Water Research 288 (2026) 124671

Contents lists available at ScienceDirect

Water Research

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

Distinct roles of granules and flocs in aerobic granular sludge processes

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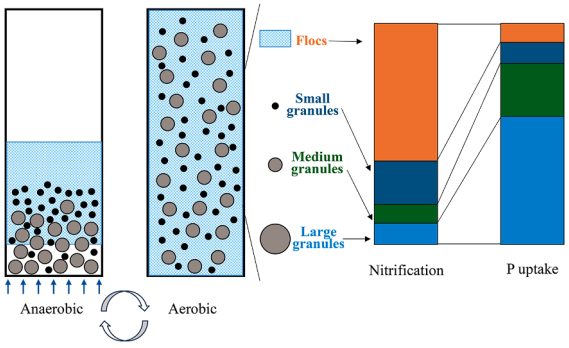
HIGHLIGHTS

- Coexistence of granules and flocs was achieved in a laboratory-scale AGS reactor fed with complex synthetic wastewater.
- Flocs contributed to about 60 % of total nitrification while large granules contributed to about 60 % of phosphorus uptake.
- Large and medium granules showed stronger mass transfer limitation of oxygen than flocs.
- Distinct microbial communities between granules and flocs were shown using metagenomics and metaproteomics.
- Balancing granules and flocs is important for optimizing nutrient removal in full-scale AGS systems.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Aerobic granular sludge
Flocculent sludge
Microbial community analysis
Nutrient removal
Simultaneous nitrification-denitrification (SND)
Enhanced biological phosphorus removal (EBPR)

ABSTRACT

Aerobic Granular Sludge (AGS) is an innovative and efficient biotechnology for wastewater treatment that has been successfully applied on full-scale worldwide. Full-scale municipal AGS systems typically contain both granular sludge (granules) and flocculent sludge (flocs). Studies on the different roles of granules and flocs remain limited. In this study, a laboratory-scale AGS reactor fed with complex synthetic wastewater was operated to simulate full-scale AGS systems and to study the different functional roles of granules and flocs. The laboratory reactor achieved a coexistence of granules and flocs with a floc mass fraction of 17 %. The activities of different size fractions were evaluated using batch experiments and compared for carbon, nitrogen, and phosphorus removal: flocs (FL; <0.2 mm), small granules (SG; 0.2~1.0 mm), medium granules (MG; 1.0~2.0 mm), and large granules (LG; >2.0 mm). During feeding, large granules and medium granules exhibited more substrate uptake than small granules and flocs due to preferential substrate access. For aerobic conversion, flocs and small granules showed higher biomass-specific nitrification rates, while medium granules and large granules showed higher phosphorus uptake and denitrification capacity. Furthermore, large granules and medium granules showed stronger mass transfer limitation of oxygen, which limits their nitrification capability. Microbial community analysis using metagenomics and metaproteomics was performed across size fractions, and distinct communities in granules and flocs were shown. Granules showed a high abundance of *Candidatus* Accumulibacter (polyphosphate-accumulating organisms, PAOs) and *Candidatus* Competibacter (glycogen-accumulating organisms, GAOs). Flocs showed a high abundance of *Nitrosomonas* (ammonium-oxidizing bacteria, AOB) and *Tetrasphaera* (fermentative PAOs) and a low abundance of *Ca.* Accumulibacter. The distribution of microbial

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https://doi.org/10.1016/j.watres.2025.124671
Received 30 April 2025; Received in revised form 17 September 2025; Accepted 23 September 2025
Available online 24 September 2025

activities and microbial community over sludge size fractions in the laboratory reactor is similar to full-scale AGS systems, indicating that this laboratory setup can simulate full-scale systems and can be used for future research. Overall, this study highlights the importance of maintaining a good balance between different granule sizes and flocs to optimize nutrient removal.

1. Introduction

Aerobic Granular Sludge (AGS) technology has emerged as an innovative and efficient approach for wastewater treatment, offering several advantages over conventional activated sludge (CAS) systems (Bengtsson et al., 2018; Hamza et al., 2022; van der Roest et al., 2013; Winkler and van Loosdrecht, 2022). AGS reactors integrate efficient biological nutrient removal, compact reactor design, and improved settling properties, leading to reduced footprint, energy savings, and enhanced treatment efficiency. Compared to CAS systems, AGS can reduce space requirements by up to 75 % and energy consumption by up to 40 % (Pronk et al., 2015b). Full-scale implementations of AGS have been successfully demonstrated across wastewater treatment plants (WWTPs) worldwide, showing the technology's robustness in handling loading fluctuations while maintaining treatment performance (Ekholm et al., 2022). The benefits of AGS technology rely on the formation of dense, well-structured granules that promote simultaneous nitrification, denitrification, and phosphorus removal (de Kreuk et al., 2005a). While granules are the dominant sludge morphology in full-scale AGS reactors, flocculent sludge (flocs) is also present in significant amount (Ali et al., 2019; Pronk et al., 2015b). The coexistence of granules and flocs in AGS systems raises important questions about their respective functional roles.

In full-scale AGS reactors, flocs are composed of influent suspended solids, biomass eroded from granular sludge, and biomass that grows within the floc fraction (Ali et al., 2019; Britschgi et al., 2025; Layer et al., 2019; van Dijk et al., 2018). Flocs are generally considered undesirable due to their low settling velocities and risk of contributing to suspended solids in the effluent (van Dijk et al., 2018; van Loosdrecht et al., 2004). Nevertheless, flocs exhibit a high surface-to-volume ratio, which leads to less mass transfer limitation compared to granules. These features suggest that flocs form a distinct ecological niche from granules within AGS systems. Full-scale AGS reactors employ a bottom-feeding strategy, introducing influent at the bottom of the settled sludge bed after settling. Because granules settle more rapidly than flocs, they accumulate at the bottom of the sludge bed and are exposed to incoming substrates earlier, giving them a competitive advantage in anaerobic substrate uptake. However, large-sized granules have a low surface-to-volume ratio, which can limit the mass transfer of substrates such as oxygen (Strubbe et al., 2022). These distinct ecological niches and characteristics between granules and flocs will result in differences in their microbial community composition and substrate conversion performance (Ali et al., 2019). Understanding the microbial community composition of granules and flocs and their respective contributions to substrate conversion is essential for optimizing full-scale AGS reactor operation.

Early research on AGS focused primarily on promoting granulation and maintaining granule stability (de Kreuk and van Loosdrecht, 2004; Nor Anuar et al., 2007; Weissbrodt et al., 2012), with limited attention paid to the floc fraction in AGS systems. More recently, the coexistence of granules and flocs, as well as the role of flocs, has received increasing attention. Several studies have investigated the microbial community differences between granules and flocs (Ali et al., 2019; Geng et al., 2023; Mohamed et al., 2025a; b; Ruiz-Haddad et al., 2025; Toja Ortega et al., 2021b). For substrate conversion, nitrification has been thoroughly studied, revealing significant differences in nitrification activity and nitrifier abundance between different sizes of granules and flocs (Britschgi et al., 2025; Haaksman et al., 2024; Nguyen Quoc et al., 2021b; Wei et al., 2021). In contrast, other important conversion processes—such as enhanced biological phosphorus removal (EBPR), denitrification, and anaerobic conversion—have not been thoroughly investigated with respect to their distribution between granules and flocs. This study aims to address this knowledge gap.

To advance understanding of EBPR, denitrification, and anaerobic conversion in AGS systems, well-controlled laboratory-scale AGS reactors can be highly effective. Although laboratory-scale reactors have been widely used in AGS research, many studies have relied exclusively on volatile fatty acids (VFAs) as the substrate (de Kreuk et al., 2005a; Geng et al., 2023; Lochmatter and Holliger, 2014). In reactors fed exclusively with VFAs, the substrates are primarily taken up during the anaerobic feeding phase, resulting in an almost entirely granule-dominated sludge, which does not accurately represent the sludge morphology in full-scale AGS reactors (Pronk et al., 2015a). To overcome this limitation, this study adopted a defined complex substrate recipe developed by Layer et al. (2019) with minor modifications, which contains VFAs, fermentable substrates, and particulate substrates. This substrate recipe is expected to better simulate the characteristics of municipal wastewater and promote floc formation (Layer et al., 2019), which is essential for reproducing the coexistence of granules and flocs in laboratory-scale reactors and for investigating their distinct functional roles.

In this study, a laboratory-scale AGS reactor fed with synthetic complex wastewater was used to investigate the different roles of granules and flocs in terms of substrate conversion and microbial community composition. Notably, optimizing reactor performance was not the aim of this study. The study aimed to address the following key research questions:

1. Laboratory AGS setup: Can a stable coexistence of granules and flocs be achieved in a laboratory-scale reactor using synthetic complex wastewater? How well does the laboratory system replicate the biomass characteristics observed in full-scale AGS reactors?
2. Substrate conversion: How do granules and flocs differ in their capacities for nitrification, denitrification, phosphorus uptake, and anaerobic conversion? What are their respective contributions to overall nutrient removal? What factors drive the difference in substrate conversion between granules and flocs?
3. Microbial community composition: How do the microbial communities differ between granules and flocs? To what extent do these community differences explain the observed variation in substrate conversion performance? What underlying factors lead to variations in microbial communities between granules and flocs?

2. Materials and methods

2.1. Laboratory reactor setup

To investigate the coexistence of granules and flocs in AGS systems, a laboratory-scale bubble column reactor was employed, operating in sequencing batch reactor (SBR) mode. The reactor had a working volume of 2.8 L with a total height of 90 cm and an internal diameter of 5.6 cm. Each SBR cycle lasted 6 h (hours) and included: 1 h of feeding (anaerobic phase), 4 h of aeration (aerobic phase), 30 min of anoxic phase, 20 min of settling, 5 min of effluent discharge, 5 min of idle. For every cycle, 1.5 L of influent was fed to the reactor from the bottom through the settling sludge bed (bottom-feeding), resulting in a volumetric exchange ratio of 54 %.

Environmental control was maintained during operation. The pH

was monitored and automatically adjusted to 7.3 ± 0.2 using 1 M NaOH or HCl solution. Dissolved oxygen (DO) concentration was precisely controlled using a gas-phase control system. During the aerobic phase, air was introduced to maintain DO at 25 ± 2 % saturation (approximately 2.3 ± 0.2 mg/L, stated as 2 mg/L in the text below). A gas recirculation system (2.5 L/min flow rate) ensured uniform oxygen distribution and mixing throughout the reactor. Reactor temperature was maintained at a constant 20 ℃ by regulating the room temperature. Sludge retention time (SRT) was controlled by periodic manual sludge withdrawal (on average 60 mL of mixed liquid per day). SRT was calculated as the ratio of biomass leaving the reactor to the total biomass within the reactor, and biomass leaving the system included both manually removed biomass and biomass discharged with the effluent. The reactor was inoculated with full-scale AGS biomass (WWTP Utrecht, Netherlands) containing granules and flocs.

Complex synthetic wastewater was fed to the reactor during the feeding phase with a volume of 1.5 L, to simulate municipal wastewater characteristics. This influent mixture was prepared by combining 900 mL of demineralized water with 300 mL of Medium A solution (C and N source) and 300 mL of Medium B solution (P and trace elements). Medium A contained complex organic substrates, based on the recipe developed by Layer et al. (2019) with minor adjustments. The organic substrates are based on chemical oxygen demand (COD)-equivalent: 1/3 of VFAs (equally divided between acetate and propionate), 1/6 glucose, 1/6 amino acids (equally among aspartate, glutamate, and leucine), 1/6 casein, and 1/6 starch (detailed in Supplementary Data). Medium A included 2000 mg/L COD and 16.4 mM NH_4Cl. Medium A solution was continuously stirred to ensure that particulate substrates were also fed to the reactor.

Medium B contained 0.86 mM K_2HPO_4, 0.43 mM KH_2PO_4, 1.80 mM $MgSO_4{\cdot}7H_2O$, and 2.35 mM KCl, supplemented with a trace element solution at 5 mL per liter, ensuring the availability of vital micronutrients. The trace element solution was composed of 4.99 g/L $FeSO_4{\cdot}7H_2O$, 2.2 g/L $ZnSO_4{\cdot}7H_2O$, 7.33 g/L $CaCl_2{\cdot}2H_2O$, 4.32 g/L $MnSO_4{\cdot}H_2O$, 2.18 g/L $Na_2MoO_4{\cdot}2H_2O$, 1.57 g/L $CuSO_4{\cdot}5H_2O$, 1.61 g/L $CoCl_2{\cdot}6H_2O$, and 50 g/L EDTA. This balanced recipe resulted in a final influent composition containing 400 mg/L of organic COD, 60 mg/L of total nitrogen (TN), and 8 mg/L of phosphate (PO_4^{3-}-P), providing consistent substrate conditions for the biological processes throughout the experiment.

2.2. Chemical analysis for bulk liquid

Mixed liquid samples were collected from the reactor and centrifuged to separate the biomass from the supernatant. The resulting supernatant was then filtered through 0.45 μm Millipore filters prior to the chemical analyses described below. Chemical oxygen demand (COD) was quantified using a cuvette-based spectrophotometric system (DR2800, Hach Lange, USA). Readily biodegradable COD (RBCOD) was estimated by subtracting the inert COD present in the effluent from the total measured COD. The concentrations of phosphate (PO_4^{3-}-P), ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) in the samples were determined using a Gallery Discrete Analyzer (ThermoFisher Scientific, USA). Simultaneous nitrification-denitrification (SND) efficiency was calculated as the ratio of TN removal to NH_4^+ removal, to indicate denitrification capacity. Acetate, propionate, and glucose were analyzed through high-performance liquid chromatography (HPLC) using a Vanquish system (ThermoFisher Scientific, USA). Separation was performed on an Aminex HPX-87H column (Bio-Rad, USA), with 1.5 mM phosphoric acid serving as the mobile phase. Detection of compounds was carried out using both refractive index (RI) and ultraviolet (UV) detectors.

2.3. Biomass and intracellular compound measurements

Sieves were used to separate different biomass size fractions: <0.2 mm fraction (flocs, FL), 0.2~1.0 mm fraction (small granules, SG), 1.0~2.0 mm fraction (medium granules, MG), >2.0 mm fraction (large granules, LG). These terms were used consistently in this paper to distinguish different size fractions. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined to assess biomass concentration. Biomass samples were collected at the end of cycles, centrifuged and the clean supernatant discarded, then dried at 105 ℃ for 24 h to obtain TSS. Subsequently, the dried samples were incinerated at 550 ℃ for 3 h in a muffle furnace to determine ash content. VSS was calculated as the difference between the TSS and the remaining ash fraction.

Freeze-dried biomass samples were used for analyzing intracellular compounds such as polyhydroxyalkanoates (PHA) and glycogen. Biomass was first separated from the supernatant by centrifugation, then frozen at -80 ℃, followed by freeze-drying and grinding into a fine powder. For PHA extraction, approximately 20 mg of the powdered biomass was placed in 15 mL glass tubes and mixed with 1.5 mL of 10 % sulfuric acid in methanol and 1.5 mL of chloroform. The mixture was incubated at 100 ℃ for 20 h, with intermittent manual vortexing to ensure complete digestion. Following incubation, 3 mL of ultrapure water was added to the glass tubes, then the tubes were centrifuged to achieve phase separation. The organic phase was then collected, filtered, and dried to remove residual water before being analyzed using gas chromatography (GC). GC analysis was carried out using an Agilent 6890 N system (Agilent Technologies, USA) to quantify PHA content. For calibration, commercial standards (Sigma-Aldrich, USA) were used: 3-hydroxybutyrate for PHB (poly-hydroxy-butyrate) and Methyl (*R*)-3-hydroxyvalerate for PHV (poly-hydroxy-valerate). All calibration samples were processed and analyzed alongside biomass samples within the same batch to ensure the reliability of the calibration curve. Benzoic acid was added to each sample as an internal standard to enhance measurement accuracy and reproducibility. Intracellular PHA content was calculated as the sum of PHB and PHV masses, normalized to the added freeze-dried biomass weight.

Glycogen extraction from the biomass was carried out following the protocol by Lanham et al. (2012). 0.9 M hydrochloric acid (HCl) was added to the freeze-dried biomass sample, which was then subjected to hydrolysis by heating at 100 ℃ for 5 h. During this period, the samples were manually vortexed at regular intervals to ensure uniform digestion. After hydrolysis, the mixture was passed through 0.45 μm Millipore filters to remove particulates. Glucose quantification of the filtrate was subsequently determined using D-Glucose Assay Kit (Megazyme, Ireland), and a glucose solution standard was used for making the calibration curve.

2.4. Batch experiments

To investigate the performance differences between different biomass size fractions during anaerobic and aerobic conversions, three different batch experiments were carried out using size-separated biomass: anaerobic conversion batch tests, aerobic conversion batch tests, and a saturated DO batch test.

For anaerobic conversion batch tests, two distinct feeding strategies were applied: bottom-feeding batch test and mixed-feeding batch test. In the bottom-feeding batch, the feeding phase replicated the normal operational cycle of the reactor. Biomass samples were collected both before and after one hour of feeding, then sieved into different size fractions. The PHA content was measured for each fraction, and the difference in PHA concentrations before and after feeding was used to calculate the amount of PHA accumulated. The mixed-feeding batch test involved combining reactor biomass with the same influent volume in the reactor and then maintaining the reactor in the anaerobic mixing condition by continuously sparging nitrogen gas. After one hour of anaerobic mixing, the PHA content was measured, and the net accumulation was determined by subtracting the initial PHA content from the PHA content after anaerobic mixing. PHA accumulation was used as

an indicator of anaerobic substrate uptake and to understand substrate distribution between size fractions.

For aerobic conversion tests, biomass was withdrawn from the reactor immediately after the feeding phase. The bulk liquid and reactor biomass were separated, and the bulk liquid was mixed with water to prepare the medium for batch experiments. The biomass was then sieved, and only one size fraction at a time was reintroduced into the reactor, which was operated under the standard cycle conditions (2 mg/L DO). During these tests, bulk liquid samples were periodically collected throughout the cycle to measure concentrations of phosphate (PO_4^{3-}-P), ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N). Volumetric ammonium and phosphate uptake rates were determined by linear regression using at least five independent data points within the linear range. TSS were also measured for each fraction. The ammonium and phosphate uptake biomass-specific rates were calculated by normalizing volumetric uptake rates with the TSS concentrations. Simultaneous nitrification-denitrification (SND) efficiency was assessed for each size fraction using the data points of the beginning and end of the aerobic phase. The contribution of each size fraction to overall ammonium and phosphate removal was calculated by multiplying the biomass-specific uptake rates by the size distribution (based on TSS).

A saturated DO batch experiment was conducted to assess the impact of oxygen mass transfer limitation. Following the feeding phase, biomass was withdrawn from the reactor and separated into different size fractions. Each fraction was placed into an individual glass bottle. Air was continuously bubbled through the bottles to maintain saturated DO conditions and ensure adequate mixing for 2 h. The medium in each bottle was formulated to replicate the composition of the reactor's bulk liquid at the start of the aeration phase. A HEPES buffer was added along with the medium to achieve a final concentration of 0.1 mM for pH stabilization. The final volume was adjusted to approximately 250 mL using demineralized water. During the experiment, bulk liquid samples were collected from each bottle over time, and TSS concentrations were measured to calculate biomass-specific ammonium uptake rates.

2.5. Microscope and microbial community analysis

Morphology of the AGS biomass was examined using a stereo zoom microscope (M205 FA, Leica Microsystems, Germany). Granule images were captured and analyzed with the assistance of the Qwin image analysis software (V3.5.1, Leica Microsystems, Germany). The images of mixed biomass samples and sieved biomass samples were taken separately to provide more visual insight.

For microbial community analysis, biomass samples were sieved into different size fractions and stored in a -80 ℃ freezer before DNA and protein extraction. Genomic DNA was extracted from biomass samples using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), following the standard protocol provided by the manufacturer. Metagenomic sequencing and raw data processing were performed by Novogene Co. (China) using standard high-throughput sequencing workflows. The resulting metagenomic data served as the reference database for subsequent metaproteomic analysis. Protein extraction and metaproteomic procedures were carried out following the protocol of Kleikamp et al. (2023), with all samples processed consistently within the same batch. Microbial community composition was estimated based on metagenomics and metaproteomics results and compared across biomass size fractions.

3. Results

3.1. Coexistence of granules and flocs

The reactor was inoculated with granular sludge sourced from a full-scale wastewater treatment plant (Utrecht, Netherlands), and fed with complex synthetic wastewater. After five months of operation, the system reached a stable state characterized by a coexistence of granules and flocs (Fig. 1). The granules displayed smooth surfaces and varied in size. The size distribution of biomass based on total suspended solids (TSS) is presented in Fig. 2, showing that large granules comprised a significant portion of the total biomass while flocs comprised 17 % of the total biomass in the reactor. The biomass concentration was maintained at 6.3 gTSS/L and 4.8 gVSS/L, with a VSS to TSS ratio of 77 %. The sludge retention time (SRT) in the system varied for different sludge size fractions, with larger aggregate sizes having a longer SRT (Fig. 2).

3.2. Reactor performance

In the stable state, the reactor performed effective carbon (C), nitrogen (N), and phosphorus (P) removal. Fig. 3 shows a typical cycle measurement in the stable state. Approximately 90 % of the RBCOD was removed by biological conversion or capturing in the sludge fraction during the 1-h anaerobic phase, with the remaining RBCOD depleted in the first 10 min of the aerobic phase. Acetate, propionate, and glucose were not detected after the anaerobic phase. PHA accumulated during the anaerobic phase and were consumed during the aerobic phase, consistent with typical polyphosphate-accumulating organism (PAO) and glycogen-accumulating organism (GAO) metabolism (Fig. 3). Glycogen levels showed minimal variation throughout the cycle.

About 28 mg/L of phosphate (PO_4^{3-}-P) was released anaerobically, which was fully taken up within 1.5 h of aerobic operation, indicating effective enhanced biological phosphorus removal (EBPR) performance. Ammonium (NH_4^+) was rapidly consumed within 2.5 h of aerobic operation, at a rate of 2.0 mgNH_4^+-N/gTSS/h. Transient nitrite (NO_2^-) accumulation occurred but was quickly converted once NH_4^+ uptake was complete. A significant concentration of nitrate (NO_3^-) remained at the end of the cycle due to low denitrification efficiency. The reactor was not operated to maximize denitrification.

3.3. Anaerobic conversion batch tests

To better understand anaerobic substrate distribution between granules and flocs, anaerobic conversion batch tests were conducted. PHA accumulation per size fraction served as an indicator of substrate uptake. The bottom-feeding batch replicated the reactor's standard feeding mode, while the mixed-feeding batch ensured thorough mixing of biomass and substrate. The PHA accumulation amount of each size fraction was determined (Fig. 4). In both batch tests, granules accounted for the majority of PHA accumulation, indicating they captured most of the substrate. Compared to the bottom-feeding batch, the mixed-feeding batch showed a shift in substrate distribution from large granules to small granules and flocs.

From Fig. 4, higher PHA accumulation in the mixed-feeding batch than the bottom-feeding batch was observed across all size fractions, likely due to improved substrate contact in the mixed-feeding condition. Improved substrate contact may have enhanced hydrolysis and fermentation processes, leading to more volatile fatty acids (VFAs) production for PHA synthesis. Interestingly, flocs and small granules exhibited higher increases in PHA accumulation compared to medium granules and large granules, suggesting that when substrate exposure is equal, smaller biomass fractions gain greater access to available substrates.

3.4. Aerobic conversion batch tests

Aerobic conversion batch tests were conducted to assess the contributions of different biomass size fractions to aerobic ammonium and phosphate uptake, as well as their denitrification capacity. Biomass was separated by size after feeding, and each size fraction was tested under conditions identical to normal reactor cycles. Biomass-specific uptake rates of NH_4^+ and PO_4^{3-} for each fraction were determined and compared (Fig. 5). The specific NH_4^+ uptake rate showed a decreasing trend with increasing biomass fraction size. Flocs exhibited a significantly higher

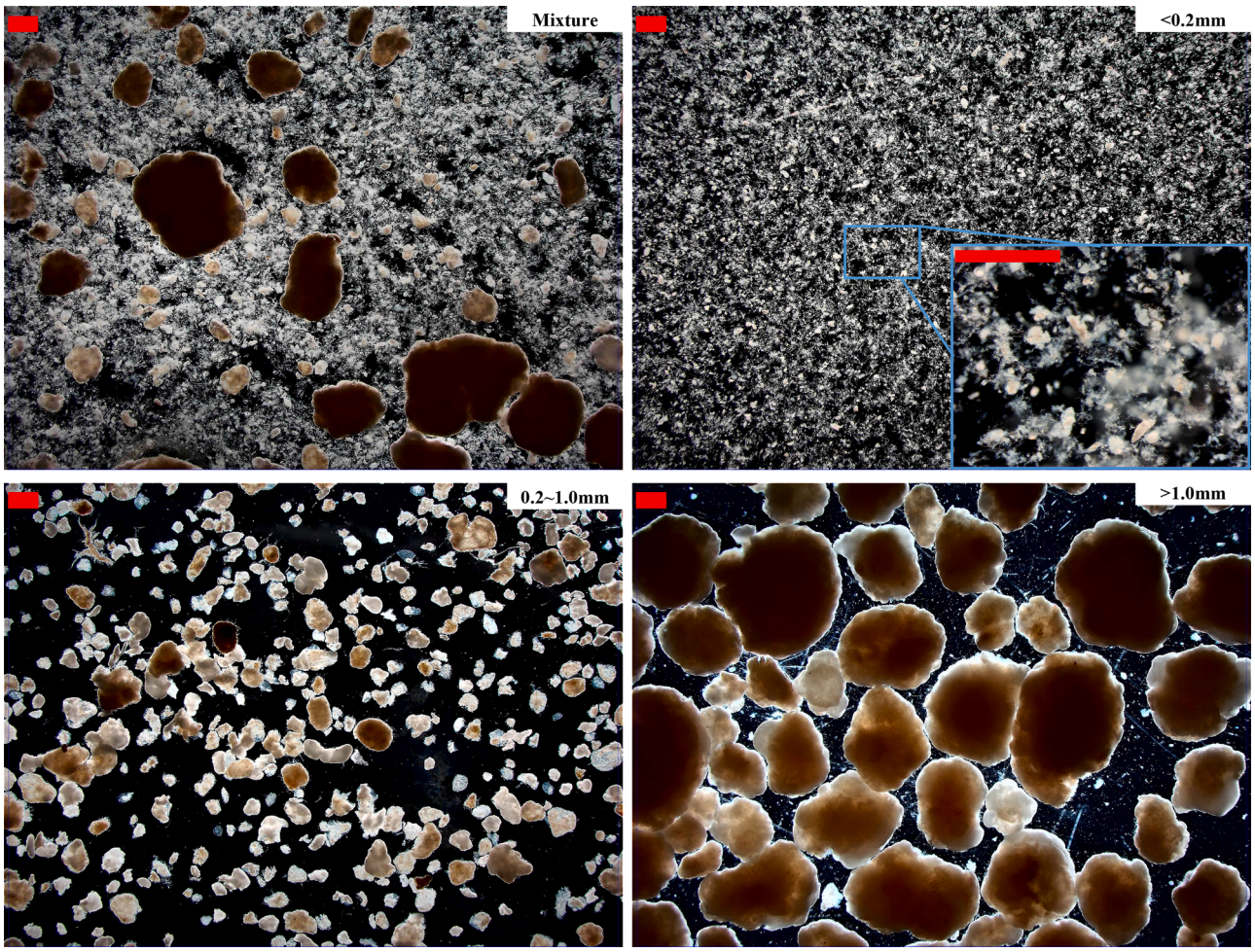

Fig. 1. Stereo zoom microscopy images of AGS laboratory reactor biomass (Day 155): a mixture of biomass and fractioned biomass using sieves. The red scale bar equals 1 mm in all images.

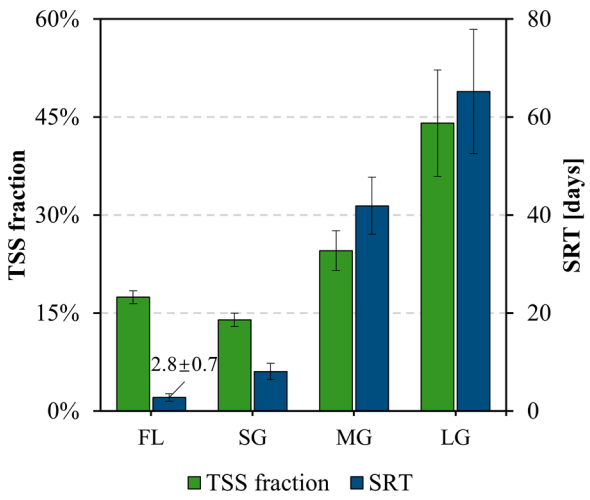

Fig. 2. Size distribution based on total suspended solids (TSS) and sludge retention time (SRT) over reactor biomass size fractions: FL (flocs; <0.2 mm fraction), SG (small granules; 0.2~1.0 mm fraction), MG (medium granules; 1.0~2.0 mm fraction), LG (large granules; >2.0 mm fraction).

NH_4^+ uptake rate compared to medium granules and large granules. In contrast, the specific PO_4^{3-} uptake rate increased with biomass size, with the highest rate observed in large granules.

Based on the biomass-specific rates and biomass size distribution, it was estimated that flocs contributed approximately 60 % of total NH_4^+ uptake, while medium granules and large granules contributed minimally (Fig. 6). Conversely, large granules accounted for 60 % of total PO_4^{3-} uptake, with flocs playing a minor role. The denitrification capability of different size fractions was assessed based on simultaneous nitrification-denitrification (SND) efficiency (Fig. 7). Flocs and small granules exhibited negligible denitrification capacity under normal cycle conditions (2 mg/L DO), while large granules achieved an SND efficiency of approximately 35 % and showed higher denitrification capacity. These findings highlight the distinct and complementary functional roles of flocs and granules in aerobic nutrient conversion.

3.5. *Saturated DO batch test*

Granules, due to their larger size, are more mass transfer limited than flocs, which affects oxygen transfer and consequently ammonium uptake rates. To assess the impact of oxygen mass transfer limitation on ammonium uptake, a batch test was conducted at saturated DO condition for different size fractions. The results are presented in Fig. 8, along with the results from the aerobic conversion batch tests (conducted at 2 mg/L DO). Under saturated DO condition, medium granules and large granules showed more than a twofold increase in specific NH_4^+ uptake rates compared to the 2 mg/L DO condition, indicating a strong mass transfer limitation. Small granules showed a moderate increase in specific NH_4^+ uptake rate. Flocs showed no change, which is likely because their small size doesn't make oxygen transfer limiting for NH_4^+ uptake

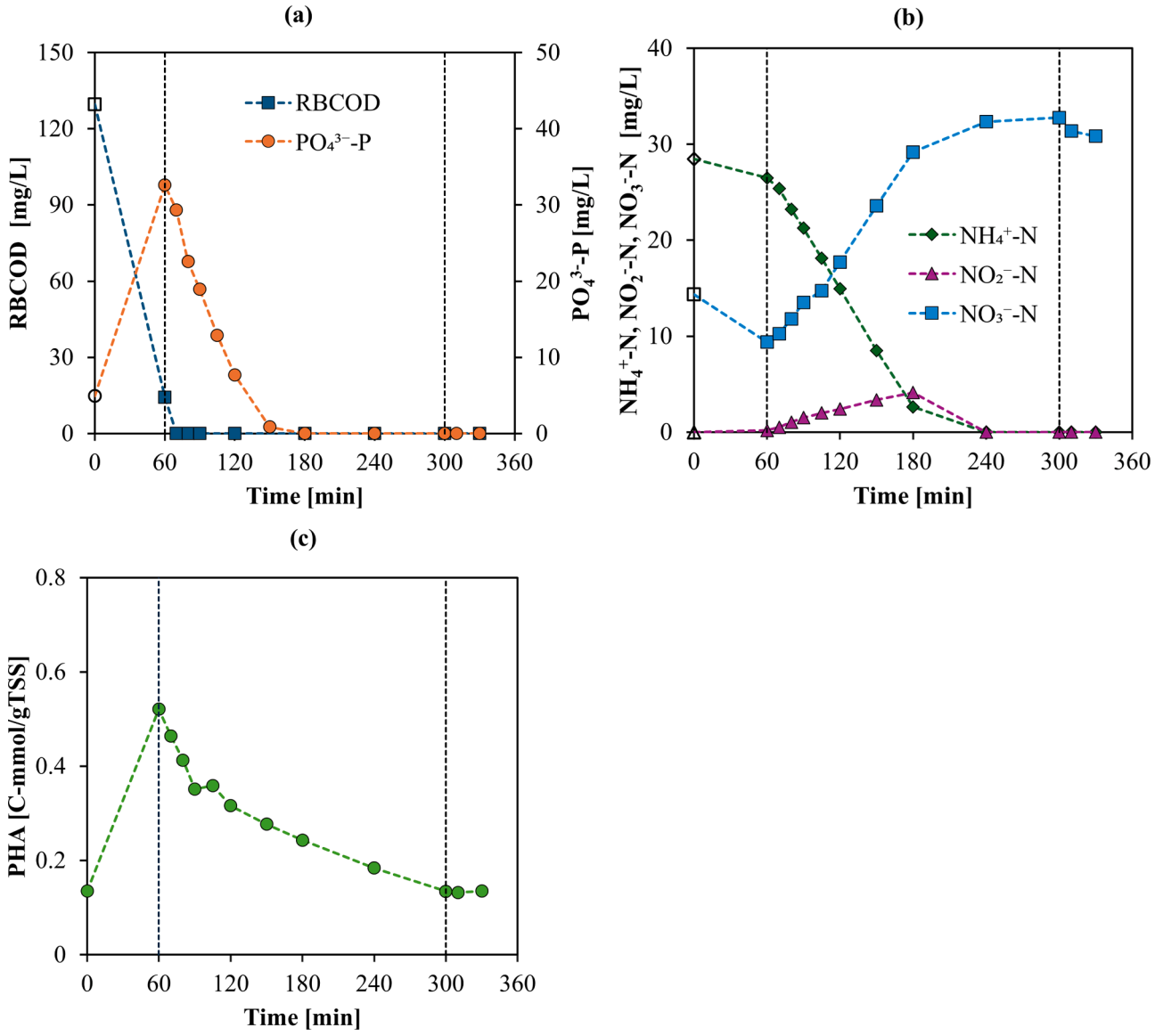

Fig. 3. Concentration profiles during a typical cycle (Day 158) of the laboratory aerobic granular sludge reactor in the stable state: (a) RBCOD (readily biodegradable chemical oxygen demand) and PO_4^{3-}-P in the bulk liquid; (b) NH_4^+-N, NO_2^--N, and NO_3^--N in the bulk liquid; (c) Intracellular PHA (poly-hydroxy-alkanoate) content. The first hollow markers represent calculated concentrations based on dilution and influent composition. The vertical dashed lines (t_{60} and t_{300}) indicate phase transitions during the cycle.

even at 2 mg/L DO condition. In terms of denitrification capacity, SND efficiency dropped to nearly zero for medium granules and large granules under saturated DO conditions, while SND for flocs and small granules was already absent at 2 mg/L DO.

3.6. Microbial community comparison

Metagenomic and metaproteomic analyses were conducted to investigate microbial community differences across various size fractions (the complete overview can be found in the supplementary data). Several key microbial genera—selected for their high relative abundance and functional importance in substrate conversion—were compared across the size fractions (Fig. 9). Overall, metagenomic and metaproteomic data revealed similar trends among the size fractions. Fig. 9 shows that *Nitrosomonas*, typically known as ammonium-oxidizing bacteria (AOB), had higher relative abundance in flocs and small granules compared to medium granules and large granules. *Candidatus* Accumulibacter, a well-known PAO, was more prevalent in granules (small granules, medium granules, and large granules) than in flocs. *Tetrasphaera*, often identified as a fermentative PAO, was significantly more abundant in flocs. These observed microbial community differences align with results from the activity batch tests, further emphasizing the distinct metabolic roles associated with different biomass size fractions.

4. Discussion

4.1. Experimental setup simulating full-scale AGS systems: substrates, operation, and biomass aggregates

The choice of substrate plays a crucial role in simulating the conditions of full-scale AGS systems in laboratory reactors. Many previous studies using laboratory AGS reactors have used VFAs, such as acetate, as the sole carbon source (de Kreuk et al., 2005a; Lin et al., 2003; Lochmatter and Holliger, 2014). While this approach is sufficient for studying granulation processes, it has limitations when it comes to understanding the complex conversion processes occurring in full-scale systems, such as hydrolysis and fermentation. When substrates are

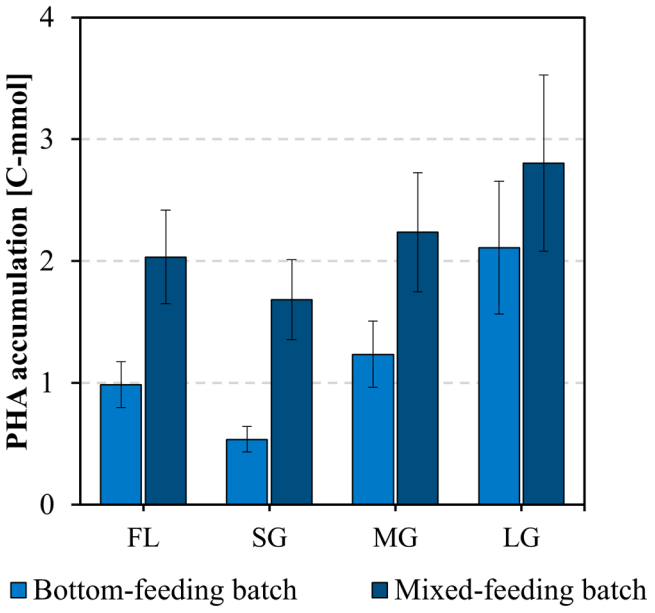

Fig. 4. Anaerobic substrate distribution indicated by PHA accumulation per size fraction in the bottom-feeding and mixed-feeding batch tests: FL (flocs; <0.2 mm), SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm).

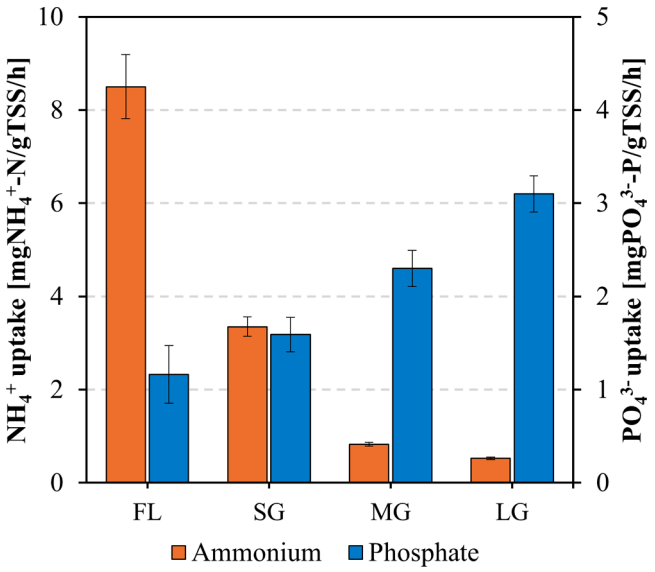

Fig. 5. Biomass-specific ammonium (NH_4^+) and phosphate (PO_4^{3-}) uptake rates by different size fractions in the aerobic conversion batch tests (at 2 mg/L DO): FL (flocs; <0.2 mm), SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm).

completely taken up during the anaerobic phase, acetate-fed lab reactors predominantly produce granules with minimal floc formation (Haaksman et al., 2020). The flocs formed in the acetate-fed reactor mainly arise from the erosion of granules, which differs fundamentally from the mechanisms driving floc formation in full-scale AGS reactors treating municipal wastewater. To incorporate fermentation or hydrolysis processes, some studies have used a single fermentative or particulate substrate. While this approach provides insights into the interaction between fermentation and granulation, it still lacks the complexity of real wastewater conditions (de Kreuk et al., 2010; Elahinik et al., 2022, 2023; Pronk et al., 2015a).

For more advanced studies, a synthetic wastewater recipe that can

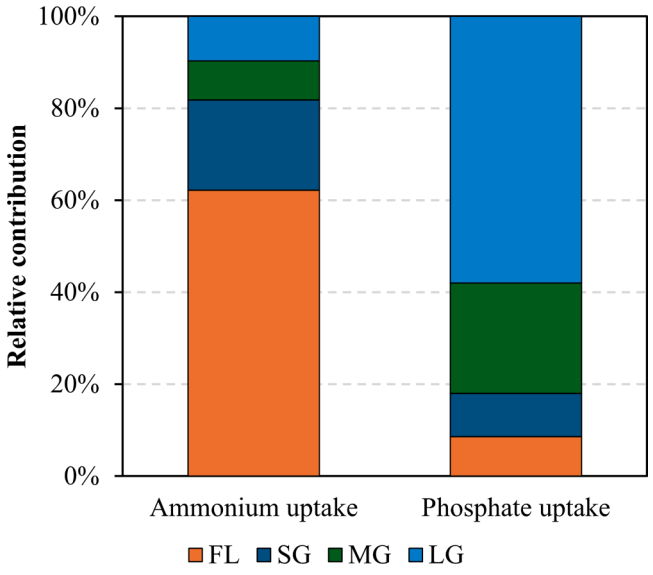

Fig. 6. Calculated relative contribution to total ammonium and phosphate uptake by different size fractions: FL (flocs; <0.2 mm), SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm).

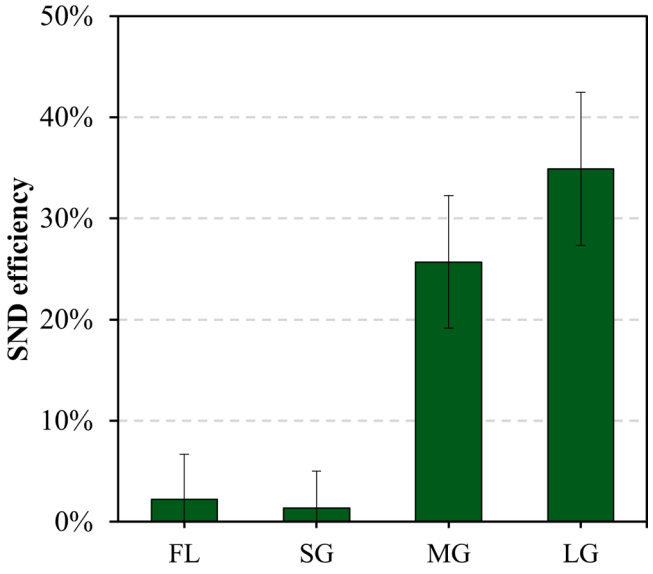

Fig. 7. Simultaneous nitrification-denitrification (SND) efficiency between different size fractions in aerobic conversion batch tests (at 2 mg/L DO): FL (flocs, <0.2 mm); SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm).

represent the complexity of municipal sewage is desired. A comparative study by O'Flaherty and Gray (2013) found that synthetic wastewater that mimics real wastewater typically includes particulate substrates such as starch and protein. In AGS systems, feeding particulate substrates leads to floc formation (Geng et al., 2025; Layer et al., 2019), and floc formation is important to replicate the sludge morphology in full-scale AGS reactors. These studies underscore the importance of incorporating particulate substrates alongside VFAs to better simulate real wastewater conditions, thus resulting in similar microbial community composition. Layer et al. (2019) used a substrate recipe containing VFAs, fermentable substrates, and particulate substrates, which supported granule formation and yielded a similar microbial community to feeding real wastewater. Following a similar approach, this study

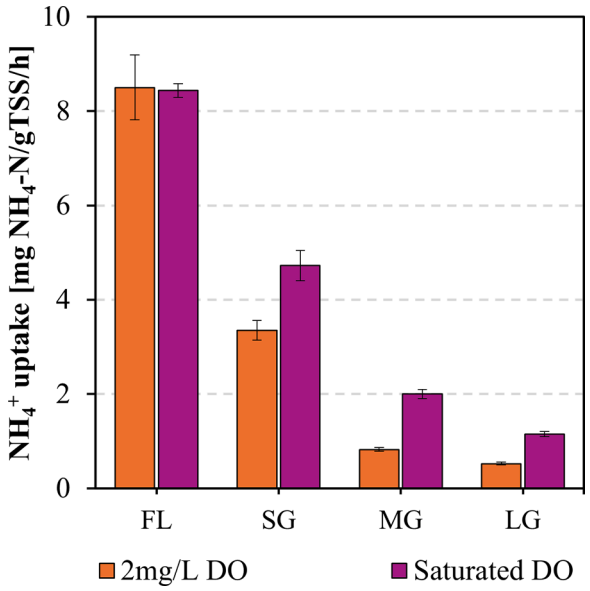

Fig. 8. Biomass-specific ammonium (NH_4^+) uptake rate of different biomass size fractions in aerobic conversion batch tests (at 2 mg/L DO) and saturated DO batch test (at saturated DO): FL (flocs; <0.2 mm), SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm).

adopted a substrate composition based on the work of Layer et al. (2019) with two minor differences. First, casein was used instead of peptone because casein serves as an undigested protein and allows for more hydrolysis. Second, fewer types of amino acids were used, which barely affects the representativeness since casein hydrolysis will yield a variety of amino acids. Overall, the complex substrates used in this study are expected to simulate real wastewater characteristics well.

Selective feeding and selective wasting are key operational strategies for successful granulation (van Dijk et al., 2022). Selective feeding of large granules ensures they receive preferential access to substrates, which is critical for granular growth and stability. To enable selective feeding, full-scale AGS systems typically employ a bottom-feeding strategy. Therefore, this laboratory-scale reactor employed the bottom-feeding strategy to simulate full-scale systems. In contrast, some laboratory studies have used mixed feeding, which fails to support selective feeding. Mixed feeding mode can lead to substrate availability favoring flocs over granules, ultimately hindering granule formation in the long term (Haaksman et al., 2024). Selective wasting involves discharging slow-settling biomass. Full-scale AGS reactors often employ a simultaneous feed and drain mode (Pronk et al., 2015b), while laboratory-scale reactors commonly use a separate feed and discharge mode (Adler and Holliger, 2020; de Kreuk et al., 2005b). Both modes can meet the requirements of selective feeding and selective wasting. The simultaneous feed and drain mode brings benefits of better effluent quality and shortened reactor cycle time, which is important for full-scale application but not needed in laboratory-scale studies. The separate feed and discharge mode is easier to operate in laboratory-scale and thus used in this study. Notably, laboratory-scale reactors are not able to simulate everything in full-scale reactors especially the large-scale hydrodynamic conditions, due to different operations like volumetric exchange ratio, feeding velocity, and height over diameter ratio.

This study's laboratory reactor demonstrated similarities in biomass aggregates to full-scale AGS systems, after employing complex synthetic wastewater and operational conditions that satisfy essential granulation mechanisms. The coexistence of granules and flocs was observed, with a floc mass fraction of 17 %, closely aligning with the 20 % reported for full-scale systems (Pronk et al., 2015b). This was achieved by including particulate substrates in the feeding and controlling the selective settling speed. The granular size distribution also matched full-scale observations. In this study, 70 % of granules were larger than 1.0 mm, comparable to 60 % at WWTP Garmerwolde (Pronk et al., 2015b) and 64 % at WWTP Utrecht (Haaksman et al., 2024). Different biomass size fractions exhibited varying SRT: flocs and small granules had shorter SRT, while medium granules and large granules had longer SRT. The finding of SRT distribution corresponds with observations from full-scale reactors (Ali et al., 2019). More importantly, the microbial community distribution across different size fractions is similar to what was reported in full-scale AGS plants (Mohamed et al., 2025b). In this study, flocculent sludge (flocs) shows a high abundance of *Nitrosomonas* and *Tetrasphaera* and a low abundance of *Ca.* Accumulibacter while granular sludge has a high abundance of *Ca.* Accumulibacter and *Ca.* Competibacter, which is in agreement with the study by Mohamed et al. (2025b). Overall, the laboratory reactor setup in this study successfully replicated key aspects of full-scale AGS systems, making it a valuable model for future research on granulation and nutrient removal processes.

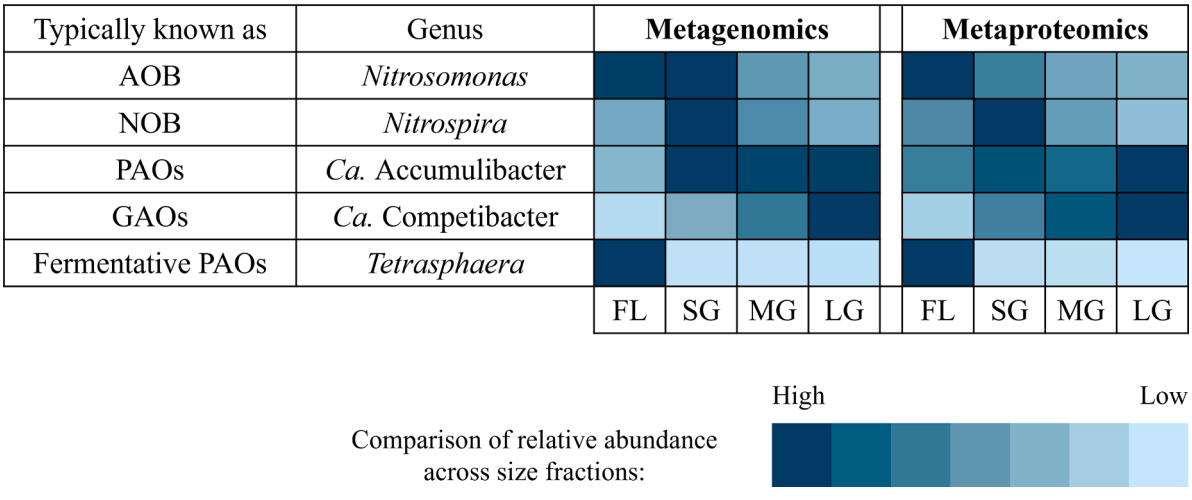

Fig. 9. Comparison of the relative abundance of key microbial genera across size fractions, based on metagenomic and metaproteomic analyses: FL (flocs; <0.2 mm), SG (small granules; 0.2~1.0 mm), MG (medium granules; 1.0~2.0 mm), LG (large granules; >2.0 mm). The blue scale refers to the comparison between different size fractions based on relative abundance for each genus: AOB (ammonium-oxidizing bacteria), NOB (nitrite-oxidizing bacteria), PAOs (polyphosphate-accumulating organisms), GAOs (glycogen-accumulating organisms).

4.2. Distinct roles of granules and flocs in anaerobic conversion

PHA accumulation provides valuable insight into substrate distribution over different biomass fractions during feeding (Haaksman et al., 2023). When using complex substrates instead of solely acetate, additional processes such as hydrolysis and fermentation occur and contribute to substrate uptake. Although hydrolysis and fermentation processes are not directly reflected in PHA accumulation, the VFAs produced in these processes are likely converted to PHA for storage. In this study, the high abundance of *Ca.* Accumulibacter and *Ca.* Competibacter indicates strong PHA accumulation potential, reinforcing the relevance of PHA accumulation as a good indicator of substrate uptake and distribution during the feeding period.

In a settling sludge bed, biomass stratification occurs naturally: large granules settle at the bottom while flocs remain near the top due to slower settling rates. Feeding the sludge bed from the bottom prioritizes substrate access for large granules, allowing them to store PHA more effectively. This is reflected in their 43 % contribution to PHA storage, compared to only 11 % contributed by small granules (Fig. 4). Metagenomics results revealed that granules had a low abundance of fermentative organisms but a high presence of PAOs and GAOs, suggesting that their PHA storage mainly results from direct VFA uptake. Despite limited substrate access, flocs still contributed 20 % to PHA storage (Fig. 4). This can be attributed to their higher abundance of fermentative organisms, such as *Tetrasphaera*, which facilitates the conversion of particulate and fermentable substrates into VFAs. The VFAs produced are subsequently stored by PAOs and GAOs as PHA. Moreover, although *Tetrasphaera*'s ability to store PHA is limited, it likely contributes partially to PHA storage in the floc fraction (Close et al., 2021). COD measurements indicated that not all substrates were consumed anaerobically. A small fraction of substrates was utilized aerobically, where flocculent sludge had better substrate access due to a larger specific surface area.

In the mixed-feeding batch test, substrates are evenly accessible among all biomass fractions during the anaerobic phase, eliminating the substrate access priority enjoyed by large granules in the bottom feeding mode. Consequently, substrate distribution shifts from large granules to small granules and flocs due to their larger specific surface area. Interestingly, higher PHA accumulation across all biomass sizes was observed in mixed feeding compared to bottom feeding (Fig. 4), likely due to better biomass-substrate contact and increased hydrolysis and fermentation. The contact time in mixed feeding (1 h) is longer than in bottom feeding (15∼20 min, depending on settled sludge bed height), which benefits the conversion of polymeric and particulate substrates (Wagner et al., 2015). The extended contact time enhances hydrolysis and fermentation, particularly for flocs with their strong fermenting capabilities (Toja Ortega et al., 2021a). Small granules also exhibit a significantly higher increase in PHA accumulation compared to flocs, despite both having minimal mass transfer limitations. This difference is likely due to the higher PAO abundance in small granules compared to flocs. The hydrolysis and fermentation processes within granule–floc coexistence systems are interesting for further investigation.

Although mixed feeding demonstrates some short-term benefits, the benefits may not be sustainable for long-term reactor performance (Haaksman et al., 2024). If mixed feeding is maintained, flocculent sludge will continue to outcompete granules for substrate, undermining the granulation process. The detrimental effect on granulation from mixed feeding will be even worse in full-scale conditions with less acidified wastewater. The anaerobic substrate distribution results underline the importance of implementing bottom feeding to maintain selective feeding operation and to support long-term stable granulation.

4.3. Distinct roles of granules and flocs in aerobic conversion: nitrification, denitrification, and phosphate uptake

Aerobic batch tests revealed differences in specific ammonium uptake rate and SND efficiency between granules and flocs (Figs. 5 and 7), reflecting their distinct roles in nitrification and denitrification. These different activities are the consequence of mass transfer limitation differences and microbial community differences. Flocs and small granules have a larger specific surface area, which leads to less mass transfer limitation and thus higher specific nitrification rates. The mass transfer limitation effect is evident from the saturated DO batch test (Fig. 8). Large granules and medium granules showed significant increases in ammonium uptake when DO was elevated, as more oxygen penetrated into the granules, enhancing nitrification. In contrast, the specific ammonium uptake rate of flocs showed no change under both DO conditions, indicating that oxygen mass transfer within the flocs does not limit nitrification when DO exceeds 2 mg/L. Small granules displayed a moderate increase in ammonium uptake rate. Microbial community composition influenced nitrification rates as well. Flocs and small granules had a higher relative abundance of *Nitrosomonas* (Fig. 9), aligning with their higher ammonium uptake rates compared to medium and large granules. The layered distribution of nitrifiers within granules may also affect nitrification performance, which can be an interesting topic for further study. The limited oxygen penetration in large granules created more anoxic zones, enhancing their denitrification performance. At 2 mg/L DO, large granules and medium granules achieved higher SND efficiency than small granules and flocs (Fig. 7). However, when DO was raised to 100 %, oxygen penetrated further within medium and large granules, eliminating anoxic zones and denitrification performance.

The findings about nitrification activity distribution align with other full-scale and pilot-scale studies (Britschgi et al., 2025; Nguyen Quoc et al., 2021b). Nguyen Quoc et al. (2021b) reported that small granules (0.33 mm) had 4.7 times the ammonium uptake rate of large granules (2.2 mm), closely mirroring this study's result, where small granules (0.2∼1 mm) showed a 4.1-fold higher rate than large granules (>2 mm) (Fig. 8). Britschgi et al. (2025) observed a very similar trend in a full-scale AGS reactor, where the maximum specific ammonium uptake rate decreased with increasing sludge size. Although they reported comparable ammonium uptake rates between flocs and small granules, this study found significantly higher specific uptake rates in flocs than in small granules. This discrepancy may be attributed to the use of a laboratory reactor and synthetic wastewater in this study, which lacks suspended solids that would typically accumulate in the floc fraction in full-scale systems. Consequently, the active biomass per gram of TSS in flocs is likely higher in this study, resulting in a higher specific ammonium uptake rate when normalized by TSS. In terms of overall nitrification (derived from ammonium uptake), flocs contributed approximately 60 % of the total nitrification activity in this study (Fig. 6), consistent with findings from a full-scale system where flocs accounted for around 50 % of nitrification activity (Britschgi et al., 2025). For estimating the contribution of each fraction to nitrification, maximum ammonium uptake rates obtained from batch tests under saturated DO conditions are commonly used (Britschgi et al., 2025; Haaksman et al., 2024). However, full-scale reactors typically operate at DO concentrations between 1 and 4 mg/L (Pronk et al., 2015b; van Dijk et al., 2021), which are well below the saturated DO concentration. As shown in this study (Fig. 8), the actual ammonium uptake rate of granules under these lower DO levels is likely reduced compared to rates measured under saturated DO conditions. This suggests that using maximum uptake rates for estimating contributions may underestimate the actual contribution of flocs to overall nitrification, even though flocs' contribution is already substantial. The similarity between this study's laboratory-based findings and full-scale reactor results underscores the laboratory setup's representativeness and the critical role of flocs in nitrification.

Granules and flocs also exhibit distinct roles in phosphate uptake (Figs. 5 and 6). *Ca.* Accumulibacter is known to utilize stored PHA for phosphate uptake during the aerobic phase (Mino et al., 1998; Smolders et al., 1994). Consequently, the abundance of *Ca.* Accumulibacter and

the amount of anaerobically stored PHA largely determine the capacity for aerobic phosphate uptake. Metagenomic analysis indicated similar *Ca.* Accumulibacter abundance across granules (small granules, medium granules, and large granules) (Fig. 9), suggesting that differences in aerobic phosphate uptake are more closely linked to anaerobic PHA storage. Since large granules store more PHA during bottom feeding (Fig. 4), they are expected to contribute more to phosphate uptake than small granules and flocs, consistent with our observations (Fig. 6). This highlights the link between anaerobic substrate storage and subsequent aerobic phosphate uptake, underscoring the critical connection between anaerobic and aerobic conversion processes.

4.4. Factors driving the distinct microbial communities of granules and flocs

The distinct microbial communities found in granules and flocs result from long-term microbial selection shaped by cumulative short-term operational conditions. Factors such as feeding strategies, mass transfer, and SRT collectively create favorable environments for different microbial groups, ultimately driving structural differences in microbial communities between granules and flocs. By using bottom feeding, granules gain preferential access to substrates, particularly VFAs, due to their position near the wastewater inlet. This advantage enriches the granules with VFA-uptake microorganisms, such as PAOs and GAOs (Fig. 9). After VFAs are depleted by granules, flocs primarily get access to particulate and fermentable substrates, promoting the growth of fermentative microorganisms such as *Tetrasphaera*. A similar distribution of microorganisms was observed in full-scale reactors (Mohamed et al., 2025b). Flocculent structure is well-suited for capturing and fermenting the particulate substrates (Toja Ortega et al., 2021a), which explains the higher abundance of *Tetrasphaera* in flocs compared to small granules (Fig. 9).

Mass transfer plays a critical role in shaping microbial communities, particularly among nitrifiers. In this laboratory reactor, *Nitrosomonas* and *Nitrospira* were used to represent ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively (Fig. 9). No other AOB, such as *Nitrosococcus*, were detected. Among NOB, *Nitrospira* was the most dominant genus, while *Nitrotoga* appeared in very low abundance, and *Nitrobacter* was not detected. The low presence of *Nitrotoga* may be attributed to the reactor's constant operating temperature of 20 °C, as this genus typically becomes more prevalent at lower temperatures (Spieck et al., 2021). Based on these findings, *Nitrospira* was used to represent the NOB population in this study. The growth of AOB is often constrained by oxygen mass transfer limitations; thus, environments with reduced mass transfer limitations are more favorable for AOB proliferation. Small-sized bioaggregates, such as flocs and small granules, experience minimal mass transfer limitation due to their high surface-to-volume ratio, which creates favorable conditions for AOB growth. As expected, flocs and small granules exhibit higher relative abundances of *Nitrosomonas* compared to medium granules and large granules (Fig. 9).

The abundance of AOB directly impacts the abundance of NOB, as AOB activity generates the nitrite that is the substrate required by NOB. Consequently, small granules with higher AOB abundance also exhibit a greater abundance of NOB. In addition to AOB availability, SRT is a critical factor for NOB growth, as NOB generally require longer SRTs to persist than AOB (Wu et al., 2016). In this reactor, flocs exhibited short SRTs (Fig. 2), which do not provide favorable conditions for sustained NOB growth. This likely explains the significantly lower NOB abundance in flocs compared to small granules, despite similarly high AOB levels (Fig. 9). Nevertheless, the presence of NOB in the floc fraction may not be solely attributed to *in situ* growth. Granular breakage and subsequent reseeding can introduce NOB into the flocs, contributing to their presence in this fraction (Mohamed et al., 2025a; van Dijk et al., 2022). This process may explain the detectable NOB population in flocs, even under SRT conditions near the lower limit required for NOB retention. Bioaugmentation of nitrifiers has been shown to be effective for enhancing nitrification (Salem et al., 2003). In AGS systems, the transfer of nitrifiers from granules to flocs through reseeding may serve a similar bioaugmentation function, supporting nitrification within the floc fraction.

4.5. Implications for full-scale wastewater treatment optimization

Operation of the AGS laboratory reactor provides valuable insights for optimizing full-scale wastewater treatment systems. A clear understanding of the distinct functional roles of granules and flocs can inform process control strategies aimed at enhancing nutrient removal and overall reactor performance. This study highlights the complementary functional roles of granules and flocs in nutrient removal. Flocs are particularly favorable for nitrification, so their presence can enhance nitrification efficiency. In contrast, granules are more effective in phosphorus uptake and denitrification, suggesting that promoting granule abundance can support improved performance in phosphorus uptake and denitrification. Selective management of biomass size fractions—through both enrichment and discharge of flocs and granules—emerges as a promising operational strategy for full-scale AGS systems. Granule enrichment requires long-term operational practices that encourage granule formation and stability (van Dijk et al., 2022), whereas selective discharge can be implemented more rapidly by adjusting settling and excess sludge removal phases. Therefore, an effective selective management strategy should integrate both short-term and long-term actions, tailored to specific treatment goals and operational conditions.

A common misconception in AGS systems is that larger granules are inherently more desirable. However, this study challenges that assumption, showing that large-sized granules are subject to significant mass transfer limitation, which can hinder efficient nutrient conversion. In contrast, small-sized granules experience less mass transfer limitation and exhibit higher nutrient removal efficiency, while still retaining good settling properties. These findings highlight the importance of managing granule size distribution to avoid the predominance of overly large granules in full-scale systems. In AGS systems where different size fractions coexist, it is natural to seek an optimal size distribution (Nguyen Quoc et al., 2021a). However, the ideal biomass size distribution is highly dependent on wastewater composition and environmental factors, both of which can vary substantially across locations and over time. Therefore, this study argues that a universally optimal size distribution is unlikely to exist. Instead, the selective management of flocs and granules should be tailored to site-specific wastewater characteristics, environmental conditions, operational strategies, and treatment objectives to achieve improved and resilient system performance.

Beyond nutrient removal, flocs and granules play essential roles in other aspects of reactor performance. Flocs contribute to the removal of influent suspended solids, enhancing effluent quality. Meanwhile, granules help create a better plug flow pattern during wastewater feeding, which promotes anaerobic conversions and fosters conditions favorable for granulation. The complementary roles of flocs and granules highlight the importance of maintaining their balanced coexistence to support robust and efficient treatment outcomes in full-scale AGS wastewater treatment systems.

5. Conclusion

- A coexistence of granules and flocs was successfully achieved in a laboratory AGS reactor. The laboratory reactor setup used in this study demonstrated a distribution of microbial activity and community composition across different biomass size fractions that closely resembles those found in full-scale AGS systems, making it valuable for future nutrient removal research.
- Granules exhibited higher PHA storage capacity due to preferential substrate access enabled by bottom-feeding, whereas flocs showed

higher potential for hydrolysis and fermentation. This functional differentiation is supported by the enrichment of PAOs and GAOs in granules and the higher relative abundance of fermentative microorganisms in flocs.
- In aerobic conditions, flocs play a dominant role in nitrification, while granules contribute significantly to phosphorus uptake and denitrification. Large granules and medium granules showed limited nitrification capability, which is attributed to mass transfer limitation of oxygen and low abundance of nitrifiers.
- Metagenomic and metaproteomic analyses revealed distinct microbial communities between granules and flocs, mainly driven by long-term effects of feeding strategy, mass transfer, and SRT.
- Granules and flocs perform distinct yet complementary functions, making their balanced coexistence crucial for optimizing nutrient removal in full-scale AGS reactors.

Declaration of generative AI in scientific writing

During the preparation of this work, the author used ChatGPT to improve the structure and readability of the article. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

CRediT authorship contribution statement

Ling-Hang Li: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martin Pabst:** Methodology, Investigation. **Mark C.M. van Loosdrecht:** Writing – review & editing, Supervision, Methodology. **Mario Pronk:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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.

Acknowledgment

This project was funded by the Spinoza Prize to Prof. van Loosdrecht by NWO (Dutch Research Council, Netherlands). The author would like to thank the staff of TU Delft (Dirk Geerts, Ben Abbas, and Dita Heikens) for their assistance in building the laboratory reactor setup, DNA extraction, and protein extraction.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.124671.

Data availability

Data will be made available on request.

References

Adler, A., Holliger, C., 2020. Multistability and reversibility of aerobic granular sludge microbial communities upon changes from simple to complex synthetic wastewater and back. Front. Microbiol. 11, 574361.
Ali, M., Wang, Z., Salam, K.W., Hari, A.R., Pronk, M., van Loosdrecht, M.C.M., Saikaly, P. E., 2019. Importance of species sorting and immigration on the bacterial assembly of different-sized aggregates in a full-scale aerobic granular sludge plant. Environ. Sci. Technol. 53 (14), 8291–8301.
Bengtsson, S., de Blois, M., Wilén, B.-M., Gustavsson, D., 2018. Treatment of municipal wastewater with aerobic granular sludge. Crit. Rev. Environ. Sci. Technol. 48 (2), 119–166.
Britschgi, L., Wei, S., Proesl, A., Morgenroth, E., Derlon, N., 2025. The critical role of flocs in nitrification in full-scale aerobic granular sludge-based WWTP. Water. Res. 274, 123021.
Close, K., Marques, R., Carvalho, V.C.F., Freitas, E.B., Reis, M.A.M., Carvalho, G., Oehmen, A., 2021. The storage compounds associated with Tetrasphaera PAO metabolism and the relationship between diversity and P removal. Water. Res. 204, 117621.
de Kreuk, M.K., Heijnen, J.J., van Loosdrecht, M.C., 2005a. Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. Biotechnol. Bioeng. 90 (6), 761–769.
de Kreuk, M.K., Kishida, N., Tsuneda, S., van Loosdrecht, M.C., 2010. Behavior of polymeric substrates in an aerobic granular sludge system. Water. Res. 44 (20), 5929–5938.
de Kreuk, M.K., Pronk, M., van Loosdrecht, M.C., 2005b. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water. Res. 39 (18), 4476–4484.
de Kreuk, M.K., van Loosdrecht, M.C.M., 2004. Selection of slow growing organisms as a means for improving aerobic granular sludge stability. Water Sci. Technol. 49 (11–12), 9–17.
Ekholm, J., Persson, F., de Blois, M., Modin, O., Pronk, M., van Loosdrecht, M.C.M., Suarez, C., Gustavsson, D.J.I., Wilén, B.-M., 2022. Full-scale aerobic granular sludge for municipal wastewater treatment – granule formation, microbial succession, and process performance. Environ. Sci.: Water Res. Technol. 8 (12), 3138–3154.
Elahinik, A., Haarsma, M., Abbas, B., Pabst, M., Xevgenos, D., van Loosdrecht, M.C.M., Pronk, M., 2022. Glycerol conversion by aerobic granular sludge. Water. Res. 227, 119340.
Elahinik, A., Li, L., Pabst, M., Abbas, B., Xevgenos, D., van Loosdrecht, M.C.M., Pronk, M., 2023. Aerobic granular sludge phosphate removal using glucose. Water. Res. 247.
Geng, M., Li, T., Qu, F., Gao, S., Tian, J., 2025. Insights into the impact of feeding with polymers on aerobic granular sludge development and stability: performance and mechanisms. Bioresour. Technol. 426, 132368.
Geng, M., You, S., Guo, H., Ma, F., Xiao, X., Ma, X., 2023. Co-existence of flocs and granules in aerobic granular sludge system: performance, microbial community and proteomics. Chem. Eng. J. 451.
Haaksman, V.A., Mirghorayshi, M., van Loosdrecht, M.C.M., Pronk, M., 2020. Impact of aerobic availability of readily biodegradable COD on morphological stability of aerobic granular sludge. Water. Res. 187, 116402.
Haaksman, V.A., Schouteren, M., van Loosdrecht, M.C.M., Pronk, M., 2023. Impact of the anaerobic feeding mode on substrate distribution in aerobic granular sludge. Water. Res. 233, 119803.
Haaksman, V.A., van Dijk, E.J.H., Al-Zuhairy, S., Mulders, M., Loosdrecht, M., Pronk, M., 2024. Utilizing anaerobic substrate distribution for growth of aerobic granular sludge in continuous-flow reactors. Water. Res. 257, 121531.
Hamza, R., Rabii, A., Ezzahraoui, F.-z., Morgan, G., Iorhemen, O.T., 2022. A review of the state of development of aerobic granular sludge technology over the last 20 years: full-scale applications and resource recovery. Case Stud. Chem. Environ. Eng. 5.
Kleikamp, H.B.C., Grouzdev, D., Schaasberg, P., van Valderen, R., van der Zwaan, R., Wijgaart, R.V., Lin, Y., Abbas, B., Pronk, M., van Loosdrecht, M.C.M., Pabst, M., 2023. Metaproteomics, metagenomics and 16S rRNA sequencing provide different perspectives on the aerobic granular sludge microbiome. Water. Res. 246, 120700.
Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., Reis, M.A., 2012. Optimisation of glycogen quantification in mixed microbial cultures. Bioresour. Technol. 118, 518–525.
Layer, M., Adler, A., Reynaert, E., Hernandez, A., Pagni, M., Morgenroth, E., Holliger, C., Derlon, N., 2019. Organic substrate diffusibility governs microbial community composition, nutrient removal performance and kinetics of granulation of aerobic granular sludge. Water. Res. X. 4, 100033.
Lin, Y.M., Liu, Y., Tay, J.H., 2003. Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. Appl. Microbiol. Biotechnol. 62 (4), 430–435.
Lochmatter, S., Holliger, C., 2014. Optimization of operation conditions for the startup of aerobic granular sludge reactors biologically removing carbon, nitrogen, and phosphorous. Water. Res. 59, 58–70.
Mino, T., van Loosdrecht, M.C.M., Heijnen, J.J., 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. Water. Res. 32 (11), 3193–3207.
Mohamed, A.Y.A., Gill, L., Monleon, A., Pronk, M., van Loosdrecht, M., Saikaly, P.E., Ali, M., 2025a. Genome-resolved metatranscriptomics provide insights on immigration influence in structuring microbial community assembly of a full-scale aerobic granular sludge plant. Environ. Sci. Technol. 59 (12), 6126–6141.
Mohamed, A.Y.A., Gill, L., Monleon, A., Pronk, M., van Loosdrecht, M., Saikaly, P.E., Ali, M., 2025b. Genome-resolved metatranscriptomics unveils distinct microbial functionalities across aggregate sizes in aerobic granular sludge. Environ. Sci. Ecotechnol. 25, 100560.
Nguyen Quoc, B., Armenta, M., Carter, J.A., Bucher, R., Sukapanpotharam, P., Bryson, S. J., Stahl, D.A., Stensel, H.D., Winkler, M.H., 2021a. An investigation into the optimal granular sludge size for simultaneous nitrogen and phosphate removal. Water. Res. 198, 117119.
Nguyen Quoc, B., Wei, S., Armenta, M., Bucher, R., Sukapanpotharam, P., Stahl, D.A., Stensel, H.D., Winkler, M.H., 2021b. Aerobic granular sludge: impact of size distribution on nitrification capacity. Water. Res. 188, 116445.
Nor Anuar, A., Ujang, Z., van Loosdrecht, M.C., de Kreuk, M.K., 2007. Settling behaviour of aerobic granular sludge. Water. Sci. Technol. 56 (7), 55–63.

O'Flaherty, E., Gray, N.F., 2013. A comparative analysis of the characteristics of a range of real and synthetic wastewaters. Environ. Sci. Pollut. Res. Int. 20 (12), 8813–8830.
Pronk, M., Abbas, B., Al-Zuhairy, S.H., Kraan, R., Kleerebezem, R., van Loosdrecht, M.C., 2015a. Effect and behaviour of different substrates in relation to the formation of aerobic granular sludge. Appl. Microbiol. Biotechnol. 99 (12), 5257–5268.
Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C., 2015b. Full scale performance of the aerobic granular sludge process for sewage treatment. Water. Res. 84, 207–217.
Ruiz-Haddad, L., Shaw, D.R., Ali, M., Pronk, M., van Loosdrecht, M.C.M., Saikaly, P.E., 2025. Characterization of the core microbial community across different aggregate sizes in full-scale aerobic granular sludge plants and their relevance to wastewater treatment performance. Water. Res. 274.
Salem, S., Berends, D.H., Heijnen, J.J., Van Loosdrecht, M.C., 2003. Bio-augmentation by nitrification with return sludge. Water. Res. 37 (8), 1794–1804.
Smolders, G.J., van der Meij, J., van Loosdrecht, M.C., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. Biotechnol. Bioeng. 43 (6), 461–470.
Spieck, E., Wegen, S., Keuter, S., 2021. Relevance of Candidatus Nitrotoga for nitrite oxidation in technical nitrogen removal systems. Appl. Microbiol. Biotechnol. 105 (19), 7123–7139.
Strubbe, L., Pennewaerde, M., Baeten, J.E., Volcke, E.I.P., 2022. Continuous aerobic granular sludge plants: better settling *versus* diffusion limitation. Chem. Eng. J. 428.
Toja Ortega, S., Pronk, M., de Kreuk, M.K., 2021a. Anaerobic hydrolysis of complex substrates in full-scale aerobic granular sludge: enzymatic activity determined in different sludge fractions. Appl. Microbiol. Biotechnol. 105 (14–15), 6073–6086.
Toja Ortega, S., Pronk, M., de Kreuk, M.K., 2021b. Effect of an Increased Particulate COD Load on the Aerobic Granular Sludge Process: a Full Scale Study. Processes 9 (8).
van der Roest, H.F., Niermans, R.P., de Bruin, L.M.M., Giesen, A., 2013. Advancements in the application of aerobic granular biomass technology for sustainable treatment of wastewater. Water Pract. Technol. 8 (1), 47–54.
van Dijk, E.J.H., Haaksman, V.A., van Loosdrecht, M.C.M., Pronk, M., 2022. On the mechanisms for aerobic granulation - model based evaluation. Water. Res. 216, 118365.
van Dijk, E.J.H., Pronk, M., van Loosdrecht, M.C.M., 2018. Controlling effluent suspended solids in the aerobic granular sludge process. Water. Res. 147, 50–59.
van Dijk, E.J.H., van Loosdrecht, M.C.M., Pronk, M., 2021. Nitrous oxide emission from full-scale municipal aerobic granular sludge. Water. Res. 198, 117159.
van Loosdrecht, M.C.M., Uijterlinde, C., van der Roest, H.F.R., de Kreuk, M.K., de Bruin, L.M.M., 2004. Aerobic granular sludge technology: an alternative to activated sludge? Water Sci. Technol. 49 (11–12), 1–7.
Wagner, J., Weissbrodt, D.G., Manguin, V., da Costa, R.H., Morgenroth, E., Derlon, N., 2015. Effect of particulate organic substrate on aerobic granulation and operating conditions of sequencing batch reactors. Water. Res. 85, 158–166.
Wei, S.P., Stensel, H.D., Ziels, R.M., Herrera, S., Lee, P.H., Winkler, M.H., 2021. Partitioning of nutrient removal contribution between granules and flocs in a hybrid granular activated sludge system. Water. Res. 203, 117514.
Weissbrodt, D.G., Lochmatter, S., Ebrahimi, S., Rossi, P., Maillard, J., Holliger, C., 2012. Bacterial selection during the formation of early-stage aerobic granules in wastewater treatment systems operated under wash-out dynamics. Front. Microbiol. 3, 332.
Winkler, M.H., van Loosdrecht, M.C.M., 2022. Intensifying existing urban wastewater. Science (1979) 375 (6579), 377–378.
Wu, J., He, C., van Loosdrecht, M.C.M., Pérez, J., 2016. Selection of ammonium oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB) based on conversion rates. Chem. Eng. J. 304, 953–961.