintaining granule stability.

3.4. COD and N removal performances of the AGS

The PWW treatment efficiency is shown in Fig. 5. During the granulation stage, the effluent COD concentration fluctuated significantly, which was expected due to intense shear forces and shortened settling times. These conditions selectively removed large amounts of sludge, thereby reducing the microbial population and causing unstable COD removal [51]. In the subsequent maturation stage, the average COD removal efficiency improved across all reactors except R-0.1, with R-0.2 and R-0.4 outperforming R-0 (Fig. S2). This improvement is attributed to the strong biocompatibility of TMBF, which rapidly promoted the aggregation of bacterial community within the granules, efficiently consuming the organic matter in the substrate. However, 0.1 mm TMBF continued to exert considerable abrasion on the microorganisms during this stage, resulting in relatively lower COD removal efficiency.

During the stable stage, R-0.1's COD removal capacity increased, as indicated in Fig. S2, with an average removal rate reaching 86.6 %. This was followed by R-0.2 (83.7 %), R-0.4 (78.5 %), and R-0 (77.4 %), indicating that the abrasive effect of 0.1 mm TMBF on microorganisms decreased in the stable stage, possibly due to its integration within granules, where it became coated with microbial cells and EPS. This encapsulation likely provided protection and minimized abrasion, allowing the microbial enrichment effect of 0.1 mm TMBF to become more evident.

By day 10, the TIN removal efficiency in R-0.1, R-0.2, and R-0.4

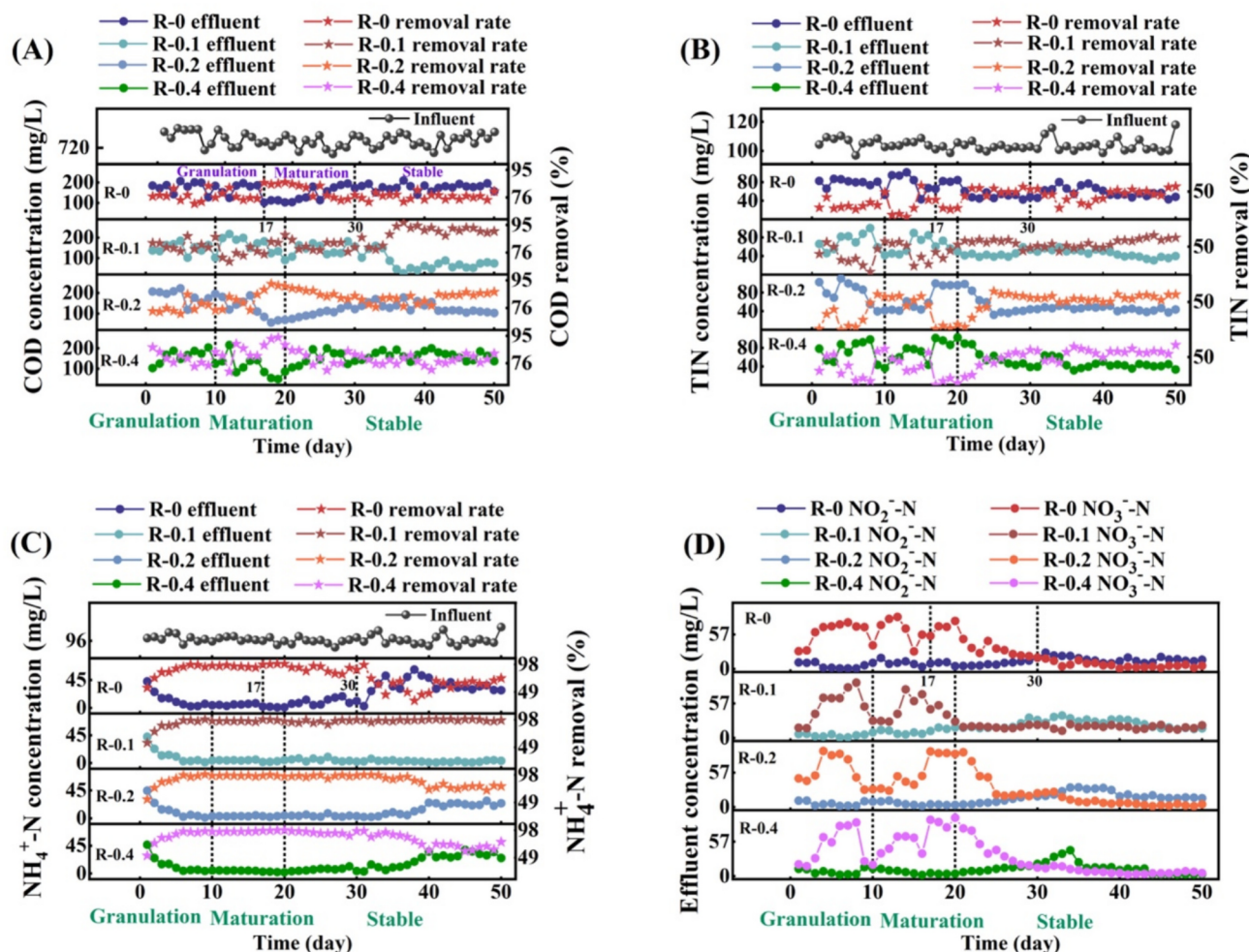


Fig. 5. Reactor performance, (A) COD, (B) TIN, (C) NH₄⁺-N and (D) NO₂⁻-N and NO₃⁻-N influent and effluent concentrations and their removal efficiency during reactor operation (data points represent single measurements without biological replicates. The data were used to show general trends). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reached 60 %, whereas R-0 achieved only 50 % (Fig. 5B). Combining this with Fig. 5 (C, D), which shows lower effluent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations on day 10 in the TMBF reactors, it can be inferred that the nitrification was more effective in these reactors. This suggests that TMBF enhances nitrogen removal by facilitating the early enrichment of nitrifying bacteria. Compared to conventional activated sludge, AGS typically harbors a greater abundance of denitrifying bacteria (DNB) [52], resulting in superior nitrogen removal.

R-0.1, R-0.2, and R-0.4 exhibited good $\text{NH}_4^+\text{-N}$ removal during the maturation stage, while R-0 peaked at 94.4 % on day 25 before declining. This decline likely resulted from a continuous decrease in MLSS in R-0, indicating that microbial growth and retention in AGS₀ could not be sustained under PWW conditions. The effluent TIN concentrations in R-0.1, R-0.2, and R-0.4 fluctuated during maturation, mainly due to variations in $\text{NO}_3^-\text{-N}$ levels, while $\text{NO}_2^-\text{-N}$ remained relatively stable. This may reflect DNB accumulation, though their limited abundance impeded a stable denitrification process.

In the stable stage, R-0.1 achieved the highest average $\text{NH}_4^+\text{-N}$ removal at 96.3 %, with TIN removal stabilizing at 70 % by the end of operation. This performance significantly exceeded that of the control (R-0), which achieved only 74 % $\text{NH}_4^+\text{-N}$ removal, and is likely due to microbial community composition. As shown in Fig. S4, AGS_{0.1} was enriched with *Thauera* (18 %) and *Chryseolinea* (13 %) at the end of the operation, suggesting a synergistic enhancement of nitrogen removal. Specifically, *Chryseolinea* is known for its heterotrophic nitrification capacity [53], while *Thauera*, a key denitrifier carrying the *nirS* gene [54], facilitated efficient denitrification.

In contrast, $\text{NH}_4^+\text{-N}$ removal in R-0.2 and R-0.4 declined to 80 % and 70 %, respectively. This reduced performance may be due to excessive PN accumulation in AGS_{0.2} (shown in Fig. 3), which likely increased granule density and reduced porosity, limiting substrate diffusion and microbial activity [49]. Moreover, although R-0.2 had a microbial community like R-0.1 in the early phase, its nitrogen removal lagged, suggesting that an optimal PN balance is crucial for maintaining mass transfer and microbial viability.

To determine whether the observed differences in pollutant removal were statistically significant, a non-parametric Kruskal–Wallis ANOVA was performed using data from days 31–50 (the stable stage). Significant differences were observed in COD ($H = 50.33, p < 0.001$), $\text{NH}_4^+\text{-N}$ ($H = 51.96, p < 0.001$), and TIN ($H = 24.15, p < 0.001$) removal efficiencies (Fig. S3). These findings confirm that TMBF addition significantly affected reactor performance during the stable phase. Although replicates were not collected due to operational limitations, the consistent reactor conditions and synchronized sampling ensured valid cross-reactor comparisons.

Notably, both R-0.1 and R-0.2 experienced $\text{NO}_2^-\text{-N}$ accumulation during the maturation stage (days 10–30), as shown in Fig. 5D, suggesting potential nitrite-oxidizing bacteria (NOB) inhibition [55]. This may reflect the sensitivity of NOB to variations in PN/EPS content within granules, which warrants further investigation.

3.5. Microbial community analysis

The microbial community composition and structure at the end of each reactor's operation were analyzed using 16S rRNA amplicon sequencing. Fig. 6A and B show the bacterial composition and relative abundance at the phylum and class levels, respectively. Proteobacteria was the dominant phylum in the AGS samples, with relative abundances of 58.4 %, 60.8 %, 66.7 %, and 65.0 % in R-0, R-0.1, R-0.2, and R-0.4, respectively. Bacteroidetes was the second most abundant phylum, with relative abundances of 37.3 %, 34.4 %, 28.4 %, and 31.6 % in R-0, R-0.1, R-0.2, and R-0.4, respectively. These findings align with previous studies indicating that *Proteobacteria* and *Bacteroidetes* are commonly dominant phyla in AGS, playing crucial roles in biological wastewater treatment [56].

At the class level, *Betaproteobacteria* and *Alphaproteobacteria* were the most abundant groups in R-0.1, R-0.2, and R-0.4. However, in R-0, the second most abundant class after *Betaproteobacteria* was *Flavobacteriia* (17.3 %), followed by *Alphaproteobacteria* (10.1 %). *Flavobacteriia* can secrete significant amounts of EPS, facilitating granule formation and

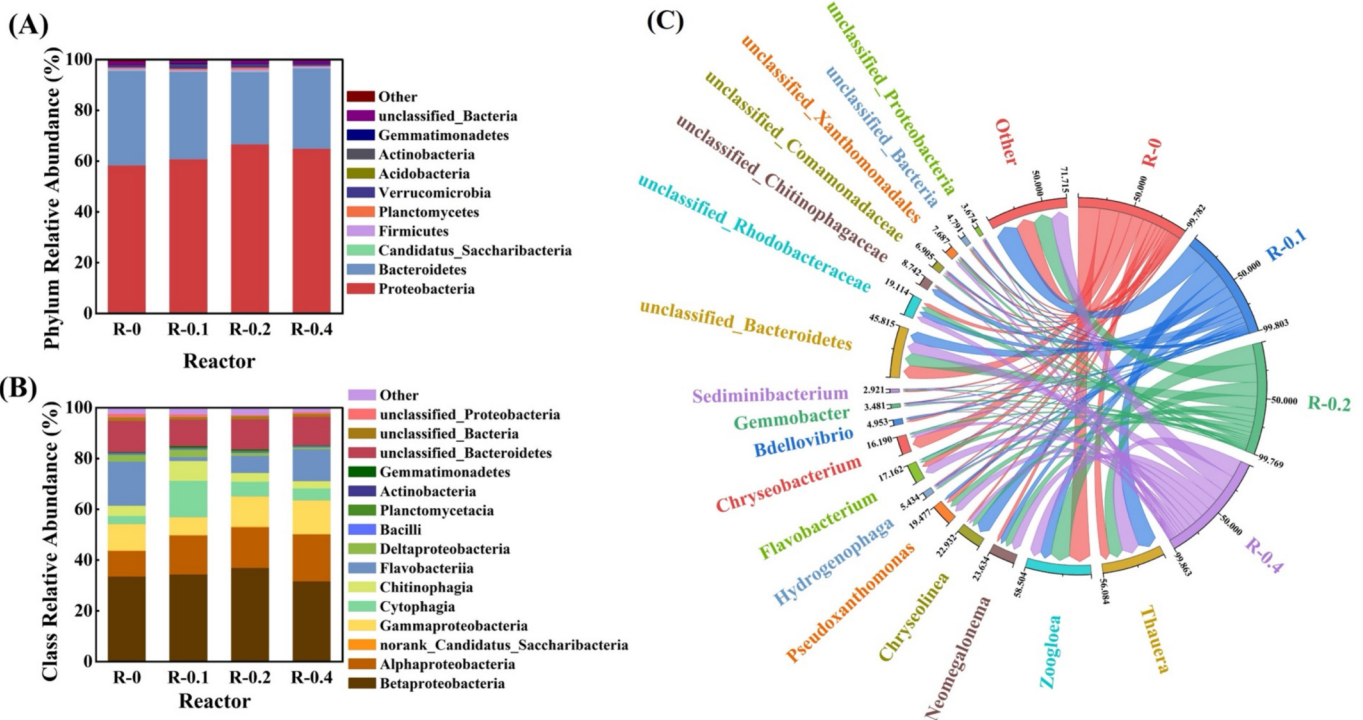


Fig. 6. Bacterial community of AGS at (A) phylum level, (B) class level, and (C) genus level (Circos image) in R0, RMB1, RMB2, RMB4 on day 50 (sampled on Day 50, corresponding to the stable stage for all reactors).

maintaining structural stability [57]. In R-0.1, the abundance of *Cytophaga* was 14.4 %, a group that degrades organic matter and contributes significantly to $\text{NH}_4^+\text{-N}$ removal [58].

To gain a comprehensive understanding of microbial functions, the dynamic changes in microbial communities at the genus level were monitored throughout the operation of each reactor. Fig. S4 presents genus-level microbial profiles at five key stages: day 1 (seed sludge), day 10 (end of granulation in TMBF groups), day 17 (end of granulation in R-0), day 30 (maturation), and day 50 (stable stage). The relative abundance of *Saccharibacteria_genera_incertae_sedis* significantly decreased by day 10, while *Thauera* and *Lactococcus* increased. This may be attributed to *Saccharibacteria*'s relevance in municipal wastewater, which limits their survival under the toxic and nutrient-variable conditions of PWW. In contrast, *Thauera* and *Lactococcus* showed greater adaptability to PWW, rapidly proliferating under favorable conditions [59].

By day 10, *Thauera* became abundant in all reactors, with relative abundances of 33.4 %, 44.0 %, and 44.5 % in AGS_{0.1}, AGS_{0.2}, and AGS_{0.4}, respectively—higher than 30.3 % in AGS₀. As an aerobic denitrifier, *Thauera* plays a significant role in nitrogen removal [60], which aligns with the elevated TIN removal efficiency observed in R-0.4 (64.6 % vs. 48.5 % in R-0). This improvement is likely related to the enhanced microbial colonization and structural support provided by TMBF. Acting as a biocarrier, TMBF improves microbial attachment and EPS accumulation, thereby fostering the proliferation of functional genera such as *Thauera* and *Chryseolinea*. These EPS- and denitrification-associated genera were enriched in the TMBF reactors, contributing to both nitrogen removal and granule stability under the challenging conditions of PWW [61]. As the operation progressed, the abundances of *Zoogloea* and *Flavobacterium* also increased, both of which are known to support granulation in PWW treatment [62]. *Zoogloea* forms flocs and biofilms, shielding inner microorganisms from antibiotics in PWW [63]. Meanwhile, *Flavobacterium*, a denitrifying phosphorus-accumulating organism (DPAO), supports nitrogen removal by facilitating simultaneous denitrification [64].

The relative abundances of *Neomegalonema* and *Chryseolinea* also increased significantly. *Neomegalonema* is typically found on the AGS surface and secretes large amounts of PN, enhancing granular structural stability [65]. *Chryseolinea* thrives in low-COD environments, explaining its enrichment in PWW [66]. The abundance of *Pseudoxanthomonas* also increased over time; this genus is known for its role in heterotrophic nitrification and aerobic denitrification during PWW treatment [67].

A Circos plot illustrated the relative abundances of 18 bacterial genera in each reactor at the end of the operation (Fig. 6 C). Compared to the other reactors, *Zoogloea* had a higher relative abundance in AGS_{0.2}, corresponding to the higher EPS content in AGS_{0.2} compared to AGS_{0.1}. *Chryseolinea* showed a significantly higher relative abundance in AGS_{0.1} and the lowest in AGS₀. This genus can perform heterotrophic nitrification-aerobic denitrification, enhancing $\text{NH}_4^+\text{-N}$ removal [68], correlating with the lowest effluent $\text{NH}_4^+\text{-N}$ concentration in R-0.1. Additionally, *Thauera* had the highest relative abundance in AGS_{0.1}, playing a crucial role in the denitrification process [69], contributing to the better TIN removal efficiency in R-0.1 and indicating that 0.1 mm TMBF has a strong ability to enrich DNB. These findings suggest that TMBF not only enhances the enrichment of denitrifying and EPS-producing bacteria but also shapes distinct microbial community profiles across different reactors. In future studies, more frequent microbial sampling during the stable stage will be conducted to enable statistical comparisons and further clarify the impact of TMBF on microbial dynamics.

3.6. Functional gene profiles related to nitrogen metabolism

To further elucidate the mechanisms behind the observed differences in nitrogen removal among reactors, key functional genes associated with nitrogen cycling were predicted and analyzed based on the sludge samples collected on day 50. In addition, to provide a temporal

perspective, PICRUSt2-based functional prediction was also performed on samples collected on day 30. The comparative heatmap (Fig. 7) reveals dynamic changes in functional gene profiles between day 30 and day 50.

As illustrated in Fig. 7, the relative abundances of genes involved in ammonia oxidation (e.g., *amoA*, *amoB*, *amoC*, and *hao*), nitrite oxidation (*nxrA*, *nxrB*), denitrification (*narG*, *narH*, *narI*, *napA*, *napB*, *nirK*, *nirS*, *norB*, *norC*, and *nosZ*), and dissimilatory nitrate reduction to ammonium (DNRA; *nirB*, *nirD*, *nrfA*, *nrfH*) showed substantial variation across reactors.

In R-0, a notable increase in *amoABC* and *hao* gene abundances from day 30 to day 50 suggests enhanced ammonia oxidation activity during the later stages. However, the concurrent decrease in *nxrAB* and *narGHI* may imply that the downstream conversion of nitrite to nitrate was weakened, potentially leading to nitrite accumulation or incomplete nitrification. Although *amoABC* and *hao* abundances were relatively high in R-0, its $\text{NH}_4^+\text{-N}$ removal rate was the lowest (73.6 %). This may be attributed to the enrichment of DNRA-related genes such as *nrfA* and *nrfH*, which mediate the reduction of $\text{NO}_2^-\text{-N}$ to $\text{NH}_4^+\text{-N}$. DNRA usually supports nitrogen removal when coupled with anammox, especially under anoxic or low-carbon conditions [70]. In such cases, $\text{NO}_2^-\text{-N}$ is first reduced to $\text{NH}_4^+\text{-N}$ by DNRA, and then $\text{NH}_4^+\text{-N}$ reacts with $\text{NO}_2^-\text{-N}$ via anammox to form N_2 . However, no *hzs* or *hdh* genes—key markers of anammox [71]—were detected in any reactor. This likely results from the high DO level (7.0–7.5 mg/L) throughout the operation. Without anammox, the $\text{NH}_4^+\text{-N}$ regenerated by DNRA could not be further removed via autotrophic nitrogen pathways, leading to $\text{NH}_4^+\text{-N}$ accumulation and lower total nitrogen removal in R-0.

The R-0.1 reactor exhibited the most enriched nitrogen cycling profile, characterized by notably high abundances of *amoABC* and *hao*, suggesting efficient ammonia oxidation (R-0.1 $\text{NH}_4^+\text{-N}$ removal on day 50 is 96.8 %). Compared to day 30, R-0.1 exhibited superior enrichment of both *nxrAB* and denitrification-associated genes, including *narGHI*, *napAB*, and *nirK/S*, which facilitate the stepwise conversion of $\text{NO}_3^-\text{-N}$ to NO . Moreover, the elevated abundance of *nosZ* in R-0.1 suggests that the final reduction of N_2O to N_2 was more complete, potentially minimizing greenhouse gas emissions. These gene patterns corroborate the higher $\text{NH}_4^+\text{-N}$ and TIN removal efficiencies observed in R-0.1 during the stable phase, reflecting a reinforced and functionally integrated nitrification-denitrification pathway.

In contrast, R-0.2 and R-0.4 experienced a decrease in *amoABC* and *hao* gene abundances from day 30 to day 50, which may underline their lower $\text{NH}_4^+\text{-N}$ removal efficiencies (R-0.2 with 77.7 % and R-0.4 with 77 %). In R-0.2, this limitation was likely due to excessive accumulation of proteinaceous EPS (PN-EPS), which could increase granule density and reduce porosity [49], thereby restricting oxygen diffusion and inhibiting AOB activity. This interpretation aligns with Section 3.4, where PN buildup was proposed as a key factor limiting nitrogen removal. To directly validate this hypothesis, future pilot-scale studies could apply oxygen microelectrode profiling to measure DO gradients within the granules. Additionally, the relatively low abundance of *nxrAB* and key denitrification genes in R-0.4 may explain its weaker nitrification and denitrification activity. Notably, R-0.4 also exhibited the highest levels of *norB* and *norC*, suggesting a potential for N_2O accumulation.

Taken together, the functional gene profiles indicate that 0.1 mm TMBF provided the most favorable conditions for enriching diverse and functionally complementary nitrogen-transforming microbial communities. Temporal trends reinforce the conclusion that microbial community functions evolved toward higher nitrogen removal capacity in R-0.1, while reactors R-0.2 and R-0.4 showed either stagnation or imbalance in key transformation pathways. These findings align with the reactor performance data and support the hypothesis that appropriate TMBF size enhances the structural and microbial configuration of AGS for optimized nitrogen removal.

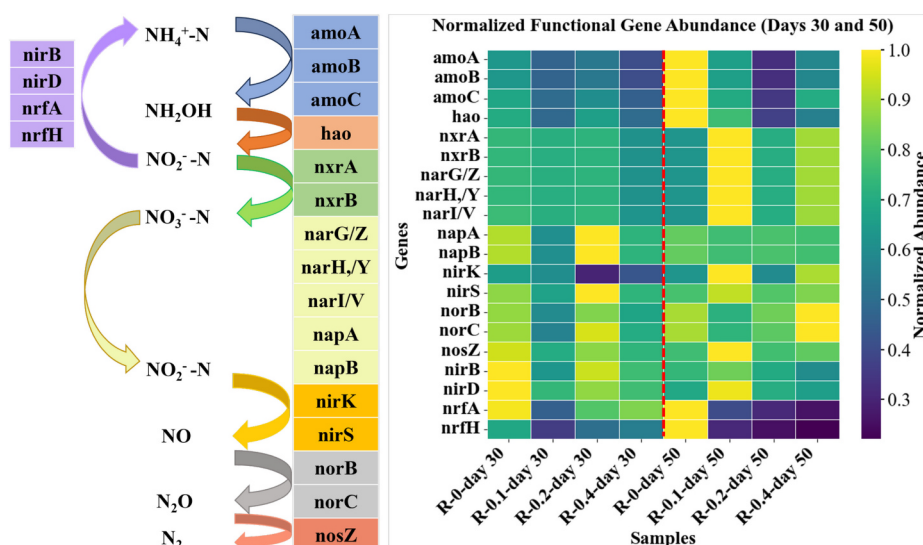


Fig. 7. Functional gene abundance heatmap (normalized) involved in nitrogen metabolism.

4. Conclusion

This study demonstrated that the addition of TMBF, especially at a length of 0.1 mm, significantly improved the granulation rate, structural stability, and treatment performance of AGS in treating PWW. Reactors with TMBF achieved faster granulation by day 10 and maturation by day 20, maintained higher MLSS levels around 3.5 g/L, and exhibited enhanced EPS secretion and microbial activity. The 0.1 mm TMBF stimulated core microbial activity and enhanced granule stability through β -PS secretion.

Functional gene and microbial community analyses further confirmed the enrichment of key nitrifiers and denitrifiers, contributing to improved nitrogen removal. Among all reactors, R-0.1 showed the best overall performance in terms of pollutant removal and granule stability. These findings suggest that 0.1 mm TMBF is an effective additive for optimizing AGS systems in real-world PWW treatment.

CRediT authorship contribution statement

Yun Zhou: Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Soares:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Xiaochun Wang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Zhiren Wu:** Writing – review & editing, Validation, Supervision, Resources. **Xiangtong Zhou:** Writing – review & editing, Resources, Funding acquisition. **Jianxun Liu:** Writing – review & editing, Resources.

Declaration of competing interest

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 paper.

Acknowledgments

This study is supported by the Open Project of the State Key Laboratory of Urban Water Resource and Environment; the Open Project of State Key Laboratory of R&D program of Zhenjiang (No. SH2021008); Key Research and transformation program of Qinghai province (No. 2022-SF-137); China Postdoctoral Science Foundation (No. 2023 M741423); Youth Program Natural Science Foundation of Jiangsu

Province (No. BK20230552); Pre-research Fund of Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment (XTCXSZ2020-4). Additionally, I would like to express my gratitude to Cranfield University, Cranfield Water Science Institute and my supervisor, Professor Ana Soares, for her invaluable guidance and support during my data analysis and manuscript preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jwpe.2025.108244>.

Data availability

Data will be made available on request.

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2025-08

Elsevier

<https://doi.org/10.1016/j.jwpe.2025.108244>

Zhou Y, Soares A, Wang X, et al., (2025) Efficacy of aerobic granular sludge treatment of piggery wastewater under length regulation of truncated modified basalt fibers. Journal of Water Process Engineering, Volume 76, August 2025, Article number 108244