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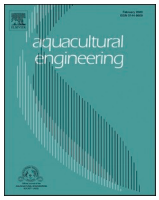

# Nascent application of aerobic granular sludge for recirculating aquaculture system effluent treatment: Performance, granule formation, and microbial community

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ABSTRACT

This study presents for the first time an evaluation of the feasibility of aerobic granular sludge (AGS) for treating recirculating aquaculture system (RAS) effluent in a sequential batch reactor configuration for nutrient removal. An AGS process was started using synthetic wastewater to grow the granules, and the feed was then switched to RAS effluent, and a systematically decreasing carbon supplementation was applied. Total nitrogen removal significantly decreased from around 75 % to as low as 13 %, but granules could restore their performance when allowed enough time (2 weeks) to acclimate to the change in feed. The dynamics of AGS microbial communities were followed by Illumina sequencing. A high abundance of microbial populations—indicating dense and stable granules—was observed after 97 days of operation with RAS wastewater. In particular, the genera *Neomegalonema, Hydrogenophaga, Thauera, Bdellovibrio, Flavobacterium,* and *Pseudomonas* represented most of the community, showing the heterotrophic, denitrifying, and phosphorus-accumulating potential of the studied operational design. The AGS showed promising results for a small-footprint solution for RAS treatment, but the energy consumption of aeration and carbon addition still requires further development.

## 1. Introduction

Recirculating aquaculture systems (RAS) is a relatively new method in the field of aquacultural food production. RAS is characterized as a booming industry globally, with a recent annual growth rate estimated at 5.3 % during 2001–2018 (Ahmed and Turchini, 2021). Such a high growth rate is attributed to the general rise in food demand associated with population increase and the growing interest in a seafood diet for its rich nutritional values (Willer et al., 2022). The attractive features of RAS, such as a conservative use of water and land, a well-controlled growth environment, nutrient recirculation, and minimal environmental impact have motivated the further flourishing of the industry (Badiola et al., 2018; Midilli et al., 2012). RAS are land-based facilities that grow fish in tanks and have a water treatment system that treats the water from the fish tanks so it can be reused in the system (Ahmed and Turchini, 2021; Turcios and Papenbrock, 2014). Water exchange of 10–20 % is required in RAS to avoid nitrate accumulation, which could negatively impact fish health (Tom et al., 2021). This amount of water requires efficient treatment. Otherwise, its discharge into an aquatic environment could cause harmful effects on the environment, such as eutrophication (Ahmed and Turchini, 2021).

RAS typically produces two types of wastewater streams: sludge containing mostly phosphorus and solids, and clearer effluent containing mostly nitrogen in the form of nitrates. The treatment of this effluent is challenging due to its low organic matter content. Currently, nitrate-containing effluents could be treated using external carbon-fed denitrification reactors, based on biofilm (van Rijn et al., 2006) or activated sludge. The more cost-efficient alternative is the application of constructed wetlands, which also has its shortcomings, such as a large footprint and low performance in cold weather (Kadlec and Reddy, 2001). However, constructed vertical wetlands have efficiently been utilized for treating RAS effluent in a hybrid system with a wood chip bioreactor and sand infiltration (Pulkkinen et al., 2021a). Wood chip bioreactors combined with only sand filtration have also been found to be effective in removing nitrogen, heavy metals, and off-flavor-inducing compounds (Lindholm-Lehto et al., 2021). Despite the promising results

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of these studies, they are relatively new, and their feasibility still requires further investigation (Pulkkinen et al., 2021a). Additionally, implementing a multi-stage treatment process requires large land usage, which might not be an attractive point for RAS facilities. Therefore, exploring other treatment options with a small footprint is still required for building compact RAS facilities. Aerobic granular sludge (AGS) is an emerging technology with great potential for treating RAS effluent while maintaining a small footprint. Denitrification of RAS effluent requires external carbon similar to the other intensified treatment. Since AGS has shown good performance for different types of effluents—including abattoir, live-stock, rubber, landfill leachate, dairy, brewery, textile, and others —it could be a promising technology for treating RAS effluent.

AGS is often defined as a round or ellipsoidal aggregated mass of microorganisms within a matrix of extracellular polymeric substances (EPS) (Corsino et al., 2019; de Kreuk et al., 2005; Gao et al., 2011). These granules, usually 0.2–5 mm in diameter, have developed a layered structure with aerobic, anoxic, and anaerobic zones, which enables granules to perform simultaneous nitrification and denitrification (Corsino et al., 2019; Pronk et al., 2015). Other benefits of AGS include high biomass retention, high resilience to toxic compounds and sudden changes in temperature and organic load, and a smaller footprint and fewer energy requirements than conventional activated sludge systems (Adav et al., 2008; Corsino et al., 2019). The layered structure of granules and the presence of slow-growing bacteria allow AGS to comprise sequential treatment processes, including aerobic, anoxic, and anaerobic steps for the efficient removal of nitrogen and phosphorus (Nancharaiah and Kiran Kumar Reddy, 2018). However, this unique structure requires a long time to develop and depends on the presence of aggregating bacterial strains. In particular, heterotrophic denitrifiers and EPS-producing bacteria are important for granule formation and stability (Szabó et al., 2017). Several studies have suggested that the start-up process of AGS formation can be triggered by bioaugmentation with AGS strains of granules (Nancharaiah and Kiran Kumar Reddy, 2018).

The application of AGS for RAS effluent treatment has not been reported in the literature. To the authors' knowledge, there are only two studies that have tested systems similar to an AGS for treating RAS water. The first is the study by Letelier-Gordo and Martin Herreros (2019) on the application of an Upflow Anoxic Sludge Bed (UASB) system for treated marine land-based RAS water. This system is different from AGS in operation and configuration. As the name of this technology suggests, it uses an anoxic environment, while for AGS, the treatment environment is aerobic. Additionally, physical mixing is used in the UASB, whereas aeration is used in the AGS for mixing and as a source of oxygen. The second study, a recent study by Santorio et al. (2022), explored the potency of a continuous flow granular reactor (CFGR) for removing nitrogen from RAS water. However, only the performance of the CFGR was evaluated in their study. Here, we report for the first time on the performance of AGS in a sequential batch reactor (SBR) to treat RAS water with a special focus on granule characterization and microbial community development, which provides useful insights for developing this technique further. The focus of this study was to operate a pilot-scale AGS reactor fed by low-strength RAS effluents and to compare its granule characteristics and microbial community with the reference reactor being fed with synthetic wastewater promoting good granular growth. The granules were characterized by size, the development of an AGS microbial community, EPS production, and granular strength. To assess the feasibility of this solution further, reactor scale-up based on the observed reaction rates and nutrient loads and flow figures of an industrial RAS system, using the Laukaa fish farm in Finland as an example is presented with a comparison to a conventional denitrification reactor.

## 2. Materials and methods

### 2.1. Wastewater and aquaculture process description

The RAS wastewater used as a feed in this study originated from an experimental fish farm of the Natural Resources Institute Finland. The fish farm is located in the Laukaa municipality in central Finland. The RAS is described in more detail in Pulkkinen et al. (2021b). Briefly, it has two identical RAS units (each 22 $m^3$), including two raceway-type fish tanks (each 5 $m^3$). The wastewater treated in this study is the effluent from the existing wastewater treatment consisting of solid removal, nitrification step, and oxygenation. Rainbow trout (*Oncorhynchus mykiss*) were reared in the system during the experiment. The origin of the make-up water is the oligotrophic Lake Peurunkajärvi, which used 500 L/kg feed when wastewater was collected.

### 2.2. Experimental design

Synthetic water simulating urban wastewater composition was used for enhanced granule formation during the start-up phase. After the granule formation, which is a critical phase for AGS, the sludge was split equally into two reactors. One was fed with the same synthetic wastewater to serve as the reference reactor (denoted as R1), and the second one was fed with the RAS effluent including external carbon addition as the test reactor (denoted as R2). Based on our previous tests with AGS we assumed the granular formation with the RAS effluent to be impossible or very slow. Preliminary startup phase testing with low strength RAS resulted in sludge disintegration and washout. Such an observation was also reported in the literature (Liang et al., 2022). This was the reason to use synthetic wastewater during the start-up phase. The presented data were collected for 14 weeks of operation and can therefore provide a primary idea of the AGS potential for RAS effluent treatment. The reference reactor allowed us to follow the impact of the RAS effluent on the granular size, strength, and microbial community. In addition, the nutrient removal performance was studied from both reactors as an indication of AGS health. The reference reactor was operated in similar conditions throughout the study and it acted as the reference for well-performing AGS. The experiment was carried out for 98 days in a temperature-controlled room set at 22 °C to simulate the temperature in Laukaa fish farm facilities. The granules were formed in a cylindrical-shaped sequencing batch reactor with a height of 100 cm, a diameter of 7.5 cm, and an operational volume of 3.36 L. Aeration was set at 2 L/min. The used seed sludge was collected from the Porvoo Hermanninsaari WWTP operated with conventional activated sludge. When granules started forming on day 22, half of the granular sludge floc mixture was moved to R2. Reactors followed a 4 h cycle with a fixed 50 min filling (1.68 L) and anoxic phase and 180 min aeration. Settling was decreased from 5 min to 4 min after a week of operation, and again to 3 min after two weeks. The sludge settlement improved with time as heavy granules formed that required little time to sink to the bottom of the reactor. Withdrawal lasted for 5 min. When the withdrawal started, the desired sludge (made mainly of granules) commenced settling and all of it settled by the end of the withdrawal time. Only the fluffs (floccules) were still suspended, and get removed through the effluent discharging pump. Throughout the whole experiment, R1 was operated with a synthetic wastewater composition similar to that reported in (Gonzalez-Martinez et al., 2017), except the MgSO4.7H2O concentration was changed to 0.03 g/L. R2 was operated with synthetic wastewater until day 43 when it was switched to RAS wastewater. RAS wastewater having gone through solid removal and nitrification contains only 15–20 mg/L of total organic carbon, 2–4 mg/L of PO4-P, 50–60 mg/L NO3-N, $<$ 1 mg/L NH4-N, and 0.2–0.3 mg/L NO2-N. Hence, CH3COONa was added to the RAS wastewater as external carbon, and its concentration was decreased gradually as follows: 1000 mg/L on days 43–62, 850 mg/L on days 63–69, 700 mg/L on days 70–89, and 500 mg/L on days 90–98. Since the role of nitrifiers in the

AGS was not known, NH4Cl was also added as follows: 200 mg/L on days 43–54 and 125 mg/L on days 54–98 to make granules adjust better to the switch between influents by maintaining nitrification. A 0.003 mg/L KH2PO4 was also added as a P source and 0.1 mg/L NaHCO3 was added to adjust the alkalinity. The synthetic wastewater fed to R1 remained unchanged all the time as the purpose of the R1 was to provide a reference to the changes caused by the RAS effluent.

### 2.3. Analytical methods

The standard methods used for measuring nutrients and sludge physiochemical characteristics are listed in Table S1. Total nitrogen (TN), NH4-N, the sum of NO3-N and NO2-N, NO2-N, total phosphorus (TP), PO4-P, chemical oxygen demand (COD), suspended solids (SS), and alkalinity and pH for reactor influent and effluent were measured 1–5 times a week from the influent and effluent samples. Mixed liquor suspended solids (MLSS) and sludge volume index for 10 min of settling (SVI10) were measured weekly after granulation. SVI10 was calculated using the method reported in (Righetto et al., 2021). Granules were also collected from the reactors once a week, and their sizes were measured. The granule sample collection was conducted during the aeration phase to ensure capturing representative samples of the sludge. Extracellular polymeric substance (EPS) extraction was done twice according to the high temperature and sodium carbonate method reported in (Felz et al., 2016) by changing the granules/sodium carbonate ratio to 1 g of granules per 10 mL of solution. Protein concentration was analyzed with the Pierce 660 nm Protein Assay (Thermofisher) and carbohydrate concentration according to the procedure described in (Dubois et al., 1956). Granule strength was measured on the same days as the EPS extraction. The stability coefficient ($S$) and percentage of change in granule diameter ($\Sigma$) were measured according to the method detailed in (Nor-Anuar et al., 2012).

Nitrification and denitrification rates were calculated using the following equation:

$$r = \frac{\text{nitrified or denitrified nitrogen } load}{\text{MLVSS V}}$$

where $r$ is the rate in gN/MLVSSh, nitrogen load is in gN/h, MLVSS is the sludge concentration in the reactor in g/L and V is the reactor volume in L. The aerobic and the anoxic retention time were the same because anoxic conditions were occurring in the granules.

### 2.4. Sample collection and DNA extraction

The samples were taken during the period when reactors were operating with different influents: the first week with RAS wastewater, around halfway through operating with RAS wastewater, and during the end of the study. Due to the workload in preparing the samples both reactors were not sampled exactly on the same day. The aim of the microbial community analysis was to study the differences between the microbial communities with synthetic wastewater and RAS wastewater and study the species differences in the outer layers and inner layers of the granules. Each AGS sample for DNA extraction included the whole granule and separated parts from the inner and outer layers of granules. Outer layer samples were collected by slicing 1–2 mm slices from 4 sides of the granules with a sterilized scalpel. For collecting the inner layers, the top layer was also sliced, and then the inner part of the granule was scooped out. After collection, samples were immediately centrifuged at 10,000 rpm for 10 min, and the pellets were stored at − 20 °C until the DNA extraction.

The DNA extraction was done using a DNeasy PowerLyzer PowerSoil DNA isolation kit (Qiagen), following the manufacturer's instructions. The extracted DNA, kept at − 20 °C, was delivered to Novogene Europe (UK) for PCR, library preparation sequencing PE250, and bioinformatic analysis.

### 2.5. Sequencing data processing and statistical analysis

Sequencing data processing was carried out at Novogene Europe (UK). DNA samples were PCR-amplified with the universal primer sets 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to target the V3–V4 regions of 16S rRNA genes. The amplicon was sequenced on the Illumina paired-end platform to generate 250 bp paired-end raw reads (Raw PE) and then merged and pretreated to obtain clean tags. The chimeric sequences in clean tags were detected and removed to obtain effective tags that can be used for subsequent analysis. Operational Taxonomic Units (OTUs) were obtained by clustering with 97% identity on the effective tags of all samples and then identified. Alpha diversity was applied through 6 indices, including observed species, Chao1, Shannon, Simpson, ACE, and Good's coverage. All these indices in our samples were calculated using QIIME (Version 1.7.0) and displayed using R software (Version 2.15.3). Beta-diversity analysis was applied for Principal Component Analysis (PCA) and plotted using OriginPro 2022 software.

## 3. Results and discussion

### 3.1. Aerobic granular sludge performance

The performance indicators for R1 and R2 represented by nutrient removal and reaction rates are summarized in Fig. 1. The seed sludge had about 70% TN removal indicating in this case the denitrification of nitrates before the formation of the first granules around the end of the first week. After that, TN removal started dropping to the 20 % range, just before the sludge was split. Following the sludge split, TN removal picked up again with a sharp increase to the ~80 % range up to day 32 and then kept on fluctuating around this level until the end of the test period. TN removal for R2 followed a similar trend as that of R1 up to day 58, but with smaller rates. This day coincided with the drop of acetate from 1000 ppm to 850 ppm. As we continued dropping C and N supplements, TN removal continued to drop to as low as 13%. It is clear that the reduction of carbon in the influent negatively affects denitrification. A similar observation was reported by Kim and co-workers, who found that reducing the C/N ratio from 20 to 5 decreased TN removal from approximately 90 % to about 50 % (Kim et al., 2021).

Another reason for this deteriorating denitrification in R2 was the too-rapid changes in the influent composition: the COD/N ratio taking into account the RAS effluent and added nutrients decreased from the initial 7.5–4.7. The granules did not have enough time to adjust to the reductions in the external carbon source. It can be seen how the effluent TN concentrations experience higher peaks after acetate reductions. After the third acetate reduction on day 70, the granules had slightly more time to adjust before the final carbon reduction, which shows a palpable increase in TN removal. Also, the reactor cycle with the long aeration phase might have affected the nitrogen removal. Ammonium was fully nitrified, and some shorter aeration phases and longer anoxic phases could have been tested to find the ideal cycle for better denitrification without disturbing nitrification. In a study by de Kreuk, et al. (2005), they found that at the low oxygen saturation of 20%, the simultaneous nitrogen, COD, and phosphorus removal was the most efficient.

Both reactors achieved nearly full NH4-N removal after 40 days of operation (Fig. 1a). The TN and NH4-N concentration variations throughout the test are shown in Fig. S1a. At the beginning of the operation, the removal was adequate with the fresh seed sludge (~73%), but it was disturbed later when granulation started. After the start of granulation, the sludge was split, and $NH_4$-N removal for R1 and R2 operating with synthetic wastewater fluctuated in the ranges of 32–99 % and 56–73 %, respectively. The difference between the two reactors' behavior before the introduction of RAS water may be explained by the inevitable uneven division of granules during sludge-splitting. After the split, $NH_4$-N removal for R2 started to increase to reach the same high

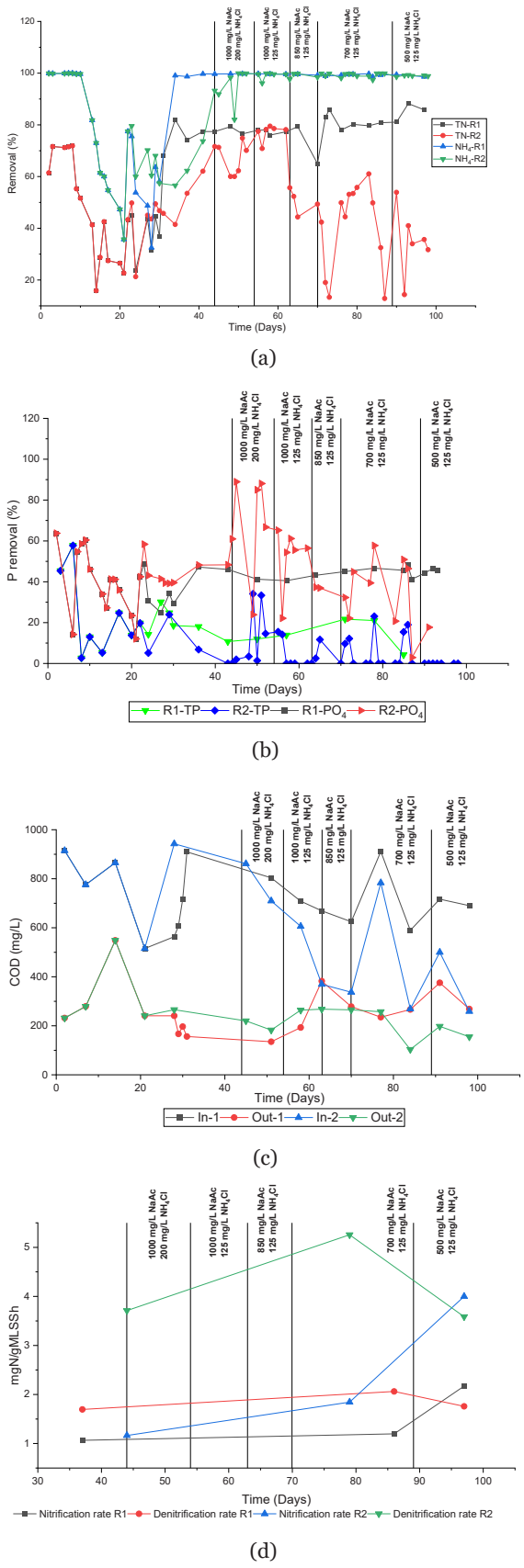


**Fig. 1.** Summary of granules performance throughout the test period for different feeding regimes: (a) N removal, (b) P removal. (c) COD variation and (d) Nitrification/Denitrification rates.

level of > 99 % achieved with R1. Switching to RAS effluent did not seem to affect $NH_4$-N removal except at the start, where $NH_4$-N removal dropped to 82 %, but it quickly recovered the next day. The change in the feeding regime also did not seem to affect $NH_4$-N. This high $NH_4$-N removal indicates good nitrification in the granules of both reactors.

Phosphorous removal for both reactors is presented in Fig. 1b. TP and PO4-P profiles for the two reactors are shown in Fig. S1b. TP and $PO_4$-P removal started at approximately 60 % and then began to decrease as the operation continued. Similar trends were observed in N removal and have been reported in the literature, where the seed sludge showed good nutrient removal followed by deterioration in the removal levels in the phase of granule formation (Lochmatter and Holliger, 2014). After the start-up phase, TP removal for R1 fluctuated between 40 % and 10 %. On average, TP removal for R2 was almost non-existent. This was due to the high effluent SS (see Fig. S2). $PO_4$-P removal, on the other hand, was stable for R1 after day 30 at 40 %. $PO_4$-P removal for R2 varied a lot throughout the operation period, dropping as low as 3 % and rising as high as 89 %. The removal cannot be directly compared to R1 because R2 received less phosphorus in the influent, 8 mg/L, and 4 mg/L respectively. Both reactors received enough phosphorus and it is possible that the observed small removal could be only due to assimilation in this case and not significantly taken up by the phosphorus-accumulating organisms.

COD levels of influent and effluent of the two reactors are illustrated in Fig. 1c. The average COD for R1 was about 700 mg/L, with a minimum of 476 mg/L and a maximum of 915 mg/L. COD for R2 was lower, with an average of 607 mg/L, dropping from 860 mg/L at the start of RAS wastewater feeding to a minimum of 259 mg/L towards the end of the operation. COD removal of R1 and R2 is presented in Fig. S3. The removal was around 75 % at the start of the operation and decreased during the granulation phase followed by an increase after splitting sludge. Decreasing the acetate supplement reduced COD removal, and as we allowed more time for granules to acclimate to the acetate drop from 850 mg/L to 700 mg/L, the removal recovered as in the case of the N and P removals. The maximum COD removal percentages reached with R1 and R2 were 83 % and 75 %, respectively.

Nitrification and denitrification rates are presented in Fig. 1d. For R1, both rates show an increasing trend. The slight increase between the first two measurements refers to the granules becoming more mature over time. The bigger increase in the last measurement is due to the loss of granules the reactor experienced due to technical errors (a glitch in the program that led to starting the withdrawing step during aeration), where MLSS decreased significantly. However, the nitrogen removal and overall reactor performance were not disturbed. The nitrification rate for R2 followed the same trend as the R1 rates: over time, the $NH_4$-N removal improved with maturing granules, and the granule loss only disturbed nitrification very slightly in the end. The R2 denitrification rate shows the effect of decreasing MLSS when the granule size decreased (Fig. 2), but TN removal was still the same between the first two measurements. The last measurement drops significantly due to the loss of granules and deterioration of denitrification (Fig. 1a).

Overall, AGS was found to be effective, with TN removal for R1 fluctuating around 80 %. However, it significantly dropped for R2 from 80 % to 13 %, with a decreasing carbon addition as acetate from 1000 mg/L to 500 mg/L. $NH_4$ removal reached almost 100 % for both reactors. No significant phosphorus removal was observed, and $PO_4$-P was around 40 % for R1 and significantly varied for R2 (3–89 %), receiving a lower phosphorus load. COD removal also varied by 40–80 % for R1 and 20–70 % for R2. TN, P, and COD removal for R2 deteriorated with the decrease in carbon addition. These observed variations in different indicators could reasonably be explained by the development of granules and changes in feeding strategies, but also part of it albeit small, could be due to the lack of experimental work repeatability. Such a lack is acceptable in the case of this study due to the extensive time and resources required for conducting such experiments and the difficulties in ensuring similar conditions are applied to all replicates.

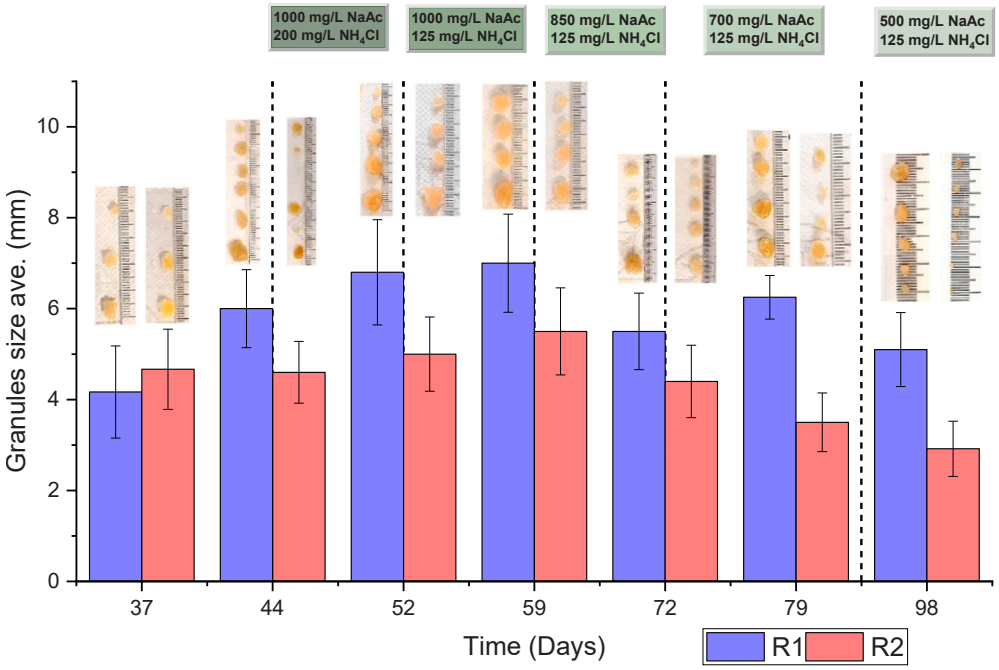


**Fig. 2.** Granules size change over time for both reactors.

### 3.2. Sludge characterization

Fig. 2 shows the evolution of the granule size during the operation for R1 and R2. First, small granules (ca. half a millimeter in diameter) were already visible on day 7 (see Fig. S4), and large granules were formed in 27 days. In the first few days, granules were distinguished from floccules by their unique whitish color and spherical structure, and they were entrapped between the floccules. Mature granules, however had color like that of floccules, but they were heavier and mostly rounded. Aerobic granules were dominating the sludge formation in less than 40 days. In R1, the average granule diameter varies between 4 and 7 mm. In R2, the average granule size dropped slightly (from 4.7 mm to 4.6 mm) after switching to RAS wastewater, but they started to adjust to the change, and the average diameter increased to 5 mm, even after decreasing the external ammonia in the influent. The decrease in acetate concentration dropped the average granule sizes during the following weeks: the first drop to 4.5 mm, and then 3.5 mm and 2.7 mm. The final acetate drop probably did not affect the granule size much because the granules had a bit longer time to adapt during the previous period. Even though the acetate was decreased from 700 mg/L to 500 mg/L, the food-to-microorganism ratio (F/M ratio) increased from 0.043 gCOD/gMLSS to 0.047 gCOD/gMLSS due to the granule loss on day 92. The granules in R1 were larger than most granules reported in other studies. The R2 granules were typical in size: 0.2–5 mm (Corsino et al., 2019). The granule size change is in line with the reported effects of C/N on granule size. It was reported that decreasing the C/N ratio resulted in a reduction in granule size (Kim et al., 2021). The small-sized granules have been reported to have a higher nitrification rate compared to bigger granules (Nguyen Quoc et al., 2021). This agrees with the results obtained in this study as shown in Fig. 1d.

The results of SVI10 measurements are provided in Fig. S5. The first measured SVI10 values were around 108 mL/g after dividing the granules into two reactors. The values started to increase, and R2 reached its peak on day 52 with 259 mL/g, and R1 on day 59 with 260 mL/g. After this, the main trend with SVI10 for both reactors was decreasing, except for a small increase for R2 on day 79 and R1 at the end of the operation. The average SVI10 values for R1 and R2 were 170 mL/g and 158 mL/g, respectively. The results in both reactors were higher than the typical SVI values for AGS, which are generally below 80 mL/g (Gao et al., 2011). For example, SVI of 24 mL/g has been measured with synthetic wastewater (de Kreuk et al., 2005), SVI of 35 mL/g with low-strength municipal wastewater (Ni et al., 2009), and 45 mL/g with a full-scale application of AGS in sewage treatment (Pronk et al., 2015). The size of the granules in all these studies was, on average, much smaller than those observed in this study, and this affected the results. In addition, the way the SVI measurements were conducted in this study might differ from other studies. Typically, SVI measurements are made with samples from the reactor, while in this study, the reactor column was used.

### 3.3. Granules structure evaluation

The results from the EPS content and strength analysis are listed in Table 1. It can be seen that R2 had higher protein and carbohydrate concentrations than R1. The higher protein and carbohydrate concentrations in R2 granules can be attributed to the harsher environment they were exposed to. The harsh environment has been proven to lead to the secretion of more EPS by microorganisms (Liu et al., 2021). Reducing the PN/PS ratio in R1 can indicate a slight weakening structure of AGS, and an increasing PN/PS ratio in R2 can indicate the AGS is getting stronger (Corsino et al., 2019). The measured PN/PS ratios in this study were closer to the typical ratios of activated sludge flocs (ca. 0.9 on average (Adav and Lee, 2008)). The reported PN/PS ratios for AGS values varied in the literature. Some are three to six times higher (Adav and Lee, 2008) than the measured values in this study, while others are on par, as was the case in Kim et al. (2021).

One factor affecting the results might be the large size of the

**Table 1**
EPS protein & carbohydrates concentration, strength analysis.

| Characteristics | Day 85 | | Day 92 * /93 * * | |
|---|---|---|---|---|
| | R1 | R2 | R1 | R2 |
| Protein (mg/g wet weight of granules) | 1.87 | 2.60 | 1.83 | 2.80 |
| Carbohydrates (mg/g wet weight of granules) | 2.05 | 2.54 | 2.12 | 2.71 |
| PN/PS | 0.91 | 1.02 | 0.86 | 1.03 |
| $S$ (%) | 14.6 | 8.8 | 3.4 | 0.6 |
| $\Sigma$ (%) | 10.5 | $\approx 0$ | 1.5 | 3.6 |

*Protein and carbohydrates measurement, * *$S$ and $\Sigma$ measurement.

granules, which leads to a smaller surface area to volume ratio than the smaller granules in other studies that report a larger PN/PS ratio. For this reason, the granules had a relatively smaller contact area with the extracting agent during the analysis. Also, the stratification of different EPS components and microbial species (Corsino et al., 2019; McSwain et al., 2005) possibly affects the results together with the size factor, when proteins that are typically located in the inner layers were not as efficiently extracted as carbohydrates that are present in the outer layers. In addition, the reported PN/PS ratios in the literature were obtained using different extraction and measurement methods.

Based on the results of the strength analysis and criteria set by Nor-Anuar et al. (2012), the granules from both reactors were categorized as strong based on $S$ and $\Sigma$ values. R2 granules seem to be stronger than those of R1, as indicated by the lower S and $\Sigma$ values. One can also notice that dropping acetate in the last week of operation made the granules stronger and more compact (Fig. 2). The strength of granules in R2 could be attributed to the increase in protein and carbohydrate secretion. The high carbohydrate concentration in granules improves the internal adhesion of microbes and other constituents resulting in a rigid structure (Liang et al., 2019). The increase in protein section as a response to fluctuation of wastewater quality was reported to increase AGS structural stability (Hou et al., 2021).

### 3.4. Study of α-diversity and β-diversity in microbial communities

The microbial α-diversity indices were calculated as shown in Table S2. The coverage of all samples was above 99%, showing good AGS community representation. Chao1 and ACE index values showed an increase in bacterial abundance over time until day 97 in R1 and day 90 in R2, with similar trends for both core and surface communities. The Shannon and Simpson index values have also shown an increase in diversity until day 90 for R1 and day 97 for R2. Therefore, 97 days might not be enough to reach a fully mature granular community, and a longer experimental period would give more insights into granular microbial composition formation.

As shown in Fig. 3, all of the samples taken on days 57 and 62 were close to each other (gray area). Samples taken during the last 3 sampling dates (days 90, 94, and 97) showed larger differences, while core communities and surface communities showed separate clusters. The surface communities of R1 and R2 were closer to each other than the core communities (light-red area). However, they clearly formed separate clusters as well (light blue and yellow areas). Overall, these results illustrated the development of microbial communities between day 57 and day 90 with the formation of core and surface sub-populations. The type of wastewater had a minor effect on the granular sludge formation compared to the operational parameters.

### 3.5. Effect of recirculating aquaculture system effluent feed on the dynamics of microbial communities

Fifteen of the most abundant phyla of AGS communities are presented in Fig. 4. Consistent with previous studies of an aerobic granule microbial community, the majority of bacteria in both reactors belonged to the phylum Proteobacteria (around 80 % of the total community), followed by Bacteroidota (formerly Bacteroidetes), representing up to 18 % of the total community, and Firmicutes (around 1–2 %) (Lv et al., 2014). In R1, the phyla Patescibacteria (up to ~10 % of the granule core community), Bdellovibrionota (up to ~7% of the granule surface community), and Myxococcota (up to ~7 % of the total community) showed high abundance but were less abundant in R2 (≤1 %). The biggest difference between the studied communities and previous studies of freshwater RAS biofilm populations at the phylum level was the lack of Planctomycetota (previously known as Planctomycetes) and Nitrospirota (previously known as Nitrospirae), which are mainly associated with anaerobic ammonium oxidation and nitrification, respectively. The abundance of these two phyla was at least 10 times lower (below 0.6 % for Planctomycetotathan and 0.1 % for Nitrospirota) than those reported in the literature (Pulkkinen, 2020; Rurangwa and Verdegem, 2015).

The relative abundance of bacterial genera representing at least 1 % of the community in at least one of the studied samples is summarized in Fig. 5. In general, the community composition of both R1 and R2 was typical for AGS previously published.

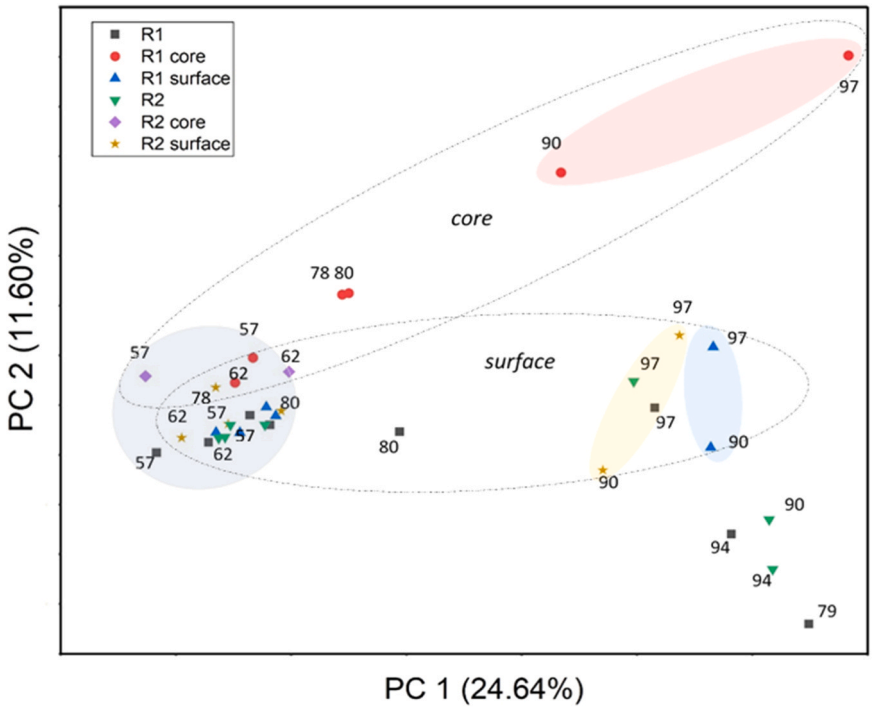


**Fig. 3.** Principal component analysis (PCA) of the bacterial community structure of granules, the granule cores and the granule surface layers from reactors R1 and R2 during the experimental period.

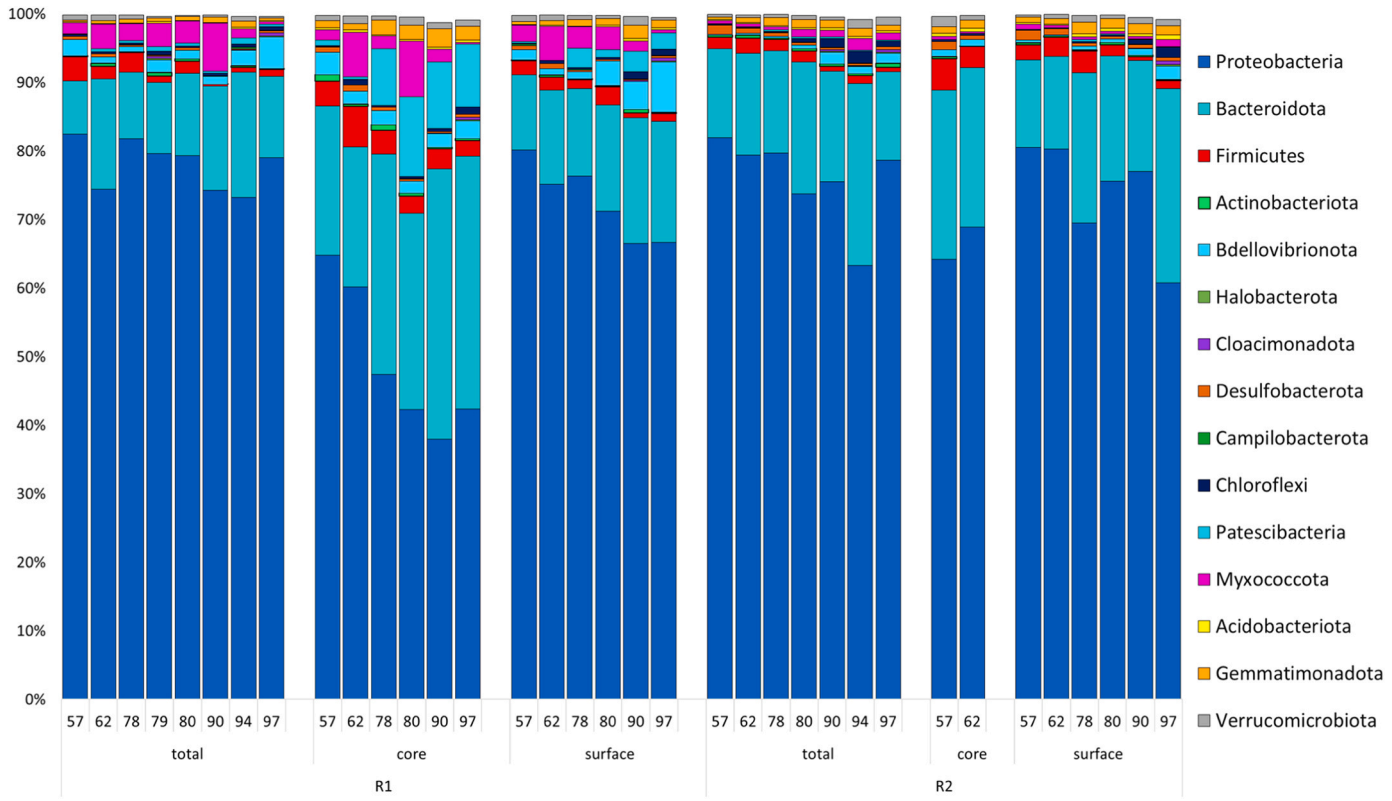


**Fig. 4.** Relative abundance of 15 most abundant bacterial phyla in studied AGS samples.

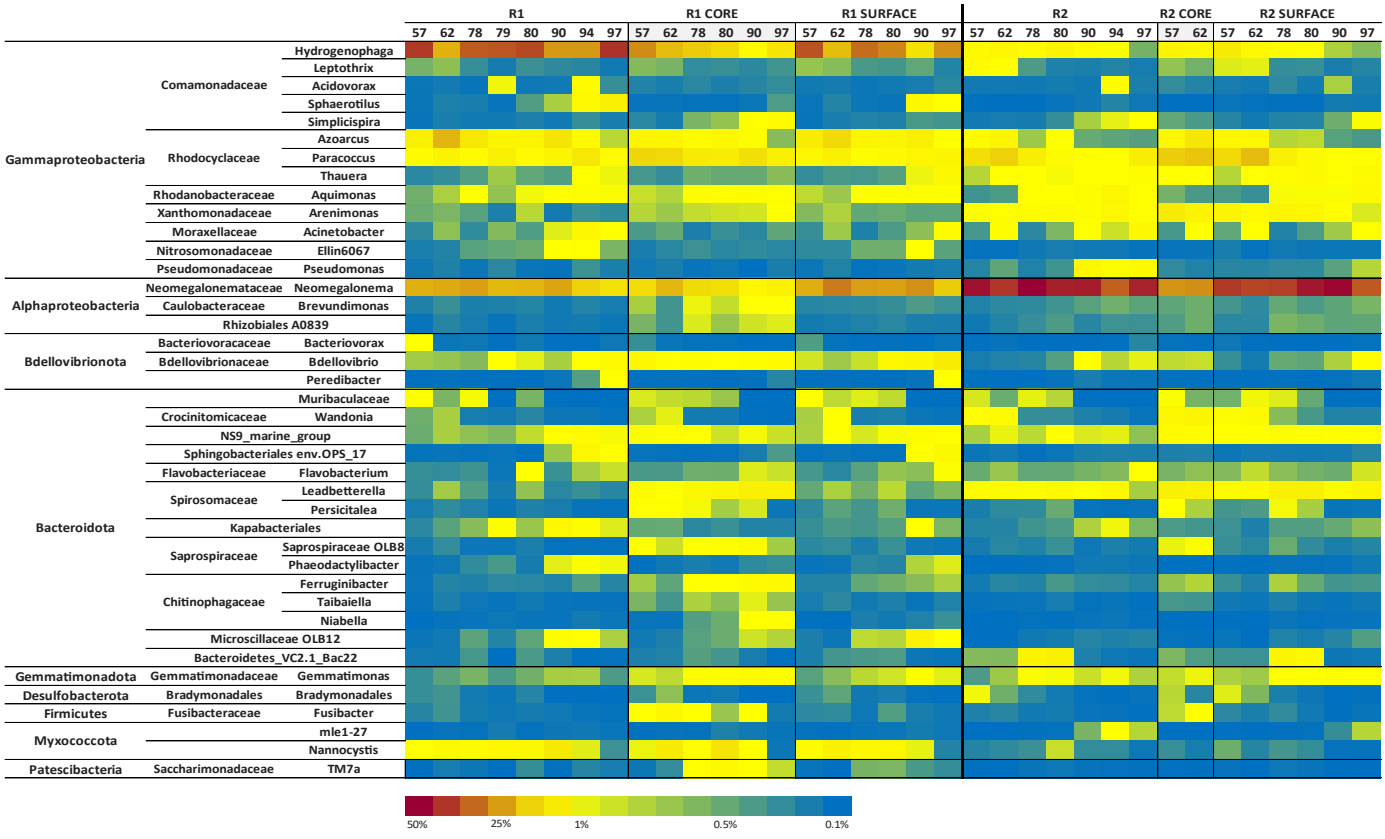


**Fig. 5.** Heat map of bacterial community structures in R1 and R2 granules (total community, core community and surface community) from day 57 till day 97 of operation at genus level (abundance ≥1 %).

The dominant phyla Proteobacteria was mostly represented by the important families Comamonadaceae, Rhodocyclaceae, Rhodanobacteraceae, Xanthomonadaceae, and Moraxellaceae, known as AGS denitrifying bacteria, while Rhodanobacteraceae and Xanthomonadaceae also play a role in extracellular polymeric substances (EPS) production in AGS (de Sousa Rollemberg et al., 2018; Ma et al., 2017).

The most abundant genus of R1 was *Hydrogenophaga* (family Comamonadaceae), representing up to 50 % of the bacterial community, followed by *Neomegalonema* (family Neomegalonemataceae) in up to 24%. The next dominant group was the Rhodocyclaceae family, represented by three denitrifying genera *Azoarcus*, *Paracoccus,* and *Thauera*. The genus *Thauera* increased in both reactors, especially in the granule surface (from 0.2 % to 3.1 % in R1, and from 0.9 % to 2.4 % in R2), while *Paracoccus* seemed to be present in higher abundance in the granular core (up to 11 % in the R1 core and up to 14 % in the R2 core). *Thauera* and *Paracoccus* are known dominant genera of aerobic granular sludge that are important for granular formation, the stability of granules, and nitrogen removal (Fall et al., 2022; Zhao et al., 2013). The identified *Thauera* species *Thauera terpenica* and *Thauera linaloolentis* have been reported as aerobic denitrifiers (Foss and Harder, 1998). The *Paracoccus* species are common in AGS as well as RAS biofilms and may take part in heterotrophic nitrification and aerobic denitrification.

In R2, the most abundant genus was *Neomegalonema* (up to 61 % of the total community, aerobic heterotrophs; some may reduce nitrate) followed by *Hydrogenophaga* (up to 33%). The dominance of *Neomegalonema* could be explained by its ability to assimilate PHA, providing an advantage under the unbalanced F:M conditions such as the COD to N:P ratio (Oren, 2017; Szabó et al., 2017). It is a known EPS producer in AGS. Szabó et al. (2017) showed that *Neomegalonema* is mostly present on the surface of the granule, which agrees with our results. Additionally, a noticeable increase in *Pseudomonas* was observed in R2 (from ~0.1 % on day 57 to about 3 % on days 90–97). Further identification of *Pseudomonas* showed that it mostly included the species *Pseudomonas peli* (heterotrophic nitrifier) and *Pseudomonas caeni* – denitrifying bacteria previously described in the sludge of an anaerobic ammonium-oxidizing bioreactor (Vanparys et al., 2006; Xiao et al., 2009). In RAS systems, *Hydrogenophaga sp*. is commonly associated with autotrophic denitrification, while *Pseudomonas sp*. is associated with heterotrophic denitrification (Rurangwa and Verdegem, 2015).

Bacteroidota, another dominant phylum, is known for including obligate anaerobes, which may represent the core of the granules (Adav et al., 2010). For instance, higher abundances of *Leadbetterella* and *Persicitalea* from the Spirosomaceae family, and the family Chitinophagaceae with genera *Ferruginibacter, Taibaiella,* and *Niabella* were observed in the samples from the granular cores.

The abundance of *Flavobacterium* (Flavobacteriaceae) increased in both reactors during the sampling period from ≤ 0.5 % to ~1 %. *Flavobacterium* is a common genus in mature AGS with known denitrification and PAO abilities and is also important for granular formation due to EPS production (Pishgar et al., 2021; Świątczak and Cydzik-Kwiatkowska, 2018).

*Bdellovibrio sp.* (Bdellovibrionaceae) showed a gradual increase in the AGS community, especially in surface samples (up to 6 % in R1 and up to 2 % in R2). It was previously reported as one of the dominant species in aerobic granules that might form a bacterial association protecting the granule surface from water turbulence (Liu et al., 2021). At the same time, as a predator species, *Bdellovibrio* were shown to decrease the diversity of AGS microorganisms and affect the microbial composition (Feng et al., 2017). Both *Flavobacterium* and *Bdellovibrio* are known to be located in the inner parts of the granules (Szabó et al., 2017).

Several important genera from other bacterial phyla are worth mentioning. *Nannocystis* (Myxococcota) was observed in up to 7 % of the total R1 community and up to 1 % of the total R2 granular community, which probably reflects the feeding since this genus includes bacteria commonly found in wastewater treatment plants (Liu et al., 2016). *Gemmatimonas* (Gemmatimonadota) represented ~2% of the mature granular community of both R1 and R2, and it has been reported as PAO bacteria in AGS (Fall et al., 2022). Although in our case, phosphorus accumulation had probably not been achieved yet and might require optimization of the operating condition, these slow-growing bacterial taxa may benefit granule formation and show better performance after the fully mature granule structure is reached (de Kreuk and van Loosdrecht, 2004). *Fusibacter* (Firmicutes) was mostly represented in the granular core (≤3.5 % in R1 and ~1.6 % in R2) and was also previously reported in AGS among dominant genera (Fall et al., 2022).

From observed communities, several bacterial genera could be attributed to ammonia-oxidizing bacteria (AOBs) (see Fig. S6), including *Nitrosomonas europaea* (Betaproteobacteria), *Ellin6067* (Gammaproteobacteria), and *N59_marine_group* (Bacteroidota). Nitrite-oxidizing bacteria (NOB) bacteria included *Nitrospira defluvii* (Betaproteobacteria) and *Candidatus Nitrotoga sp.,* which represent two key NOB genera in bacterial communities of nitrogen-removing systems (Lücker et al., 2015; Spieck et al., 2021). The abundance of AOB bacteria in R1 was between 0.7% and 3.8 % of the total community, with the tendency to increase from day 57 to day 97 due to the increase of *Ellin6067* and *N59_marine_group* bacteria on the surface of the granules. In R2, AOB remained at 0.8–1.7 % of the abundance, mostly represented by *N59_marine_group* (about 1 % of the total sludge community) and *Ellin6067* (0.1 %), while *Nitrosomonas* was only at around 0.03%, which can be explained by lower ammonium concentration in the feeding wastewater of R2. In both reactors, *Nitrosomonas* was presented in higher abundance in the granule core, suggesting that the granular core was aerobic. The abundance of NOB bacteria was below 0.1% in all of the studied samples. The decrease in AOB and NOB presence, with granule formation and maturation despite the high removal performance, was previously reported by Świątczak and Cydzik-Kwiatkowska (2018). Szabó et al. (2017) demonstrated efficient nitrification in AGS despite the abundance of AOB and NOB below 0.1 %.

Despite the differences in wastewater content, both reactors established microbial communities, representing maturing aerobic granules with common AGS dominant denitrifiers (*Hydrogenophaga, Pseudomonas, Paracoccus*), EPS producers (*Neomegalonema, Thauera, Bdellovibrio, Flavobacterium*), PAO bacteria (*Thauera, Gemmatimonas, Flavobacterium*), and nitrifiers (*Nitrosomonas*, *Ellin6067*, *N59_marine_group*, *Nitrospira, Candidatus Nitrotoga,* etc.*)*. These microbial populations may provide stable granulation and provide the potential for high performance of AGS, while the start-up period could be shortened by primary growth of the granules on higher strength wastewater.

## 4. Comparison of an aerobic granular sludge sequencing batch reactor and a conventional denitrification reactor at the Laukaa fish farm

The dimensioning of AGS SBR to be placed in the Laukaa fish farm was done for the treatment of the RAS effluent flow of 9 $m^3$/d (other design parameters are presented in Table S3). Based on the observed denitrification rate during our tests, the MLSS required to denitrify 80 % of the influent nitrate in Laukaa was estimated. The external carbon addition as acetate was kept at 500 mg/L, which was estimated as not yet limiting the reactions. The design MLSS is 3.86 g/L, which is still clearly below the typical MLSS values in AGS reactors of ca. 7 g/L (Nguyen Quoc et al., 2021; Wei et al., 2013). The AGS SBR reactor for this fish farm resulted in a total volume of 1500 L. Operation would be carried out with similar sequences as in the study (50 min filling, 180 min aeration, 3 min settling, 7 min withdrawal and idle). A storage tank was included. The added ammonium chloride was also decreased and could be decreased more if the granules adjusted. This AGS design was compared to continuous-flow conventional activated sludge denitrification based on an assumed typical denitrification rate of 5 mgN/gMLVSSh with acetate and surface loading of 0.3 m/h in the settler. The lower assumed denitrification rate in AGS can be explained

by the diffusion needed in the granules. This comparison shows that AGS resulted in a total reactor volume that was 55 % of the volume needed for the conventional activated sludge process. Nevertheless, it can be estimated that the aeration energy consumption of AGS in this case will clearly be higher than the mixing energy needed in the conventional denitrifying process. This is partly due to the fact that additional nitrification was taking place in the AGS reactor. The nitrification capacity of AGS could be used to replace the existing nitrification step. Further optimization of the operating conditions in the future would be beneficial for gaining more insight into the effective application of AGS for RAS wastewater treatment and reuse. More feasible solutions for external carbon also exist. Running longer tests would also help in forming a conclusive evaluation of AGS feasibility for this application.

## 5. Conclusions

AGS potency for treating RAS effluent in an SBR configuration was evaluated by monitoring nutrient profile and sludge characteristics and thorough microbial community analysis. A reference reactor with well-balanced nutrient content wastewater was run in parallel. AGS achieved high nitrogen removal (about 80 % for both reactors), but COD removal was average (40–80 % for R1 and 20–70 % for R2) and there was no P removal. The uneven quality of the sludge after splitting the grown granules and possibly the lack of measurements repeatability led to a noticeable disparity of nitrogen removal between R1 and R2 even before the introduction of Laukaa effluent. It was noticed that AGS performance recovered when granules were allowed sufficient time to adapt to the carbon drop. Granules produced a compact and stronger structure with a higher PN/PS than the reference reactor. Microbial communities of both reactors represented maturing granules with common AGS dominant denitrifiers, EPS producers, PAO bacteria, and nitrifiers. A preliminary dimensioning based on the denitrification rate revealed that AGS had a smaller footprint than activated sludge with a higher energy requirement. Following this investigation, it would be useful to examine the impact of other parameters such as salinity on AGS performance with RAS effluent in future research work.

## CRediT authorship contribution statement

**T.S.:** Investigation, Methodology, validation, data curation, formal analysis, writing original draft, **RA.A.:** Conceptualization, methodology, formal analysis, supervision, writing original draft, **A.K.:** Formal analysis, validation, visualization, writing original draft, **J.P:** Resources, project administration, funding acquisition, **J.V.:** Resources, project administration, funding acquisition, **A.M.:** Conceptualization, formal analysis, supervision, writing original draft, project administration, funding acquisition.

## 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.

## Data availability

All data are presented in the manuscript and SI.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquaeng.2023.102361.

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