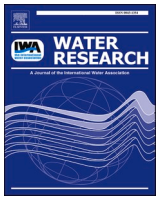
Water Research 274 (2025) 123021

Contents lists available at ScienceDirect

Water Research

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



The critical role of flocs in nitrification in full-scale aerobic granular sludge-based WWTP

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ARTICLE INFO

Keywords:
Aerobic granular sludge
Flocs
Size distribution
Nitrifying community distribution
Nitrifying activity distribution

ABSTRACT

Aerobic granular sludge (AGS) is usually considered to be a biofilm system consisting of granules only, although practical experience suggests that flocs and granules of various sizes co-exist. This study thus focused on understanding the contribution of flocs and granules of various sizes to nitrification in a full-scale AGS-based wastewater treatment plant (WWTP) operated as a sequencing batch reactor (SBR). The size distribution in terms of total suspended solids (TSS) and the distribution of the nitrifying communities and activities were monitored over 14 months. Our results indicate that AGS is a hybrid system in which flocs (<0.25 mm) play a critical role in nitrification. AGS consisted of 36 % flocs and 50 % large granules (>2 mm) at a TSS concentration of 4.7 ± 0.7 gTSS L^{-1}. The growth of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in large granules was limited due to the high mass transfer limitation in biofilm and the high solids retention time (SRT) of flocs, where favorable conditions for the growth of nitrifiers were maintained during the warm season. The specific activities of the small aggregates (<1 mm) were 5 to 15 times higher than those of large granules. As a result, flocs contributed >50 % to nitrification during the warm season, whereas granules >1 mm contributed <20 %. Such predominance of flocs in nitrification became problematic in the cold season when the minimum SRT of NOB increased to values similar to the floc SRT, resulting in 79 % loss of the NOB. Consequently, NOB activities dropped, and elevated effluent nitrite concentrations of several mgN L^{-1} were monitored. We suggest operating AGS systems similarly to hybrid systems in order to promote the enrichment of NOB in the granules by controlling the floc SRT at low values smaller than the minimum SRT of NOB throughout the year.

1. Introduction

The development of advanced treatment technologies is essential for effective and intensified nitrogen removal from wastewater (WW). Aerobic granular sludge (AGS) is a rather novel technology with significant potential benefits over conventional biological wastewater treatment processes (de Bruin et al., 2004). Although AGS systems are considered to be a biofilm-only process (Ali et al., 2019), some recent studies acknowledge that AGS also contains bio-aggregates <0.25 mm, termed flocs (Layer et al., 2019; Pronk et al., 2015; van Dijk et al., 2018). However, it remains unclear to what extent AGS systems function similarly to hybrid systems such as integrated fixed-film activated sludge (IFAS) systems: for instance, whether nitrification occurs mostly in granules or is distributed among flocs and granules. Furthermore, AGS plants are typically designed for a defined sludge loading rate that is relative to the overall amount of sludge maintained in the system and thus does not differentiate between granules and flocs (Lopez-Vazquez et al., 2023). If flocs and granules coexist within AGS, it is crucial to understand their implications for the operation and design of AGS-based plants. Further research is therefore required to assess the roles of flocs and various-sized granules in nitrification in AGS from full-scale plants.

Many studies on AGS have focused on the granulation mechanism and on optimizing the settling properties of the sludge and have overlooked the presence of flocs and their effect on process performances. Flocs are small bio-aggregates (<0.25 mm) of loose and irregular shape. Unlike granules, flocs tend to flocculate and settle slowly. Consequently, flocs are selectively removed from AGS systems to provide a competitive advantage to granules and to maintain the settling properties of the

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https://doi.org/10.1016/j.watres.2024.123021
Received 4 October 2024; Received in revised form 4 December 2024; Accepted 20 December 2024
Available online 20 December 2024
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sludge. Nevertheless, the presence of flocs in AGS systems treating municipal wastewater is increasingly recognized, and significant fractions of 16 to 40 % of total suspended solids (TSS) have been reported (Layer et al., 2019; Pronk et al., 2015; van Dijk et al., 2018). However, these studies do not provide a detailed and long-term monitoring of flocs and granules' distribution and dynamics over time. Consequently, two questions that remain open are whether flocs are an integral component of AGS and whether AGS systems should be considered hybrid systems.

The flocs and various-sized granules, referred to as *size-classes*, allow various local growth conditions, for instance for electron donor/acceptor availability (Liu & Tay, 2012; Strubbe et al., 2022) and solids retention time (SRT) (Ali et al., 2019; Winkler et al., 2012). These local growth conditions strongly influence the assembly of microbial communities among the size-classes (Ali et al., 2019; Liu & Tay, 2012; Wei et al., 2021). In AGS, slow-growing organisms such as nitrifiers are intuitively expected to grow preferentially in granules, where high SRT conditions are maintained (de Kreuk & van Loosdrecht, 2004; Layer et al., 2019; Onnis-Hayden et al., 2011; Winkler et al., 2012). This intuition assumes that the SRT of the flocs is too short to allow the nitrifiers to grow there. However, Ali et al. (2019) found a considerable abundance of nitrifiers in both granules and flocs, challenging the assumption that the floc SRT is lower than the minimum SRT required for the nitrifiers to grow. In contrast to activated sludge or hybrid systems, the floc SRT in AGS is not a controlled parameter. Because the removal of excess sludge in AGS systems is intended to maintain good sludge settleability by selectively removing the flocs, a low floc SRT is generally expected. However, long-term monitoring of the floc SRT data from full-scale AGS WW treatment plants (WWTPs) is lacking. Additionally, species migration may influence microbial distribution (Ali et al., 2019; Daigger et al., 1993; Ekholm et al., 2022). Therefore, flocs may contain debris and thus microorganisms from large granules as a result of their disintegration, making flocs relevant to nitrification. Notably, the different mass-transfer limitations of flocs and granules mean that their associated microbial activity cannot be predicted from their microbial abundance data. This highlights the need for further investigation of the distribution of both nitrifiers and nitrifying activities in AGS.

Nitrification is a key microbial conversion process in biological wastewater treatment. Although it is a relatively well understood process (Gujer, 2010; Ward, 2011), nitrification failures during cold and rainy weather periods are common in various process configurations (Bollon et al., 2016; Gruber et al., 2024; Gruber, Niederdorfer, et al., 2021; Hoang et al., 2014; Johnston et al., 2019; Ju et al., 2014). Consequently, maintaining stable nitrification throughout all seasons remains an important challenge in wastewater treatment. Nitrification in biofilm systems has been reported to be more robust and less affected by low temperatures than that in activated sludge systems (Salvetti et al., 2006; Zhu & Chen, 2002). Granules in AGS may provide AGS with the resilience required to avoid seasonal nitrification failures (Chen et al., 2020; de Kreuk et al., 2005). In full-scale AGS systems where flocs are present, we expect that such resilience against cold temperatures is provided if a major proportion of the nitrification occurs in granules. Although simultaneous nutrient removal has been linked to granule size (Layer et al., 2020; Quoc et al., 2021), understanding of the distribution of nitrifying activities between the different size-classes of AGS is limited. Using a lab-scale continuous-flow system, Wei et al. (2021) reported that small bio-aggregates (<0.4 mm) contribute significantly to nitrification, accounting for approximately 35 % and 25 % of ammonia-oxidizing bacteria (AOB) and nitrogen-oxidizing bacteria (NOB) activity, respectively. But in full-scale systems operating as sequencing batch reactors (SBRs), how nitrifying activities are distributed among the flocs and granules of different sizes remains unclear, as does how this distribution evolves or remains stable over seasons. Such knowledge is essential to assessing whether AGS systems could be more resilient to temperature variations than conventional activated sludge systems.

The main aim of this study was to gain a better understanding of the co-existence of flocs and various-sized granules in full-scale AGS systems and of their respective impacts on nitrification performance over the seasons. Therefore, the study addressed four research questions:

1. *Aggregate size:* What is the size distribution of the bio-aggregates in full-scale AGS plants?
2. *Nitrifying populations:* How are the AOB and NOB populations distributed among the size-classes? To what extent does the distribution of AOB and NOB among the flocs and granules correlate with growth conditions such as the floc SRT?
3. *Nitrifying activities:* How are the nitrifying activities (ammonium oxidation and nitrite oxidation) distributed among the size-classes? What is the contribution of the flocs to the overall performances of AGS?
4. *Dynamics:* To what extent are the size distribution, nitrifying communities, and nitrifying activities subject to seasonal variations, and what are the implications for effluent quality in terms of nitrogen species?

2. Material and methods

2.1. Overall approach

AGS from the full-scale SBR at Kloten–Opfikon WWTP (Switzerland) was monitored over 14 months for (i) size distribution, (ii) floc SRT, (iii) distribution of AOB and NOB populations, and (iv) distribution of AOB and NOB activities among the size-classes. The monitoring period was divided into three phases: (1) start-up based on the wastewater load, (2) increasing temperature, and (3) decreasing temperature (Table 1).

2.2. Experimental approach

2.2.1. Kloten–Opfikon WWTP

Kloten–Opfikon WWTP receives wastewater, including industrial wastewater, from two municipalities. The WWTP is equipped with primary clarifiers (hydraulic retention time = 2.3 h) prior to biological treatment. The SBR monitored in our study was started in November 2022 with an AGS inoculum from a parallel AGS SBR. The monitored SBR has a volume of 4750 m^3 and treated the wastewater of a population equivalent of 44,000, corresponding to an average WW flow of 10,227 ± 2735 $m^3\ d^{-1}$. The main characteristics of the WW are detailed in Table 2. Kloten–Opfikon WWTP was under commissioning during this study, i.e., the full WW load was treated in half of the design volume. But both the

Table 1
Phases of the monitoring campaign.

	Date	Wastewater load	Temperature
1) Start-up	December 22–March 23	Increase from 50 to 100 %	16.9 ± 1.3 °C
2) Increasing temperature	April 23–September 23	100 %	21.4 ± 1.4 °C
3) Decreasing temperature	October 23–January 24	100 %	17.1 ± 3.2 °C[a]

[a] November 23–January 24: 15.5 ± 1.7 °C

Table 2
Primary effluent characteristics and loads on the monitored SBR between April 2023 and January 2024.

Parameter	Min. [mg L^{-1}]	Max. [mg L^{-1}]	Average [mg L^{-1}]	Load [kg d^{-1}]
COD	182	610	335	3414
COD dissolved/COD			34 %[a]	
Total phosphorus	3.6	9.0	5.9	59
PO_4^3-P dissolved	2.3	7.9	4.4	43
Total nitrogen	27.0	68.0	50.6	497
NH_4^+–N dissolved	15.6	51.8	36.1	357

[a] Average of measurements from 21.05.24 to 25.06.24 for reference (n = 8).

organic sludge loading rate of 0.15 kgCOD $kgTSS^{-1}$ d^{-1} and the nitrogen sludge loading rate of 0.016 kgN $kgTSS^{-1}$ d^{-1} agreed with the design values of the Kloten–Opfikon WWTP during this period. The organic sludge loading rate is comparable to the value reported for Garmerwolde WWTP by Pronk et al. (2015) (0.10 kgCOD $kgTSS^{-1}$ d^{-1}) and well below the standard design value of 0.40 kgCOD $kgTSS^{-1}$ d^{-1} proposed by Lopez-Vazquez et al. (2023).

2.2.2. Sludge collection

AGS samples were always collected 5 min after the start of the aeration phase during maximum-capacity air flow to ensure complete mixing. The sampling port is located 4.4 m below the surface (reactor water depth is 6.5 m). Tubing was always flushed for 1 min before sampling to ensure that a representative sample was collected.

The excess sludge removal rate is required for calculating the SRT of flocs (Section 2.5.1). At Kloten–Opfikon WWTP, excess sludge was collected in a sludge buffer tank prior to final dewatering. In our study, the excess sludge removal rate was determined from the load of sludge leaving the sludge buffer.

2.2.3. Sieving

Sieving was conducted to determine the size distribution of bio-aggregates based on TSS, and to collect samples of various size-classes for subsequent DNA analysis and activity tests. AGS was sieved into four size-classes using woven wire mesh sieves (Retsch): flocs (<0.25 mm), small granules (0.25–1 mm), medium granules (1–2 mm), and large granules (>2 mm). In literature, the cutoff for flocs is normally at 0.2 mm (de Kreuk et al., 2007) or at 0.25 mm (Layer et al., 2019). For this study, we chose the latter, given that at Kloten–Opfikon WWTP, relatively large particles exhibited the morphology of flocs.

A critical aspect of the separation of the size-classes by sieving is the formation of a cake on the sieve. Applying a controlled protocol for rinsing the filtration cake is particularly important to ensure reproducibility when separating flocs from small granules, and this aspect is often overlooked in studies focusing on densification or granulation. At each step of sieving, the sludge cake formed on the sieve was carefully rinsed at a controlled flowrate of 1 L min^{-1} and washing volume of 15 L $gTSS^{-1}$ of expected flocs on the sieve. The rinsing flowrate and volume were selected according to the results of sieving tests conducted under a wide range of rinsing conditions (flowrate varied from 0.5 to 2 L min^{-1} and volume varied from 13 to 70 L $gTSS^{-1}$). To determine the size distribution and sample storage for DNA analysis, demineralized water was used for rinsing. To separate the size-classes for activity tests, presettled effluent was used to avoid any change in the bulk liquid composition that would impact microbial activities. The sample volume was adapted to the type of analysis (size distribution: 0.2 L for flocs and small granules, 1 L for medium and large granules; sample storage for DNA analysis: volume not critical; activity tests: larger volumes in order to receive 1 gTSS L^{-1} per size-class).

2.2.4. Determination of AOB and NOB activities

AOB and NOB activities were quantified for each size-class. AGS was first collected and sieved into the four size-classes. A sample of each size-class of sludge was then placed into a 1 L beaker and filled with primary effluent from the same WWTP. A TSS concentration of roughly 1 gTSS L^{-1} was maintained in each beaker. The beakers were left idle at room temperature overnight. Aeration and mixing were then started to reactivate the nitrifiers. Oxygen concentration was maintained near saturation by constant aeration. Mechanical stirrers were used for mixing. The pre-aeration phase also ensured that the nitrification tests were performed in the absence of organic substrate, so that the nitrite uptake rate could be directly derived from the nitrate production rate (no denitrification). After a reactivation phase of 1.5 h, the NH_4^+ concentration of each beaker was measured with cuvette tests (LCK303, Hach Lange GmbH, Switzerland). An ammonia spike (NH_4Cl solution) was added to obtain approximately 50 mgNH_4^+–N L^{-1} in each beaker. Samples were then collected at 20 min intervals for 2 h and analysed for NH_4^+, NO_3^-, and NO_2^-. Ammonia uptake rate (AUR) and nitrite uptake rate (NUR) (based on nitrate production due to low bulk NO_2^- concentrations) were determined by linear regression.

Flux and penetration depth calculations were performed according to Chen et al. (2020) to compare the limiting substances in the activity tests with those in full-scale operation, and to confirm that the growth conditions maintained during the activity tests mirrored those applied during normal full-scale operation (Supplementary Information S1). Results from calculations indicated that for ammonia oxidation, dissolved oxygen (DO) was limiting during the activity tests similarly to full-scale operation, while ammonia oxidation rate was slightly higher due to the oxygen saturation (as opposed to dissolved oxygen set-point of 2.5 mgO_2 L^{-1} during normal full-scale operation). For the nitrite oxidation, nitrite was limiting during both the activity test and duringfull-scale operation where similar nitrite concentrations were measured. The AUR and NUR values measured were also compared with the rates of the full-scale AGS SBR, which were calculated from sensor data. Overall, both the comparison of the activity tests results with full-scale nitrification rates and the flux and penetration calculations confirm the relevance of the activity tests to understanding the distribution of nitrifying activities among flocs and different-sized granules.

2.3. Physical and chemical analysis

TSS was quantified according to the standard procedure described in APHA (2023). Glass fibre filters were used with a pore size of 1.4 μm and with diameters of 90 mm for flocs and small and medium granules and 240 mm for large granules (MN GF-4, Macherey–Nagel). Cations (NH_4^+–N) and anions (NO_3^-–N and NO_2^-–N) were measured with ion chromatography (930 Compact IC Flex and 881 Compact IC pro, respectively; Metrohm).

2.4. Molecular microbiology analysis

2.4.1. DNA extraction

Samples for digital droplet polymerase chain reaction (ddPCR) were collected at intervals of 2 to 4 weeks and then sieved into the four size-classes. Biological duplicates were transferred into 1.5 mL microtubes (Protein LoBind Tubes, Eppendorf) and centrifuged at 10,000 RPM for

Table 3
Primers and ddPCR conditions. All primer sets target 16S rRNA genes. The coefficient of variation indicates the ratio of the standard deviation relative to the mean.

Target organism	Primers	Reference	Annealing temperature	Sample dilution	Coefficient of variation of positive controls
Nitrosomonas	CTO189FA/B CTO189FC[a] RT1R	(Hermansson & Lindgren, 2001)	52 °C	1:1000	16 %
Nitrospira	NSR 1113F NSR 1264R	(Dionisi et al., 2002)	60 °C	1:10	34 %
Nitrotoga	NTG200f NTG840r	(Alawi et al., 2007)	57 °C	1:10	49 %

[a] The forward primers CTO189FA/B and CTO189FC were added in a 2:1 ratio as described in (Hermansson & Lindgren, 2001).

2 min (uniCFUGE 5, LLG Labware). The supernatant was discarded and the pellet stored at -80 °C until further processing. DNA was extracted from each using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). The manufacture's protocol was followed except that samples were lysed four times at 4 °C using a Cryo 24 & Bead Ruptor 24 (Omni) with an idle phase of 2 min in between. Then, 5–620 mg of wet biomass was used for each DNA extraction. Concentrations of the DNA extractions were determined with the Qubit dsDNA HS Assay on a 96-well plate using the Spark 10M plate reader (Tecan), then normalized to a working concentration of 10 ngDNA μL^{-1} with DNase/RNase-free distilled water.

2.4.2. Droplet digital PCR

Our study applied ddPCR for absolute quantification of targeted organisms (Hindson et al., 2011) rather than 16S sequencing, which only provides information about relative abundances (van Loosdrecht et al., 2016). The ddPCR was performed on a QX200™ (Bio-Rad). *Nitrosomonas* (AOB), *Nitrospira* (NOB), and *Nitrotoga* (NOB) are the dominant nitrifying genera in activated sludge (Wegen et al., 2019), which was confirmed by 16S rRNA gene amplicon sequencing to be the case for the Kloten–Opfikon WWTP (Fig. S1). The three genera were quantified using primers targeting the 16S genes (Table 3). For each DNA extraction, technical duplicates were conducted in a 22-µL reaction mix containing 11 µL QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad), 1 µL 10 ng μL^{-1} template, 0.275 µL each of 20 µM forward and reverse primers for a final concentration of 250 nM, and 9.45 µL DNAse/RNAse-free water. Then, 20 µL of reaction mix and 20 µL of Automated Droplet Generation Oil for EvaGreen (Bio-Rad) were used to generate droplets with the Automated Droplet Generator (BioRad). PCR was performed (T100™ Thermal Cycler, Bio-Rad) following the protocol suggested by the manufacturer: 95 °C for 5 min, 40 cycles of 95 °C for 30 s (denaturation) and 1 min at the respective annealing temperature (annealing/extension) (Table 3), 4 °C for 5 min and 90 °C for 5min. Annealing temperature was optimized by preliminary tests applying a temperature gradient between 52 °C and 66 °C, aiming for optimal separation between positive and negative droplets (Huggett, 2020). Similarly, PCR inhibition was eliminated by testing various sample dilutions. After PCR, positive and negative droplets were counted (QX200™ Droplet Reader, Bio-Rad). Data were then analysed with the QX Manager software 2.1 (Bio-Rad). Automatic thresholding was applied to separate positive and negative droplets, confirmed visually, and where necessary adjusted manually for each sample.

In each run, positive and negative controls confirmed the robustness and reproducibility of the protocol. For positive controls, 16S gene sequences of *Nitrosomonas europaea* (RefSeq ID: NC_004757.1) and of *Nitrospira fluvii* (RefSeq ID: NZ_CAJNBJ010000003.1) were obtained from NCBI and synthetic fragments were ordered from IDT, and for *Nitrotoga*, one of the samples from the Kloten–Opfikon WWTP was used. For negative controls, both DNA extractions without biomass and PCR reactions with water were used.

2.4.3. Data analysis

Measurements from technical duplicates were averaged. If the relative difference of the technical duplicates was higher than the coefficient of variation of the positive controls of the specific primer set (Table 3), the measurement was omitted. Both measurements of the biological duplicates were used for further analysis. The concentrations were converted from copies μL^{-1} to copies $gTSS^{-1}$. For the time series of each size-class and target genus, a nonparametric model was fitted with a local polynomial regression (R, method = loess, span = 0.5).

2.5. Calculations

2.5.1. Solids retention time of flocs

The aerobic SRT of the flocs was approximated using Eq. (1) and without accounting for seeding. We expect that in AGS, biomass in the flocs partly results from seeding from granules (Zhou et al., 2014), similar to seeding from the biofilm in IFAS systems (Houweling & Daigger, 2019). Therefore, the values we report are actually an underestimation of the true SRT of the biomass of the flocs.

$$SRT_{flocs,aerobic} = \frac{V \cdot X_{flocs}}{(Q - Q_W) \cdot x_{e,flocs} + Q_{W,\ buffer} \cdot X_{W,\ buffer,flocs}} \cdot f_{aerobic} \quad (1)$$

where $SRT_{flocs,aerobic}$ is the aerobic solids retention time of flocs [d], V is the reactor volume [L], X_{flocs} is the floc concentration in the reactor [gTSS L^{-1}], Q is the influent wastewater flow rate [L d^{-1}], Q_W is the excess sludge flow rate [L d^{-1}], $X_{e,flocs}$ is the effluent flocs concentration [gTSS L^{-1}], $Q_{W,buffer}$ is the flow rate leaving the buffer tank [L d^{-1}], $X_{W,buffer,flocs}$ is the floc concentration in the excess sludge leaving the buffer tank [gTSS d^{-1}], and $f_{aerobic}$ is the fraction of aeration phase per cycle [-]. Values for X_{flocs} stem from TSS measurements. Values for $X_{e,flocs}$ were derived from an online optical turbidity sensor placed in the effluent line, with all the solids in the effluent assumed to be flocs. Values for $X_{W,buffer,flocs}$ were obtained either from sampling and sieving of the excess sludge samples from the buffer tank and subsequent TSS measurements or from solids measurements taken with an online optical turbidity sensor placed in the effluent line of the pre-thickener and the flocs to small granule ratios, which was determined for the biological

Table 4
Parameters for SRT_{min} calculation.

		AOB	NOB
Kinetic parameters (SUMO2, Dynamita)			
$u_{max,20°C}$	[d^{-1}]	0.9	0.65
$b_{20°C}$	[d^{-1}]	0.17	0.15
K_N	[mgN L^{-1}]	0.7	0.1
K_{O2}	[mgO_2 L^{-1}]	0.25	0.25
$\theta_{\mu max}$	[-]	1.072	1.06
θ_b	[-]	1.03	1.03
Electron acceptor/donor bulk concentrations			
S_N (NH_4+ or NO_2)	[mgN L^{-1}]	12[a]	0.3[b]
S_{O2}	[mgO_2 L^{-1}]	2.5[c]	2.5[c]

[a] 1/3 of the average influent NH_4^+ concentration (volume exchange ratio at Kloten–Opfikon WWTP = 1/3).

[b] Nitrite effluent concentration from composite samples of Kloten–Opfikon WWTP followed a log-normal distribution with a mean value of 0.3 mgN L^{-1} during summer. Although the effluent nitrite was usually below 0.3 mgN L^{-1}, we chose to use this mean value assuming that during aeration in the SBR, the nitrite concentrations were actually above the effluent concentrations. The value 0.3 mgN/L^{-1} is the maximum nitrite effluent concentration recommended by the Swiss legislation.

[c] Operating DO concentration at Kloten–Opfikon WWTP.

reactor. Both Q_W and $Q_{W,buffer}$ were obtained from the flow measurements of the sludge buffer.

2.5.2. Minimum SRT of AOB and NOB

Minimum SRT values for AOB and NOB were determined with Eqs. (2) to (5) (Metcalf et al., 2014). Bulk electron acceptor/donor concentrations were used as in operating conditions, and kinetic values were from the biological model SUMO2 (Dynamita) (Table 4). Calculations using kinetic parameters from Metcalf et al. (2014) yielded similar $SRT_{X,min}$ (Supplementary Information S3).

$$\mu_X = \mu_{max,X,T} \cdot \left(\frac{s_N}{s_N + k_N}\right) \cdot \left(\frac{s_{O2}}{s_{O2} + k_{O2,X}}\right) - b_{X,T} \quad (2)$$

$$SRT_{X,min} = \frac{1}{\mu_X} \quad (3)$$

The subscript X indicates AOB or NOB, the subscript N indicates NH_4^+ or NO_2^-, μ_X is the specific growth rate [gVSS gVSS^{-1} d^{-1}], $\mu_{max,X,T}$ is the maximum specific growth rate at temperature T [gVSS gVSS^{-1} d^{-1}], $b_{X,T}$ is the specific endogenous decay rate at temperature T [gVSS gVSS^{-1} d^{-1}], S_N is the NH_4^+ or NO_2^- concentration [mgN L^{-1}], K_N is the half-saturation constant for NH_4+ or NO_2^- [mgN L^{-1}], S_{O2} is the DO concentration [mgO_2 L^{-1}], and $K_{O2,X}$ is the half-saturation constant for DO for AOB or NOB [mgO_2 L^{-1}]. $u_{max,X,T}$ and $b_{X,T}$ were corrected for temperature:

$$\mu_{max,X,T} = \mu_{max,X,20°C} \cdot \theta_{\mu max,X}^{(T-20)} \quad (4)$$

$$b_{X,T} = b_{X,20°C} \cdot \theta_{b,X}^{(T-20)} \quad (5)$$

$\theta_{\mu max,X}$ and $\theta_{b,X}$ are the temperature coefficients [-]. Temperature values were obtained from an online sensor in the AGS reactor at Kloten–Opfikon WWTP.

3. Results

3.1. What is the size distribution in AGS?

The change in size distribution in AGS was monitored over 14 months (Fig. 1). AGS from Kloten–Opfikon WWTP consists of bioaggregates with a highly heterogeneous size distribution, from flocs (<0.25 mm) to granules between several hundred µm and 6 mm in diameter (Fig. S4). During the start-up phase (Fig. 1, white background), the biomass concentration gradually increased from 3.6 to 5.0 gTSS L^{-1}. After this start-up phase, a rather stable total TSS concentration of 4.7 ± 0.7 gTSS L^{-1} was maintained. The variation in May and June 23 resulted from short operational changes. After the start-up phase (Fig. 1, bright and dark brown background), the relative AGS composition remained stable throughout the monitoring campaign. Overall, large granules of 2 to 6 mm diameter represented the most abundant biomass fraction (50 ± 11 %), and flocs were the second most abundant fraction with a high proportion of 36 ± 5 %. The remainder were smaller granule sizes.

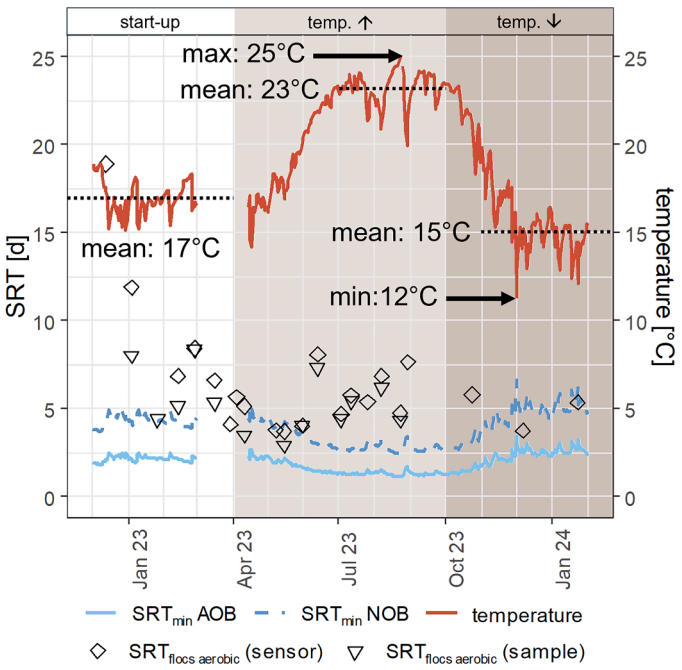

Fig. 2. Trend of temperature, SRT_{min} values for AOB and NOB and the $SRT_{flocs,aerobic}$ values, during start-up and increasing and decreasing temperature. The SRT_{min} were derived as described in Section 2.5.2. The $SRT_{flocs,aerobic}$ were calculated as described in Section 2.5.1 from either excess sludge sensor (diamond markers) or sample data (triangle markers).

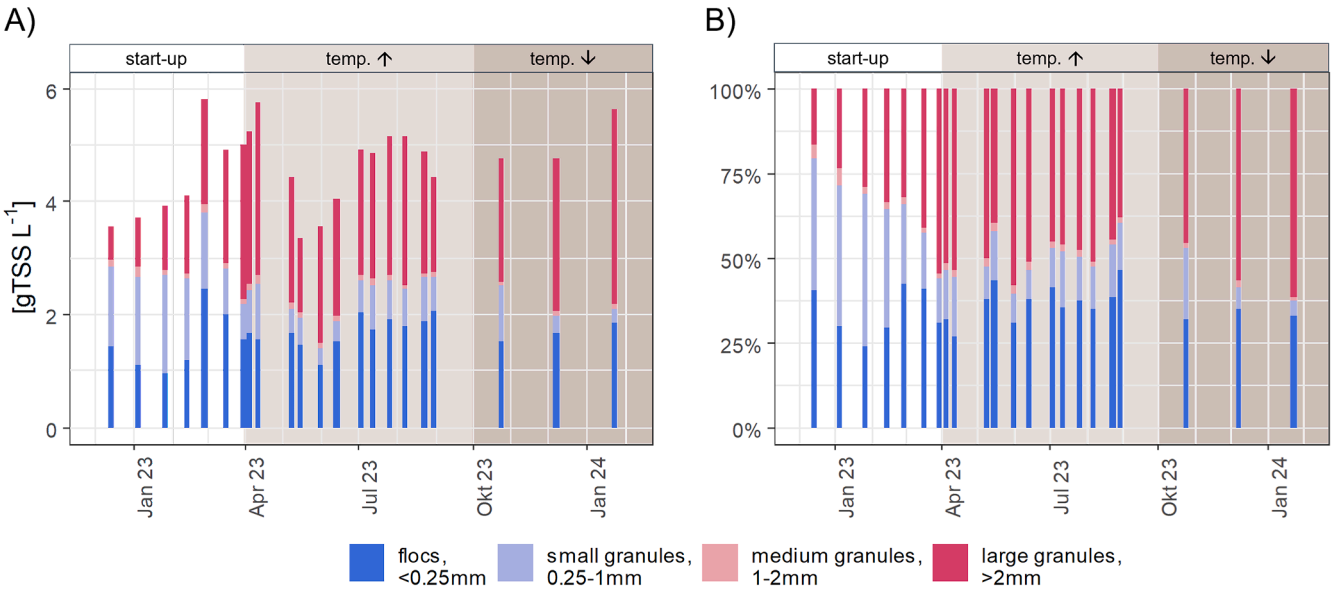

Fig. 1. Change in the TSS size distribution of AGS in (A) absolute and (B) relative values, during start-up and increasing and decreasing temperature.

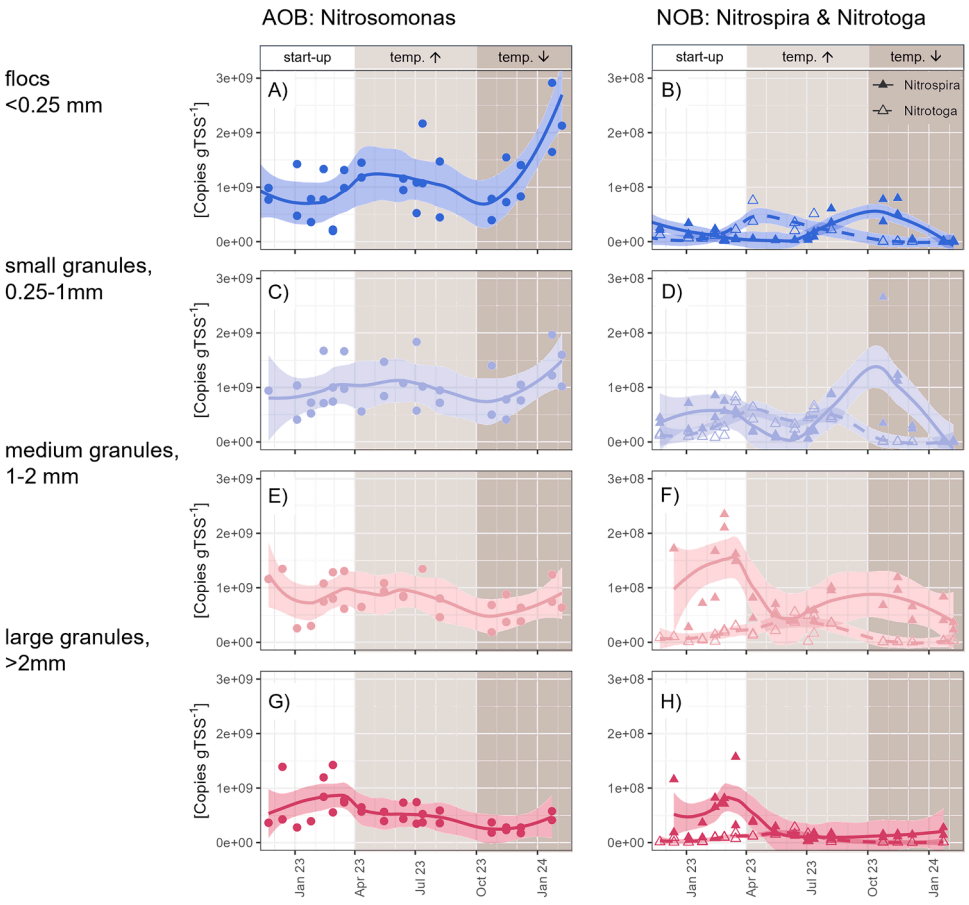

Fig. 3. Specific copy numbers [Copies $gTSS^{-1}$] of *Nitrosomonas* (AOB) (A, C, E, G) and *Nitrospira* and *Nitrotoga* (NOB) (B, D, F, H). Nonparametric models and 95 % confidence intervals are shown for each size-class and each genus.

3.2. What is the SRT of flocs?

The aerobic floc SRT was determined and compared to the minimum SRT of AOB and NOB, which were calculated as a function of temperature (Fig. 2). Overall, the $SRT_{flocs,aerobic}$ was well above the SRT_{min} of AOB, and only slightly above and occasionally equal to the SRT_{min} of NOB. After start-up, the excess sludge consisted of 80 % flocs and 20 % small granules (n = 8). The average floc SRT was 5.1 ± 1.4 d (n = 23) and was intermittently <5 d in April and May 23 due to operational changes. The mixed liquor temperature was stable at 17 °C during the start-up phase and was then gradually increased to 25 °C during the increasing temperature phase. During the decreasing temperature phase, the temperature then dropped on average to 15 °C with minimum values of 12 °C during rain and snow events. As the temperature decreased, the SRT_{min} value of AOB increased from 1.3 to 2.5 d and that of NOB from 2.5 to 5.0 d

3.3. How are the nitrifying communities distributed among the size-classes?

To assess the roles of flocs and granules in nitrification, we first monitored the distribution of the dominant nitrifying genera among the size-classes over seasons (Fig. 3). The total copy numbers and actual distribution of AOB and NOB at reactor-scale were derived from the size distribution and from the specific copy numbers for each size-class (Fig. 4). Overall, AOB preferentially enriched in flocs and small granules and were never washed out of the system, resulting in relatively stable total AOB copy numbers. In contrast, NOB (first *Nitrotoga*, then *Nitrospira*) were washed out from flocs and small granules, and their enrichment in large granules remained low throughout the study. As a result, the total NOB copy numbers showed considerable variability over seasons.

At the beginning of monitoring, similar *Nitrosomonas* copy numbers were observed in all four size-classes (Fig. 3). Over the course of the study, *Nitrosomonas* copy numbers in flocs and small granules increased, whereas a decrease was measured in the medium and large granules. By the end of the study, the enrichment was considerably higher in the small size-classes (<1 mm) than in the large size-classes (>1 mm). At reactor scale, the total copy number of AOB remained rather stable, despite an increase during the decreasing temperature phase (relative standard deviation after start-up: 34 %, Fig. 4A). Two thirds of the AOB were found in the small size-classes <1 mm, and one third was present in large granules (Fig. 4C). AOB were negligible in medium granules throughout the study.

The dominant NOB genera, *Nitrospira* and *Nitrotoga*, were successively enriched and washed out in the <2 mm size-classes (Fig. 3). By the end of the monitoring period, both *Nitrotoga* and *Nitrospira* were nearly absent as a result of their washout during the increasing and decreasing temperature phases. In large granules, *Nitrospira* copy numbers remained low after start-up and slowly increased only during decreasing temperature phase, whereas *Nitrotoga* were almost entirely absent throughout the study. At reactor scale, the total copy numbers of NOB exhibited a greater variability than did those of AOB (relative standard deviation after start-up: 52 %, Fig. 4B). During the cold months from October to December, when NOB were nearly completely washed out from the flocs and small granules, the total NOB copy numbers declined by 79 %. Throughout the study, the distribution of NOB among the flocs and small and large granules was highly variable (Fig. 4D). Similarly to AOB, medium granules contained negligible NOB throughout the study.

Notably, AOB were around 10 times more abundant than NOB, likely

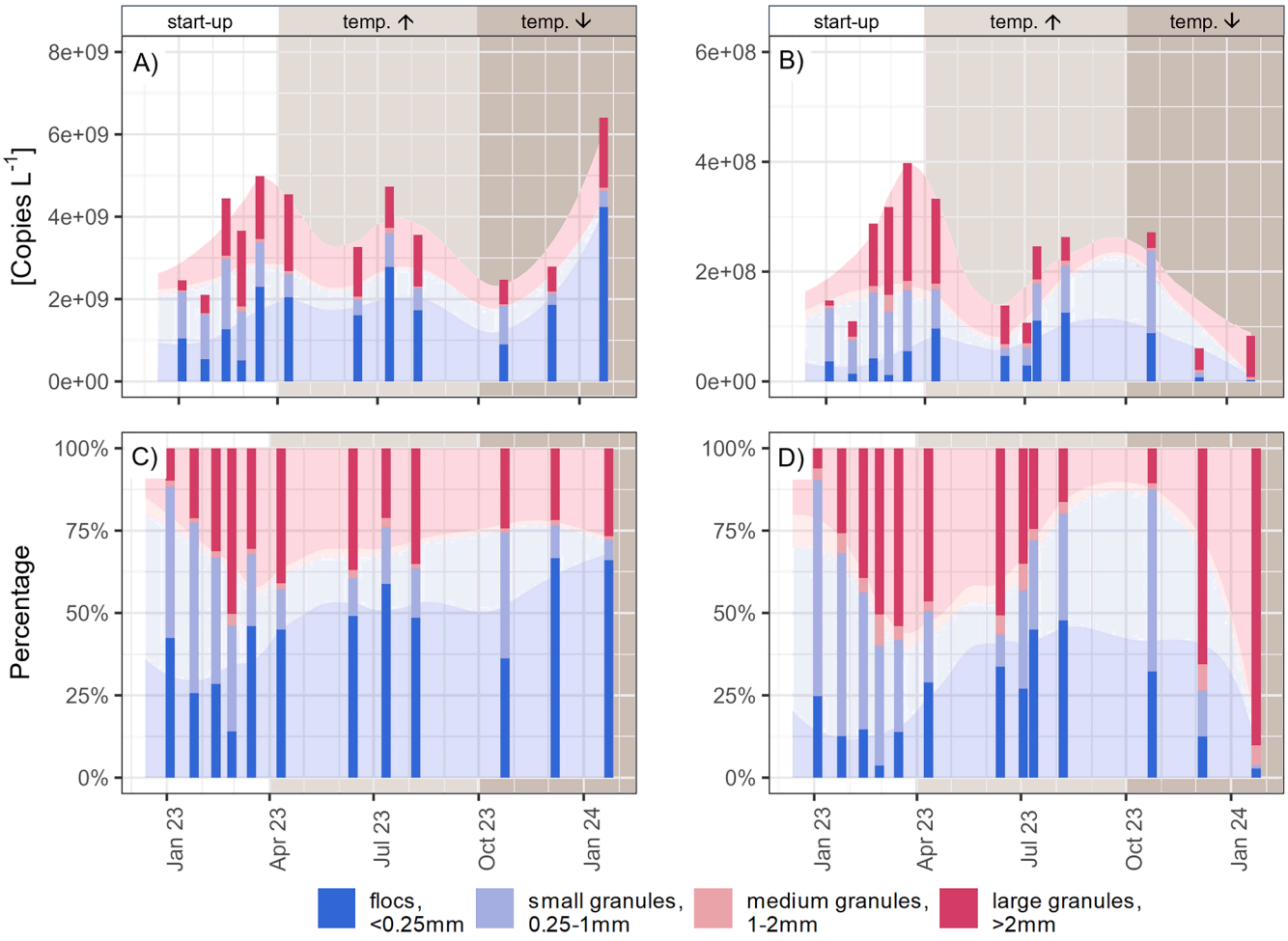

Fig. 4. Volumetric copy numbers [Copies L^{-1}] of *Nitrosomonas* (AOB) (A absolute, C relative) and *Nitrospira* + *Nitrotoga* (NOB) (B absolute, D relative), during start-up and increasing and decreasing temperature phases. The specific copy numbers were weighted by the TSS size distribution at reactor scale and cumulated. The bars show the volumetric copy numbers derived from the average of the measurements of the biological duplicates and the TSS measurements. The shaded areas in the background show the volumetric copy numbers derived from the nonparametric model for the specific copy numbers and for the TSS measurements.

due to a combination of true difference, difference in primer specificity, and possibly other unknown factors. Nevertheless, the quality check on the ddPCR analysis and the comparison with the trend of activities (Section 3.4) confirmed the validity of our measurements.

3.4. How are the nitrifying activities distributed among the size-classes?

3.4.1. What are the specific activities of the size-classes?

Diffusion limits the microbial conversion rates of biofilm systems. Hence, the activities of nitrifiers among the size-classes in AGS are not proportional to their abundance. To gain further insight, activity tests were performed on all the size-classes (Fig. 5). Our results indicate that the specific activities of large granules are significantly lower than those of flocs and small granules (Fig. 5).

During the increasing temperature phase, specific AOB activities were similarly high in flocs and small granules (3.7 ± 0.9 and 3.7 ± 0.4 mgN $gTSS^{-1}$ h^{-1}, Fig. 5A and C, respectively) and substantially lower in medium and large granules (2.1 ± 0.7 and 0.8 ± 0.4 mgN $gTSS^{-1}$ h^{-1}, Fig. 5E and G, respectively). During the same phase, specific NOB activities were the highest in small granules (4.5 ± 2.1 mgN $gTSS^{-1}$ h^{-1}, Fig. 5D), followed by those in flocs (3.1 ± 0.6 mgN $gTSS^{-1}$ h^{-1}, Fig. 5B). Specific NOB activities found in medium and large granules were very low (1.3 ± 0.3 and 0.3 ± 0.2 mgN $gTSS^{-1}$ h^{-1}, Fig. 5F and H, respectively). Notably, specific NOB activities were zero in all size-classes in January 24.

The specific activities of the total mixed liquor suspended solids (MLSS) were calculated as the sum of the weighted specific activities of each size-class. Similar specific activities of the total MLSS of around 2 mgN $gTSS^{-1}$ h^{-1} were quantified for both AOB and NOB (Fig. 5I and J). The specific activities were compared with the corresponding specific copy numbers and as a visual aid, a linear regression through the origin was fitted for each size-class (Fig. 6). The specific activities increase with increasing copy numbers for all size-classes <2 mm, while the specific activities of large granules remain low even with increasing copy numbers. At similar copy numbers, specific AOB activities in large granules represent around one third of those of the smaller size-classes. At similar copy numbers, specific NOB activities in medium and large granules represent around one fifth of those of the flocs and small granules.

3.4.2. What are the contributions of the various size-classes to the overall nitrification rates at reactor scale?

The total nitrification rates and the distribution of nitrifying activities at the reactor scale are the products of the specific activities and the TSS size distribution. The change in volumetric AOB and NOB activities is shown in Fig. 7. Most of the nitrifying activity (>50 %) is found in flocs, although they represent only one third of the biomass, whereas large granules (50 % of the biomass) contribute less than 20 %.

During the increasing temperature phase, the total AURs and NURs were 10.2 ± 2.1 and 9.1 ± 3.2 gN m^{-3} h^{-1}, respectively. These

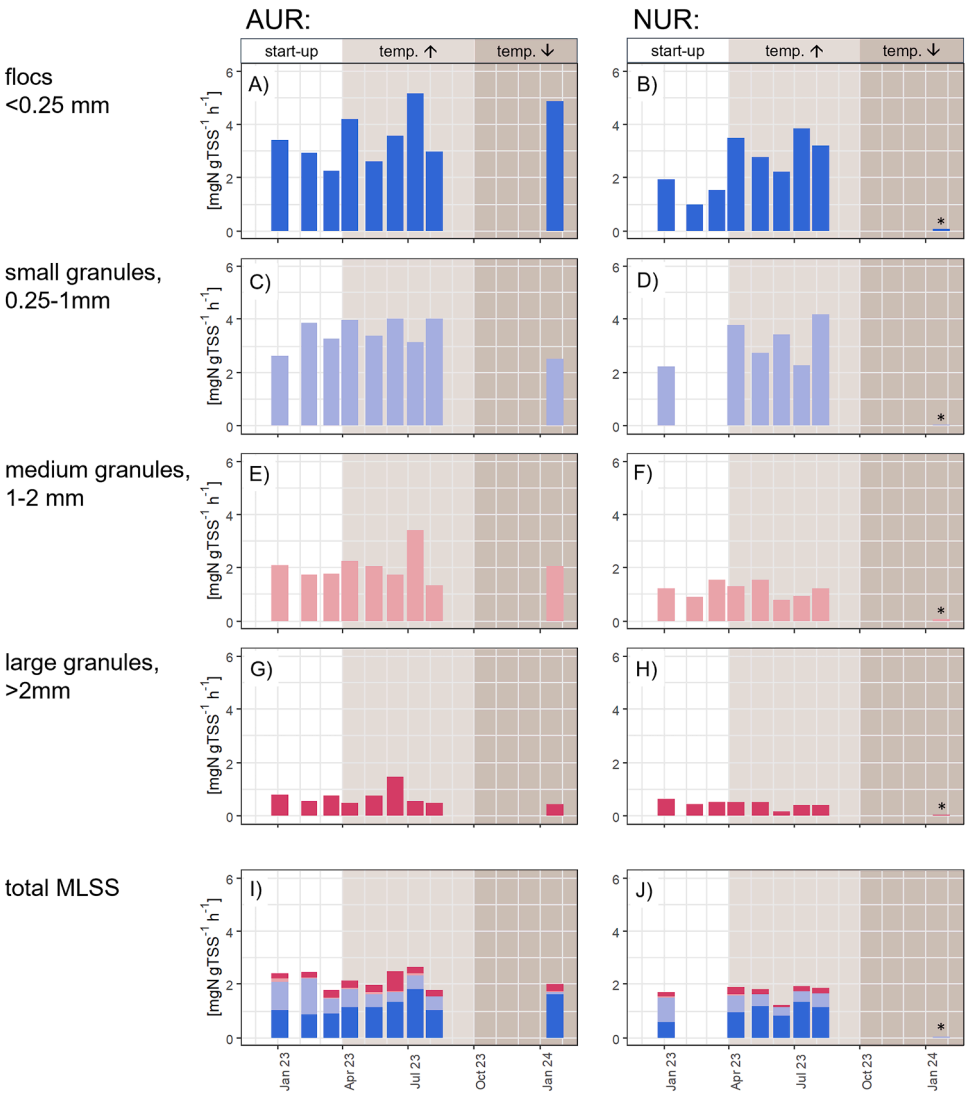

Fig. 5. Specific activities [mgN $gTSS^{-1}$ h^{-1}] of AOB, i.e., AUR (A, C, E, G, I), and NOB, i.e., NUR (B, D, F, H, J), during start-up, increasing and decreasing temperature. The total MLSS activities were estimated from the sum of the weighted specific activities based on the size-classes TSS in the reactor. *Zero activity measured.

experimentally determined activities align with the nitrification rates observed at the full-scale WWTP of Kloten–Opfikon (AUR = NUR = 9 gN m^{-3} h^{-1}). During this phase, more than 50 % of the total AURs and NURs were attributed to the flocs. Specifically, 83 % of the total AURs and 91 % of the total NURs were found in the flocs and small granules, whereas granules >1 mm contributed to the remaining minor activities. Similar AURs were monitored during the decreasing temperature phase (11.4 gN m^{-3} h^{-1}), whereas the NURs dropped to zero, consistent with the wash-out of NOB indicated by the copy numbers (Fig. 4).

3.5. *What are the consequences of the roles of flocs and granules of various sizes in nitrification performance at reactor scale?*

Ammonia and nitrite effluent concentrations were monitored by analysing composite samples of the effluent from both the monitored and a parallel AGS reactor. Ammonia effluent concentrations complied with the Swiss guideline of 2 mgN L^{-1} during the monitoring campaign. Until October 23, the Kloten–Opfikon WWTP also complied with the Swiss nitrite effluent recommendation of 0.3 mgN L^{-1} (GSchV, 2023), except for a few minor nitrite events between January and May 23 that occurred due to operational changes. Elevated nitrite concentrations up to 10 mgN L^{-1} were observed when temperature dropped during winter 23/24 (minimum temperature of 15 °C as opposed to 17 °C, during winter 22/23).

4. Discussion

4.1. *In AGS, flocs play a critical role in nitrification*

A main result of our study is that flocs represent a significant solids fraction of AGS and play a critical role in nitrification in full-scale aerobic granular sludge (AGS) WWTPs. Monitoring of a full-scale AGS WWTP in Switzerland over 14 months, that was in the commissioning phase but still operated below design / standard AGS loading rate, revealed that flocs contributed more than 50 % to the nitrifying activities during warm summer months. The predominant role of flocs became problematic during the cold season, when the NOB activity in flocs diminished, resulting in critical nitrite accumulation. These observations raise three questions: (i) Are flocs an integral part of AGS, and if so, to what extent? (ii) How can the fairly high specific activities of flocs be explained despite their selective removal? (iii) What are the implications for the resulting activity distribution?

AGS systems have long been referred to as biofilm-only systems consisting only of granules (de Kreuk et al., 2007; Giesen et al., 2013). But our study challenges this conventional conception. Our results reveal that flocs represent a high fraction of the sludge, approximately

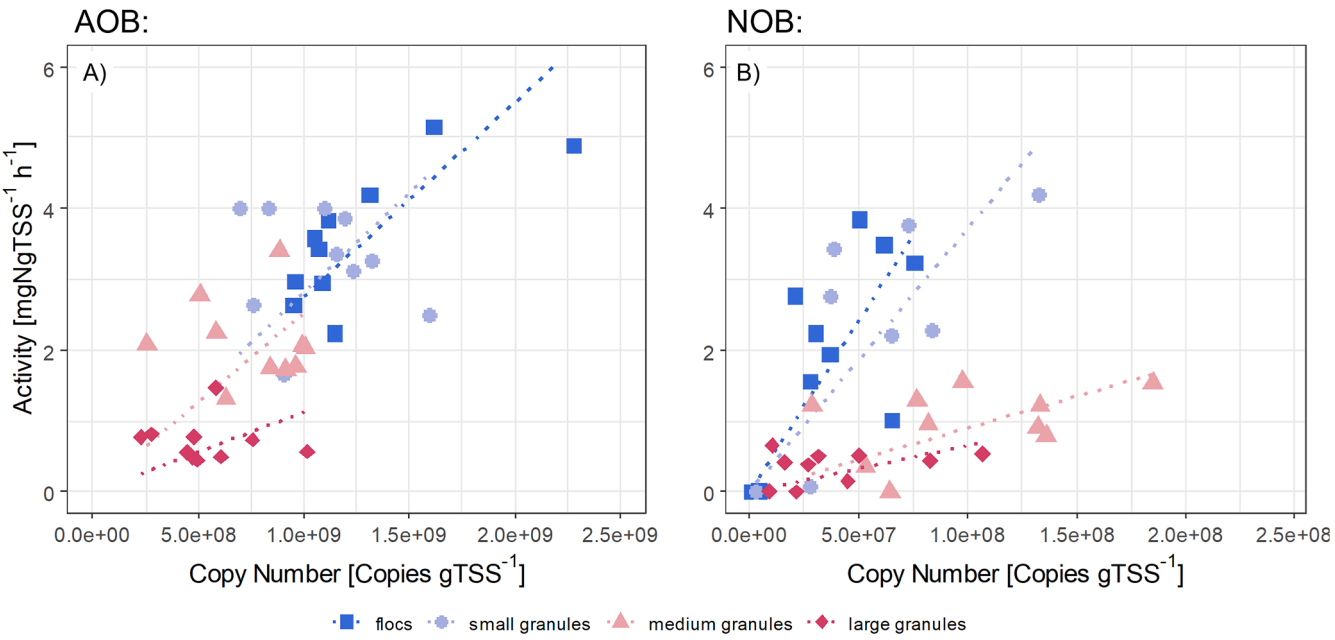

Fig. 6. Specific activities [mgN $gTSS^{-1}$ h^{-1}] vs. copy numbers [copies $gTSS^{-1}$] for AOB (A) and NOB (B). Mass-transfer limitation is "lumped" into the values of specific activity and therefore, different slopes indicate different extent of mass transfer limitation. Linear regressions through the origin were fitted as a visual aid.

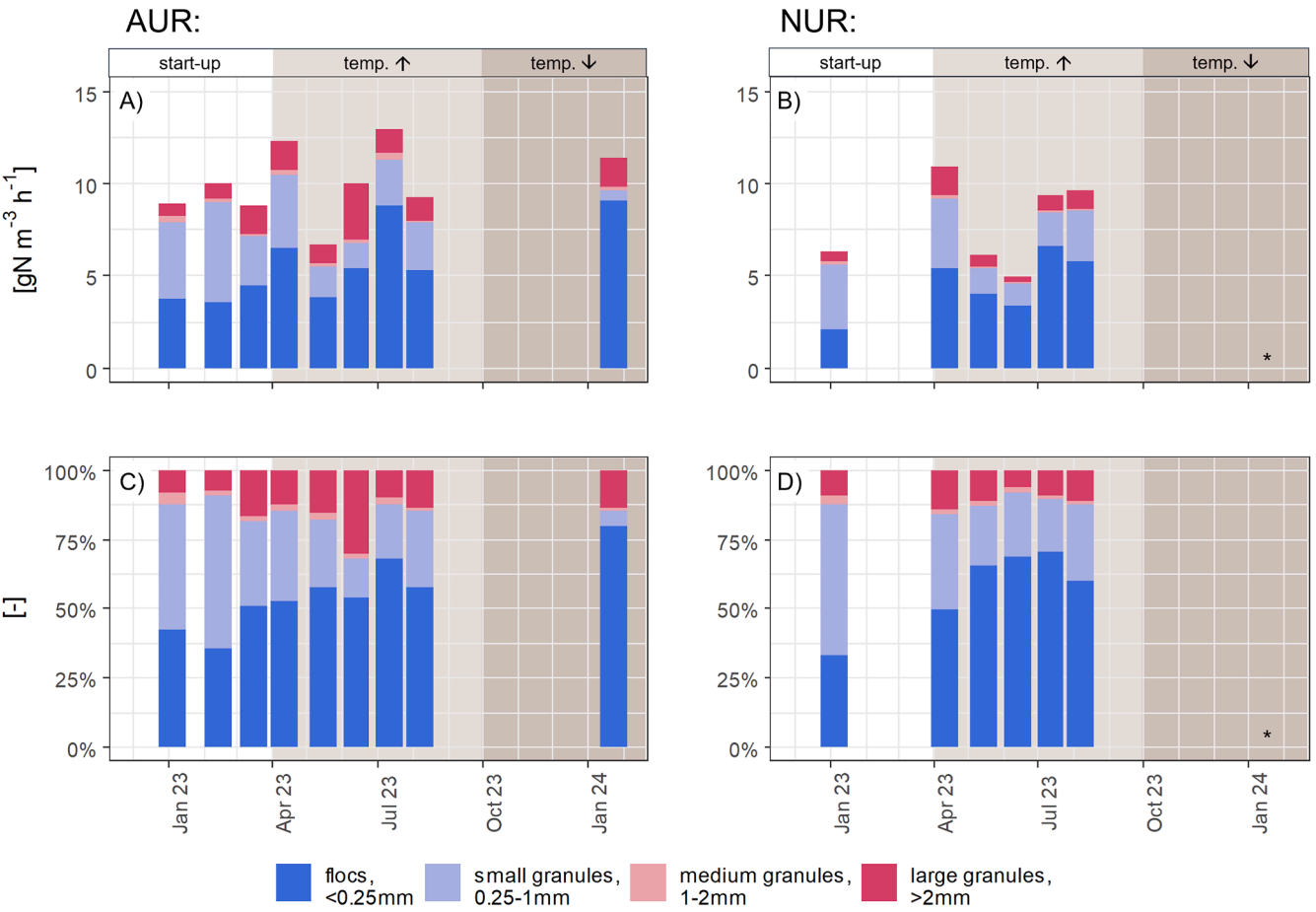

Fig. 7. Distribution of volumetric activities of AOB, i.e., AUR (A absolute, C relative) and NOB, i.e., NUR (B absolute, D relative), during start-up and increasing and decreasing temperature. The specific activities weighted based on the TSS size distribution at reactor scale and cumulated. *Zero activity measured.

one-third, and that this fraction was maintained in the system throughout the monitoring campaign. Flocs in AGS comprise debris from granules, biomass growing on residual COD under aerobic conditions, and influent particulate materials (van Dijk et al., 2018; Wagner et al., 2015; Zhou et al., 2014). The proportion of flocs in AGS depends on the composition of influent wastewater (Layer et al., 2019) and the operating conditions of the reactor, such as the selective pressure on slow-settling biomass (van Dijk et al., 2022). Our study is the first to quantify the floc proportion regularly over an extended period of >1 year in a full-scale WWTP. At the Kloten–Opfikon WWTP, we found a non-negligible average floc (<0.25 mm) content of 36 %, which is consistent with floc contents reported in literature ranging from 16 % to 40 % of TSS (Layer et al., 2019; Pronk et al., 2015; van Dijk et al., 2018). Considering both our observations and recent literature, we propose to revise our conception of AGS applied to the treatment of municipal WW: AGS should be viewed as a hybrid system comprising both suspended and biofilm biomass similar to IFAS systems (Houweling & Daigger, 2019). At present, however, the aims of excess sludge removal differ significantly between AGS and IFAS systems. Excess sludge removal in AGS systems seeks to selectively remove the flocs and retain the fast-settling granules, whereas in IFAS systems, the aim is to maintain a low floc SRT to promote nitrifying activity on the biofilm. Understanding the distribution of activities among AGS size-classes and the underlying mechanisms is crucial for determining the extent to which AGS systems operate like IFAS systems in terms of nitrifying activity distribution.

Another significant result of our study is the high specific activities observed in the small bio-aggregates (<1 mm). The specific AOB and NOB activities of the flocs and small granules were five to fifteen times higher than those of the large granules (Fig. 5). Similarly, Quoc et al. (2021) observed that specific nitrifying activities of small granules were five times higher (0.212–0.425 mm) than those of large granules (>1.4 mm). These high specific activities of small bio-aggregates can be explained (i) by their high surface-to-volume ratio, which minimizes the mass-transfer limitation compared to large granules (Fig. 6) (Strubbe et al., 2022) and (ii) by the preferential enrichment of nitrifiers in the small size-classes (Fig. 3).

As a result of the non-negligible floc proportion in the biomass and of the high specific activities of the small size-classes, we found that most nitrification occurred within the flocs during the warm season (>50 %, Fig. 7). Although flocs represented only one third of the total biomass (Fig. 1), their contribution to nitrification was crucial. In contrast, granules >1 mm (51 % of the biomass) contributed to only 17 % and 9 % of the AOB and NOB activities, respectively. Although some nitrifying activity in flocs is expected in IFAS, the extent of their contribution observed in our study is surprising. In full-scale IFAS systems, flocs can contribute 25–36 % to nitrifying activities, and most nitrification thus occurs within the biofilm, resulting in robust nitrification (Onnis-Hayden et al., 2011; Regmi et al., 2011). Although AGS systems are similar to IFAS systems in their sludge composition, our results highlight significant differences between AGS and IFAS systems in the distribution of nitrifying activity between flocs and granules/biofilm. The significantly higher contribution of flocs to nitrification observed in our study likely resulted in an unstable nitrification of the AGS system, leading to effluent nitrite concentrations above the Swiss legal recommendation. In our study of the Kloten–Opfikon AGS WWTP, NOB activities in flocs and small granules decreased as the temperature dropped, resulting in incomplete nitrification and elevated nitrite concentrations.

The comparison of the total nitrifying activities measured from AGS to those of other hybrid systems is not straightforward and can be misleading. The activities in a hybrid system depend on several factors such as design (e.g., area available for biofilm formation), operation (e.g., floc SRT control), wastewater composition, and loading conditions. Even comparing different AGS systems requires caution: Although the TSS concentration at the AGS WWTP in Garmerwolde was 60 % higher than that at Kloten-Opfikon WWTP (7.5 gTSS L^{-1} vs. 4.7 gTSS L^{-1}), the total volumetric activities were lower (AUR=6 and NUR=3 gN m^{-3} h^{-1}) than those measured at Kloten-Opfikon WWTP (experiments: AUR=10 and NUR=9 gN m^{-3} h^{-1}; full-scale: AUR=NUR=9 gN m^{-3} h^{-1}, Section 3.4.2). This is due to the rather low total specific activities found at Garmerwolde WWTP (AUR=0.8 and NUR=0.4 mgN $gTSS^{-1}$ h^{-1}) in comparison to those at Kloten-Opfikon WWTP (AUR=NUR=2 mgN $gTSS^{-1}$ h^{-1}). It is likely that the discrepancy between the nitrifying activities arises from differences in size distribution and microbial distribution, both of which can be linked to the history of AGS. In contrast, the total volumetric rates of densified activated sludge (DAS) with 50 % of flocs and 50 % of small granules <0.5 mm at up to 5 gTSS L^{-1} are potentially up to 50–140 % higher than AGS rates (Fumasoli et al., 2024; Regmi et al., 2022), which results from the high specific activities that can be found in flocs and small granules (Fig. 5). Higher AGS nitrification rates could be achieved in the future with if higher biomass concentrations were reached or if nitrifier enrichment could be enhanced.

4.2. Mechanisms behind the distribution of nitrifying communities

A second important finding of our study is that nitrifiers would rather grow in the flocs than in the large granules (>2 mm) as we had anticipated. The preferential growth of nitrifiers in flocs resulted in a highly dynamic NOB assembly, which caused instabilities in the NOB activity of the reactor and in turn resulted in transient incomplete nitrification. Two key questions emerge from these observations: (i) Why was the enrichment in large granules limited? (ii) What are the mechanisms behind the dynamic NOB assembly?

Contrary to our expectations, nitrifiers were primarily found in flocs rather than in large granules, where they would benefit from higher SRT conditions than in the flocs (Layer et al., 2019; Onnis-Hayden et al., 2011). Although sloughing from biofilm can be the reason for nitrifier abundance in the flocs of a hybrid system (Houweling & Daigger, 2019), we argue that at Kloten–Opfikon WWTP, the nitrifiers were actually actively growing in the flocs and not merely the result of seeding. In a hybrid system such as AGS, microorganisms grow where the conditions are optimal: where the SRT is larger than their minimum SRT and where access to substrate is easier. The limited access to oxygen in the biofilm may result in a competition between slow and fast-growing organisms, i.e., between ordinary heterotrophic organisms (OHO) and nitrifiers (Houweling & Daigger, 2019). Under high organic loading on the biofilm, the growth of AOB may be limited by heterotrophic activity due to the limitation by oxygen. Consequently, although NOB growth is limited by nitrite and not oxygen, NOB growth may also be inhibited indirectly by heterotrophic activity if it induces limited AOB growth and thus limited nitrite production in the biofilm. Nevertheless, two observations suggest that heterotrophic activity occurred in the flocs and that competition between OHO and nitrifiers was not responsible for the limited growth of nitrifiers in granules at Kloten–Opfikon WWTP: 1) The organic loading on the flocs was 0.14 kgsCOD $kgTSS_{flocs}^{-1}$ d^{-1} and thus in a typical range for nitrifying activated sludge (Metcalf et al., 2014). 2) The floc SRT was 5.1 ± 1.4 d and thus considerably higher than the SRT_{min} of OHO of 1 to 3 d (Chen et al., 2020). However, the access to substrate in granules is limited by mass transfer, whereas substrate is easily accessible in the flocs. We therefore hypothesize that the relatively high SRT of the flocs allowed nitrifiers to grow there and thus limited their enrichment in the large granules. Although the monitored SBR was frequently operated at short cycles due to rain weather conditions, we argue that this did not cause the limited nitrifier growth in granules. Even when operating at longer cycles, growth conditions in flocs remain more favourable than in granules if the floc SRT is larger than the minimum SRT of nitrifiers due to easier access to substrate. Indeed, during the warm season, the operational floc SRT was normally above the minimum SRT of AOB and NOB (Fig. 2). Consequently, both AOB and NOB preferentially enriched in the flocs, and their growth in the large granules was limited. This pattern persisted for AOB but not for

NOB during the subsequent cold season. NOB were washed out from flocs, likely because their minimum SRT increased with the drop in temperature, therefore converging with the floc SRT (Fig. 2). The SRT in flocs was no longer sufficient for NOB growth in flocs during the cold temperature season. Meanwhile, the loss of nitrite oxidation activity in flocs was not compensated by an increase in the large granules, where the NOB growth was still limited despite their long SRT. The continued limited enrichment of NOB is likely due to the limited diffusion of oxygen into the large granules, resulting in a microbial competition between heterotrophs and nitrifiers. Oxygen diffusion within the granules is very dynamic over the aeration phase of an SBR (Layer et al., 2020). The resulting competition for oxygen between heterotrophs and nitrifiers might have been influenced by several factors such as cycle length (due to rain weather conditions) or bulk concentrations / surface area (as a result of the commissioning phase).

In addition to insufficient SRT, other factors such as nutrient or oxygen availability can lead to the loss of NOB. We acknowledge that both the minimum SRT and the floc SRT are approximations. The kinetic values for $SRT_{min,NOB}$ are for unknown NOB genera, although our evaluation of other sets of kinetic parameters confirmed the SRT_{min} values. The SRT_{flocs} were underestimated because seeding is neglected. However, influent NH_4^+, pH, and oxygen levels in winter remained similar to those in summer and were therefore excluded as a cause of NOB loss. This suggests that the convergence of the SRT values with decreasing temperatures is likely to explain the washout of NOB observed.

The abundance of NOB in the small size-classes was associated with a highly dynamic NOB community, similar to patterns observed for activated sludge systems (Gruber, Niederdorfer, et al., 2021; Ju et al., 2014). Throughout the study, *Nitrospira* and *Nitrotoga* dominated the NOB population alternatively, suggesting the occurrence of competition between the two NOB genera. *Nitrospira* were initially washed out and replaced by *Nitrotoga* before the reverse then happened (Fig. 3B and D). Although the exact factors governing the competition between *Nitrospira* and *Nitrotoga* are not fully understood (Nowka et al., 2015), previous studies indicated that *Nitrotoga* tend to dominate at low temperatures (Alawi et al., 2007; Alawi et al., 2009; Wegen et al., 2019) and at high nitrite concentrations (Kinnunen et al., 2017; Zheng et al., 2020). In contrast, *Nitrospira* grow preferentially at higher temperature and are characterized by a low nitrite half-saturation constant (Nowka et al., 2015). In our study, the replacement of *Nitrospira* by *Nitrotoga* coincided with slightly elevated nitrite levels from March to May 23. As nitrite level decreased and temperature rose from June to September 23, *Nitrotoga* was gradually washed out and outcompeted by *Nitrospira* (Fig. 3).

Nitrification remained robust during the warm season due to the high abundance and activities of both AOB and NOB in the small size-classes. However, during the cold season, the slow NOB enrichment in large granules was insufficient to compensate for the loss of NOB from flocs and small granules, which led to a significant loss of NOB activity (Fig. 7B) and elevated nitrite concentrations in the effluent. Curiously, NOB activities decreased not only in flocs and small granules, where NOB were almost completely absent by the end of the study, but also in medium and large granules despite their continued presence in these aggregates (Figs. 3 and 5). Similar to Wei et al. (2021) observations, we suspect a dependency of large granules on the smaller bio-aggregates to maintain their nitrification activities.

Due to the limited enrichment in large granules and the dynamics shift in the NOB community within the smaller size-classes, NOB activity was lost across all size-classes and elevated effluent nitrite concentrations were measured when temperature started to decrease. In the short-term, the DO setpoint can be increased to directly enhance diffusion into the granules and thus the activity. However, if NOB are not present, this measure will only have a limited effect. In the long-term, preventing incomplete nitrification under similar conditions may require a revised approach to the operational strategy of AGS wastewater treatment.

4.3. Optimization potential for the operation of AGS

Our study provides insights into optimizing the operation of AGS-based WWTP. Granules from AGS have good settling properties throughout the year and are associated with high SRT conditions. AGS therefore has the potential to ensure robust nitrification through all seasons, provided that a significant proportion of nitrifying activity occurs in the granules. To make use of the full potential of the AGS technology, we recommend to limit the contribution of flocs to nitrification in order to avoid transient accumulation of nitrite. Short periods of elevated nitrite concentrations in the effluent are often observed in Swiss WWTP during winter. Some 50–80 % of Swiss WWTPs regularly exceed the Swiss nitrite guideline value of 0.3 $mgNO_2$–N L^{-1} (GSchV, 2023) as a result of the transient washout of NOB and possibly the population dynamics of NOB (Gruber et al., 2024; Gruber, von Känel, et al., 2021). Nevertheless, the nitrite accumulation observed in our study was higher and lasted longer than is typically seen in Switzerland (Gruber et al., 2024) and cannot be attributed solely to a seasonal effect. In activated sludge systems, increasing the SRT is frequently advised to prevent partial nitrification related to the washout of NOB (Johnston et al., 2019). In AGS systems, however, the SRT required is already provided in the granules. Therefore, the challenge is to shift the nitrifiers from flocs, where they are susceptible to seasonal washout, to the granules, where they would benefit from high SRT conditions. Similar to the operation of IFAS systems, we suggest reducing the floc SRT to limit the nitrifier enrichment in the flocs and push them into the biofilm (Di Trapani et al., 2013; Houweling & Daigger, 2019). We therefore recommend minimizing the contribution of flocs to nitrification by controlling the floc SRT at a value below the minimum SRT over an extended period, and well in advance of the critical cold season. In contrast to the conventional excess sludge removal strategy, which aims to maintain good sludge settleability, excess sludge removal should therefore depend on a target floc SRT.

A shorter floc SRT might however have a detrimental effect on the long-term granulation. If large granules would mostly originate from densified flocs (van Dijk et al., 2022), more extensive floc removal might hamper the granulation process. A better understanding of the life cycle of AGS is therefore required. Second, a shift of the nitrifying population from flocs to large granules might not result in the desired increase of activity of the large granules if the effect of mass transfer limitation is too dominant (Strubbe et al., 2022). The correlation of specific activities and specific copy numbers (Fig. 6) demonstrated that, even at similar copy numbers, three and five times higher AOB and NOB activities can be achieved in small size-classes than in large granules. This indicates that if a similar activity needs to be reached after pushing the NOB on the large granules, then a very high degree of enrichment is required. One optimization could also be to engineer the size distribution of AGS to increase the surface area available for mass transfer and thus to increase the activities (Liu & Tay, 2012).

5. Conclusions

1. AGS consists of both biofilm-type biomass in granules and suspended biomass in flocs, and thus resembles a hybrid system such as IFAS rather than a biofilm-only system. The AGS monitored in our study consisted of 50 % large granules (>2 mm) and 36 % flocs. But unlike IFAS systems, a significant proportion of nitrification occurs in the flocs of AGS during the warm season (more than 50 % of the AUR and NUR), whereas less than 20 % occurs in granules >1 mm.
2. The limited growth of AOB and NOB in large granules during the warm summer season was likely due to the relatively high SRT of the flocs. The floc SRT of 5.1 ± 1.4 d was well above the minimum SRT for nitrifiers during the warm temperature season. As a result, nitrifiers preferentially grew in the flocs, as opposed to in the large granules, where access to substrate is mass-transfer limited.

3. The high abundance of nitrifiers in flocs, where they are more susceptible to seasonal changes, resulted in unstable NOB assembly, particularly during the cold season. Approximately 80 % of NOB were washed out, eventually resulting in a complete loss of NOB activity. As a result, nitrification was incomplete during a transient period (elevated effluent nitrite concentrations up to few mgN L^{-1}). NOB growth in large granules of AGS system is essential to maintaining a stable NOB community and robust nitrification.
4. The distribution of nitrifiers among the different size-classes should be controlled to avoid transient periods of partial nitrification and to use the full potential of the AGS technology. It is therefore recommended to control the floc SRT at a lower value of around 3 d to limit the growth of nitrifiers in flocs and to promote their enrichment and thus nitrifying activity in large granules. Further investigations are needed into the effect of such an operating strategy on the life cycle of AGS and on the activities in large granules.

CRediT authorship contribution statement

Livia Britschgi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stephany Wei:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. **Andreas Proesl:** Writing – review & editing, Conceptualization. **Eberhard Morgenroth:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Nicolas Derlon:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, 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.

Acknowledgments

This work was financed by the Swiss National Science Foundation (SNSF), grant number 200021_212210. Special thanks go to Michael Kasper, Philip Rüegsegger and Tino Furrer, from Kloten–Opfikon WWTP, for giving us access to their facilities, providing us with material for this research and the helpful discussions. We also thank Mercedes Garcia for the invaluable discussions. We would like to extent our gratitude to the technical staff of Eawag for their support, particularly Sylvia Richter, Aline Hobi, Marco Kipf and Lea Caduff. Finally, we thank Andreas Scheidegger for the discussions on the statistical analysis and Simon Milligan for carefully reviewing the manuscript.

Guidance for the molecular analysis conducted in this study was provided by the Genetic Diversity Centre (GDC), ETH Zurich.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.123021.

Data availability

Data is provided on an online repository and can be found at https://doi.org/10.25678/000DEQ.

References

Alawi, M., Lipski, A., Sanders, T., Eva-Maria-Pfeiffer, Spieck, E, 2007. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. ISME J. 1 (3), 256–264. https://doi.org/10.1038/ismej.2007.34.

Alawi, M., Off, S., Kaya, M., Spieck, E., 2009. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. Environ. Microbiol. Rep. 1 (3), 184–190. https://doi.org/10.1111/j.1758-2229.2009.00029.x.

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. https://doi.org/10.1021/acs.est.8b07303.

APHA. (2023). *Standard Methods for the Examination of Water and Wastewater* (A. P. H. Association, A. W. W. Association, & W. E. Federation, Eds. Vol. 24). APHA Press.

Bollon, J., Filali, A., Fayolle, Y., Guerin, S., Rocher, V., Gillot, S., 2016. N2O emissions from full-scale nitrifying biofilters. Water Res. 102, 41–51. https://doi.org/10.1016/j.watres.2016.05.091.

Chen, G., Ekama, G.A., van Loosdrecht, M.C.M., Brdjanovic, D., 2020. Biological Wastewater Treatment: Principles, Modeling and Design. IWA Publishing. https://doi.org/10.2166/9781789060362.

Daigger, G.T., Norton, L.E., Watson, R.S., Crawford, D., Sieger, R.B., 1993. Process and kinetic analysis of nitrification in coupled trickling filter/activated sludge processes. Water Environ. Res. 65 (6), 750–758. https://doi.org/10.2175/WER.65.6.7.

de Bruin, L.M.M., de Kreuk, M.K., van der Roest, H.F.R., Uijterlinde, C., van Loosdrecht, M.C.M, 2004. Aerobic granular sludge technology: an alternative to activated sludge? Water Sci. Technol. 49 (11-12), 1–7. https://doi.org/10.2166/wst.2004.0790.

de Kreuk, M.K., Kishida, N., van Loosdrecht, M.C.M., 2007. Aerobic granular sludge – state of the art. Water Sci. Technol. 55 (8-9), 75–81. https://doi.org/10.2166/wst.2007.244.

de Kreuk, M.K., Pronk, M., van Loosdrecht, M.C.M, 2005. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Res. 39 (18), 4476–4484. https://doi.org/10.1016/j.watres.2005.08.031.

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. https://doi.org/10.2166/wst.2004.0792.

Di Trapani, D., Christensson, M., Torregrossa, M., Viviani, G., Ødegaard, H., 2013. Performance of a hybrid activated sludge/biofilm process for wastewater treatment in a cold climate region: Influence of operating conditions. Biochem. Eng. J. 77, 214–219. https://doi.org/10.1016/j.bej.2013.06.013.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira*spp. from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68 (1), 245–253. https://doi.org/10.1128/AEM.68.1.245-253.2002.

Dynamita. SUMO2. https://dynamita.com/.

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. Roy. Soc. Chem. 8.

Fumasoli, A., Probst, M., Wett, B., Derlon, N., Wenger, R., Rérolle, M., 2024. inDENSE®-Verfahren für granulierten Schlamm. Aqua Gas (1), 32–39. https://www.aquaetgas.ch/wasser/abwasser/20240109_indense-verfahren-f%C3%BCr-granulierten-schlamm/.

Giesen, A., de Bruin, L.M.M., Niermans, R.P., van der Roest, H.F., 2013. Advancements in the application of aerobic granular biomass technology for sustainable treatment of wastewater. Water Pract. Technol. 8 (1), 47–54. https://doi.org/10.2166/wpt.2013.007.

Gruber, W., Niederdorfer, R., Ganesanandamoorthy, P., Bürgmann, H., Morgenroth, E., Joss, A., 2024. Wie kann der Nitrit-Richtwert eingehalten werden? Aqua Gas 1.

Gruber, W., Niederdorfer, R., Ringwald, J., Morgenroth, E., Bürgmann, H., & Joss, A. (2021). Linking seasonal N2O emissions and nitrification failures to microbial dynamics in a SBR wastewater treatment plantHYPERLINK https://doi.org/10.1016/j.wroa.2021.100098.

Gruber, W., von Känel, L., Vogt, L., Luck, M., Biolley, L., Feller, K., Moosmann, A., Krähenbühl, N., Kipf, M., Loosli, R., Vogel, M., Morgenroth, E., Braun, D., Joss, A., 2021b. Estimation of countrywide N2O emissions from wastewater treatment in Switzerland using long-term monitoring data. Water Res. X 13, 100122. https://doi.org/10.1016/j.wroa.2021.100122.

GSchV. (2023). Gewässerschutzverordnung.

Gujer, W., 2010. Nitrification and me – A subjective review. Water Res. 44 (1), 1–19. https://doi.org/10.1016/j.watres.2009.08.038.

Hermansson, A., Lindgren, P.E., 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR [Article]. Appl. Environ. Microbiol. 67 (2), 972–976. https://doi.org/10.1128/AEM.67.2.972-976.2001.

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A. J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Colston, B.W., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analyt. Chem. 83 (22), 8604–8610. https://doi.org/10.1021/ac202028g.

Hoang, V., Delatolla, R., Laflamme, E., Gadbois, A., 2014. An Investigation of Moving Bed Biofilm Reactor Nitrification during Long-Term Exposure to Cold Temperatures. Water Environ. Res. 86 (1), 36–42. https://doi.org/10.2175/106143013X13807328848694.

Houweling, D., Daigger, G.T., 2019. Intensifying Activated Sludge Using Media-Supported Biofilms.
Huggett, J.F., 2020. The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020. Clin. Chem. 66 (8), 1012–1029. https://doi.org/10.1093/clinchem/hvaa125.
Johnston, J., LaPara, T., Behrens, S., 2019. Composition and dynamics of the activated sludge microbiome during seasonal nitrification failure. Scientif. Rep. 9 (1), 4565. https://doi.org/10.1038/s41598-019-40872-4.
Ju, F., Guo, F., Ye, L., Xia, Y., Zhang, T., 2014. Metagenomic analysis on seasonal microbial variations of activated sludge from a full-scale wastewater treatment plant over 4 years. Environ. Microbiol. Rep. 6 (1), 80–89. https://doi.org/10.1111/1758-2229.12110.
Kinnunen, M., Gülay, A., Albrechtsen, H.-J., Dechesne, A., Smets, B.F., 2017. Nitrotoga is selected over Nitrospira in newly assembled biofilm communities from a tap water source community at increased nitrite loading. Environ. Microbiol. 19 (7), 2785–2793. https://doi.org/10.1111/1462-2920.13792.
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. https://doi.org/10.1016/j.wroa.2019.100033.
Layer, M., Villodres, M.G., Hernandez, A., Reynaert, E., Morgenroth, E., Derlon, N., 2020. Limited simultaneous nitrification-denitrification (SND) in aerobic granular sludge systems treating municipal wastewater: Mechanisms and practical implications. Water Res. X 7, 100048. https://doi.org/10.1016/j.wroa.2020.100048.
Liu, Y.-Q., Tay, J.-H., 2012. The competition between flocculent sludge and aerobic granules during the long-term operation period of granular sludge sequencing batch reactor. Environ. Technol. 33 (23), 2619–2626. https://doi.org/10.1080/09593330.2012.673011.
Lopez-Vazquez, C.M., Damir, B., Volcke, E.I.P., van Loosdrecht, M.C.M., Wu, D., Chen, G., 2023. Biological Wastewater Treatment: Examples and Exercises. IWA Publishing. https://doi.org/10.2166/9781789062304.
Metcalf, L., Eddy, H.P., Abu-Orf, M., Bowden, G., Burton, F., Pfrang, W., Stensel, H., Tchobanoglous, G., Tsuchihashi, R., 2014. AECOM (2014) Wastewater engineering: Treatment and Resource Recovery. McGraw Hill Education.
Nowka, B., Daims, H., Spieck, E., 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. Appl. Environ. Microbiol. 81 (2), 745–753. https://doi.org/10.1128/AEM.02734-14.
Onnis-Hayden, A., Majed, N., Schramm, A., Gu, A.Z., 2011. Process optimization by decoupled control of key microbial populations: Distribution of activity and abundance of polyphosphate-accumulating organisms and nitrifying populations in a full-scale IFAS-EBPR plant. Water Res. 45 (13), 3845–3854. https://doi.org/10.1016/j.watres.2011.04.039.
Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M, 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207–217. https://doi.org/10.1016/j.watres.2015.07.011.
Quoc, B.N., Wei, S., Armenta, M., Bucher, R., Sukapanpotharam, P., Stahl, D.A., Stensel, H.D., Winkler, M.-K.H., 2021. Aerobic granular sludge: Impact of size distribution on nitrification capacity. Water Res. 188, 116445. https://doi.org/10.1016/j.watres.2020.116445.
Regmi, P., Sturm, B., Hiripitiyage, D., Keller, N., Murthy, S., Jimenez, J., 2022. Combining continuous flow aerobic granulation using an external selector and carbon-efficient nutrient removal with AvN control in a full-scale simultaneous nitrification-denitrification process. Water Res. 210, 117991. https://doi.org/10.1016/j.watres.2021.117991.
Regmi, P., Thomas, W., Schafran, G., Bott, C., Rutherford, B., Waltrip, D., 2011. Nitrogen removal assessment through nitrification rates and media biofilm accumulation in an IFAS process demonstration study. Water Res. 45 (20), 6699–6708. https://doi.org/10.1016/j.watres.2011.10.009.
Salvetti, R., Azzellino, A., Canziani, R., Bonomo, L., 2006. Effects of temperature on tertiary nitrification in moving-bed biofilm reactors. Water Res. 40 (15), 2981–2993. https://doi.org/10.1016/j.watres.2006.05.013.
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, 131427. https://doi.org/10.1016/j.cej.2021.131427.
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. https://doi.org/10.1016/j.watres.2022.118365.
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. https://doi.org/10.1016/j.watres.2018.09.052.
van Loosdrecht, M.C.M., Nielsen, P.H., Lopez-Vazquez, C.M., Brdjanovic, D., 2016. Experimental Methods in Wastewater Treatment. IWA Publishing.
Wagner, J., Weissbrodt, D.G., Manguin, V., Ribeiro 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. https://doi.org/10.1016/j.watres.2015.08.030.
Ward, B.B., 2011. Nitrification: an introduction and overview of the state of the field. Nitrification, pp. 1–8. https://doi.org/10.1128/9781555817145.ch1.
Wegen, S., Nowka, B., Spieck, E., 2019. Low temperature and neutral pH define "*Candidatus*Nitrotoga sp." as a competitive nitrite oxidizer in coculture with nitrospira defluvii. Appl. Environ. Microbiol. 85 (9), e02569. https://doi.org/10.1128/AEM.02569-18. -02518.
Wei, S.P., Stensel, H.D., Ziels, R.M., Herrera, S., Lee, P.-H., Winkler, M.-K.H., 2021. Partitioning of nutrient removal contribution between granules and flocs in a hybrid granular activated sludge system. Water Res. 203, 117514. https://doi.org/10.1016/j.watres.2021.117514.
Winkler, M.K.H., Kleerebezem, R., Khunjar, W.O., de Bruin, B., van Loosdrecht, M.C.M., 2012. Evaluating the solid retention time of bacteria in flocculent and granular sludge. Water Res. 46 (16), 4973–4980. https://doi.org/10.1016/j.watres.2012.06.027.
Zheng, M., Li, S., Ni, G., Xia, J., Hu, S., Yuan, Z., Liu, Y., Huang, X., 2020. Critical factors facilitating candidatus nitrotoga to be prevalent nitrite-oxidizing bacteria in activated sludge. Environ. Sci. Technol. 54 (23), 15414–15423. https://doi.org/10.1021/acs.est.0c04192.
Zhou, D., Niu, S., Xiong, Y., Yang, Y., Dong, S., 2014. Microbial selection pressure is not a prerequisite for granulation: dynamic granulation and microbial community study in a complete mixing bioreactor. Bioresour. Technol. 161, 102–108. https://doi.org/10.1016/j.biortech.2014.03.001.
Zhu, S., Chen, S., 2002. The impact of temperature on nitrification rate in fixed film biofilters. Aquacult. Eng. 26 (4), 221–237. https://doi.org/10.1016/S0144-8609(02)00022-5.