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The granular biomass properties and the acclimation period affect the partial nitritation/anammox process stability at a low temperature and ammonium concentration

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ABSTRACT

Extensive research on the anammox-based processes under mainstream conditions is currently in progress. Most studies have used a long acclimation period for the partial nitritation-anammox (PN-An) sludge at a low temperature and ammonium concentration. However, in this study, the results demonstrated that PN-An granular biomass produced under sidestream conditions (30 °C and 1000 mg NH_4^+-N/L) can operate at 15 °C and 50 mg NH_4^+-N/L without acclimation. The nitrogen removal efficiency was 70% and was stable for 60 days. The long-term operation of the system with progressive adaptation provided important information for process optimization. Control of the dissolved oxygen (DO) concentration was crucial to maintain the balance between ammonia oxidizing bacteria (AOB) and anammox bacteria activities. A calculation of the oxygen penetration depth inside the granules is proposed to estimate an adequate DO level, which allows for the definition of the aerobic and anoxic zones that depend on the temperature, the size distribution and the granule density. However, the development of NOB was difficult to avoid with DO control alone. The selective washing-out of the floccular biomass, which contains mainly NOB, is proposed, leaving the granular fraction with the AOB and anammox bacteria in the system.

1. Introduction

Treatment systems based on the anammox process have been increasingly applied recently to nitrogen removal from effluent produced in anaerobic digesters, treating either industrial wastewater or activated sludge (i.e., sidestream) in wastewater treatment plants (WWTPs). These systems treat effluents that have temperatures in the mesophilic range (>30 °C) and relatively high ammonium concentrations (500–2000 mg NH_4^+-N/L) as the residual water from municipal digestates [1,2]. Validation of the operation of the anammox process at low temperature (10–20 °C) and a low ammonium concentration (20–60 mg NH_4^+-N/L) is required in order to profit from its advantages, such as maximization of the recovery of the energy in the wastewater via methane production, because no organic carbon is consumed for nitrogen removal [3] when large amounts of urban wastewater (i.e., mainstream in the WWTP) are treated [2]. WWTP energetic self-sufficiency is feasible if the wastewater is treated via a process to remove organic matter followed by the removal of nitrogen via partial nitritation-anammox (PN-An) processes [4].

Currently, approximately 88% of the technologies used in sidestream conditions combine the PN-An process into a single unit [1]. Study results appear to indicate that operation of PN-An process in single-stage systems makes the treatment of higher nitrogen loads feasible, requiring simpler operation and control strategies than the two-step configurations [5] and offering a more sustainable and economical process for nitrogen removal [6]. Consequently, most research efforts have been focused on the use of

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a single-stage configuration to evaluate applications of the PN-An process for the treatment of the WWTP mainstream [7].

Because the anammox bacteria have a low growth rate and strongly reduced activity under mainstream conditions, most experiments performed with the PN-An process [2,8–12] or the anammox process alone [13,14] have been carried out with a long operational acclimation period (i.e., several months). The temperature and/or the ammonium concentration were progressively decreased during the acclimation period, but it is a bottleneck to be considered in a full scale-up of the process.

Furthermore, studies have indicated that partial nitritation is a limiting step because it is difficult to avoid the activity of the nitrite oxidizing bacteria (NOB) under mainstream conditions [10,15]. The suppression of NOB activity has been evaluated in different types of biofilm reactors, most of which use granular sludge, by controlling the dissolved oxygen (DO) concentration and/or employing intermittent aeration. However, required DO levels of supply have not yet been identified, nor have appropriate conditions for NOB removal and the avoidance of the inhibition of anammox activity during stable operation [2,7,8,16]. Furthermore, the effects of the physical properties of the granular biomass, such as particle size or density, have not been intensely studied either. Granules with different properties might cause different responses of the applied DO. The definition of appropriate operational conditions to suppress NOB activity is crucial to maximize nitrogen removal and produce an effluent that conforms to the discharge requirements.

In summary, the application of the PN-An process under mainstream conditions is not completely resolved, and further research to understand the optimum operational conditions for its successful performance is necessary.

This study aimed at determining the required operational conditions for the stable performance of the PN-An process in a single-stage reactor operated at a low temperature and a low ammonium concentration. The effects of an acclimation period and the properties of the granular sludge on reactor performance were also evaluated.

2. Materials and methods

2.1. Experimental set-up

Two glass sequencing batch reactors (SBR1 and SBR2) with working volumes of 1.5 L and 4.0 L, respectively, were used. A set of two peristaltic pumps was used to introduce the feeding solution continuously during the reaction phase through the top of the reactor and to discharge the effluent, at a medium height in SBR1 and at 75% of the height in SBR2. The volume of the influent and effluent reservoir tanks was 40 L. A thermostatic bath was installed to control the reactor temperature. The air was supplied from the bottom of the reactors through diffusers using an air pump. Complete mixing and the provision of the desired DO concentration inside the reactor was achieved using a mixture of the flow of N_2 gas and the recycled reactor off-gas (SBR1, stages A-I–A-V), combined with the addition of a fresh air flow. Furthermore, a mechanical stirrer operated at 100 and 150 rpm in stages A-VI (SBR1) and B-I (SBR2), respectively, was used. Both reactors operated in 3-h cycles, as indicated in the Supplementary material (Fig. S.1), where the type of flow for the influent and the effluent can be found. The values of the hydraulic retention time (HRT) were fixed at 0.25 d and 0.5 d for SBR1 and SBR2, respectively. The settling times defined the minimum settling velocity ($V_{s,min}$) to guarantee granular biomass retention in the reactor (Table 1). The minimum settling velocity was calculated as the height of the liquid column discharged divided by the settling time.

2.2. Reactor operation description

Both reactors were fed with the supernatant from an anaerobic sludge digester, located in a municipal WWTP, with a composition as described by Vázquez-Padín et al. [17]. The supernatant was diluted with tap water to achieve the desired concentrations of approximately 200 and 50–75 mg NH_4^+-N/L.

SBR1 was operated for 1676 days (Stages A-I to A-VI) with acclimation to low temperature and ammonium concentration. During the start-up, the temperature was fixed at 20 °C, and the ammonium concentration was fixed at 216 ± 48 mg NH_4^+-N/L. The values of both parameters progressively decreased during the five operational stages described in Table 1 to reach 15 °C and 50 mg NH_4^+-N/L. SBR2 was operated for 186 days (Stage B-I) without an adaptation process for the biomass. In this case, the temperature was fixed directly at 15 °C, and the ammonium concentration at 50 mg NH_4^+-N/L.

In total, three operational conditions were tested: (1) Operation at 20 °C and moderate ammonium concentrations of approximately 200 mg NH_4^+-N/L (A-I and A-III), (2) operation at 15 °C and moderate ammonium concentrations of approximately 200 mg NH_4^+-N/L (A-II and A-IV), and (3) operation at 15 °C and low ammonium concentrations of 50 − 75 mg NH_4^+-N/L (A-V, A-VI and B-I).

SBR1 was inoculated with granular biomass collected from a laboratory-scale reactor where simultaneous partial nitritation/anammox occurred (operated at 20 °C and fed with 200 mg NH_4^+-N/L, [18]). SBR2 was inoculated with granular biomass sampled from a pilot-scale reactor (200 L) used to treat the supernatant from an anaerobic sludge digester of a municipal WWTP (1000 mg NH_4^+-N/L), where the ELAN® process occurred at 30 °C [17].

2.3. Analytical methods

The pH and the concentration of ammonium, nitrite, nitrate, the biomass as volatile suspended solids (VSS) and the total suspended solids (TSS) were determined according to standard methods [19]. Total organic and inorganic carbon concentrations (TOC and IC) were measured with a Shimadzu analyser (TOC-5000). The DO concentration was determined during stages A-I and A-II using a DO membrane meter (CellOx 325, WTW) and with a luminescent DO probe (LDO, Hach Lange) from stage A-III on. The biomass density of the granules was determined as the mass of the granule per granule volume using the blue dextran method [20]. A stereomicroscope (Stemi 2000-C, Zeiss), incorporating a digital camera (Coolsnap, Roper Scientific Photometrics), was used to take the images of the granules. These images were processed using Image Pro Plus software for the determination of the granule size distribution [21]. Batch assays were used to estimate the Specific Anammox Activity (SAA) [22]. Respirometric tests were performed using a Bench-model Oxygen Meter (YSI 5300) with oxygen-selective probes (YSI 5331) for the determination of the NOB activity [23]. The bacterial community in the biomass was identified by a 16S rRNA gene-based amplicon analysis (Illumina®) according to a procedure described by Regueiro et al. [24].

2.4. Calculations

Removal rate: The aerobic ammonium and nitrite oxidation rates (AOR and NOR, respectively), the nitrogen removal rate by the anammox bacteria (ANR), and the ammonium (AR) and total nitrogen removal efficiencies (NR) were estimated based on the nitrogen balance and the anammox process stoichiometry. The equations suggested by Vázquez-Padín et al. [25], modified to include the nitrite or nitrate present in the influent, were used.

Table 1
Operational conditions and influent characteristics corresponding to the operational stages of SBR1 and SBR2.

	Stage	Days	DO (mg O_2/L)	T (°C)	$NH_4^+{}_{Inf}$ (mg N/L)	pH	TOC (mg/L)	$V_{s,min}$ (m/h)
SBR1	A-I	1–506	3.8 ± 1.4	20	216 ± 48	7.8 ± 0.1	30.4 ± 10.7	8.1
	A-II	507–623	2.8 ± 1.3	15	193 ± 26	7.8 ± 0.1	20.1 ± 14.4	
	A-III	624–945	2.0 ± 0.8	20	186 ± 32	7.7 ± 0.1	25.6 ± 12.8	
	A-IV	946–1120	1.5 ± 0.3	15	185 ± 24	7.7 ± 0.2	26.0 ± 11.9	
	A-V	1250–1585	1.2 ± 0.8		74 ± 19	7.6 ± 0.2	6.4 ± 3.5	
	A-VI	1600–1676	0.25 ± 0.13		48 ± 7	8.0 ± 0.3	12.5 ± 4.2	0.3–8.1
SBR2	B-I	1–186	0.17 ± 0.21	15	53 ± 5	8.0 ± 0.2	12.3 ± 7.4	0.1–0.3

DO: dissolved oxygen; T: temperature; TOC: total organic carbon; $V_{s,min}$: minimum settling velocity.

Biomass production: The biomass production corresponding to each bacterial population in the reactor was estimated based on the AOR, NOR, and ANR rates and on the yield coefficients.

Oxygen penetration depth: The oxygen penetration depth in the PN-An granules was determined by considering the internal mass transfer and the aerobic ammonium oxidation reaction rate (zero order kinetic) within the volume of the granules, assuming that they were spherical particles under steady state conditions and had no external mass transport resistance (see Fig. S.2).

The equations used to perform these calculations are described in the Supplementary material.

3. Results

The results obtained using progressively adapted (SBR1) and non-adapted (SBR2) granular biomass to low temperature and ammonium concentrations are described below. The evolution of the nitrogen species concentrations for the entirety of the operation of SBR1 (Fig. S.3) and pictures of the granules (Figs. S.4 and S.5) are presented in the Supplementary material.

3.1. Biomass gradual acclimation (SBR1)

3.1.1. Operation at 20 °C and 200 mg NH_4^+-N/L (A-I and A-III)

Stable nitrogen removal was achieved in SBR1 operated at 20 °C and fed with concentrations of approximately 200 mg NH_4^+-N/L (Stages A-I and A-III) (Table 2). The average ammonium and nitrogen removal efficiencies were 78 and 56% in Stage A-I, respectively. In Stage A-III, despite the previous operation of the system at lower temperature (15 °C) (Stage A-II), the results were similar, and the ammonium and nitrogen removal efficiencies were on average 71% and 47%, respectively. The DO concentration was maintained less than 4 mg O_2/L (Table 1) to limit the AOR so that the anammox bacteria could cope with the nitrite produced while simultaneously limiting the NOB activity. However, the latter proved impossible, and the calculated NOR/AOR ratio indicated that 28% of the nitrite generated in Stage A-I and approximately 18% in stage A-III were consumed by the NOB (Table 2).

In both stages, the biomass was successfully retained in the reactor, and its concentration increased from 6.7 g VSS/L (inoculum) to values as high as 10.7 g VSS/L in Stage A-I and 11.5 g VSS/L in Stage A-III (Table 3). Furthermore, the estimated sludge retention time (SRT) was approximately 100 days (Table 3), and the average solids concentration in the effluent was approximately 33 mg VSS/L. Granules with a similar average granular size (of approximately 3.50 mm) were measured in both stages.

In spite of the different initial conditions of stages A-I and A-III, similar results were obtained at the end of both stages, which indicates that under the operational conditions imposed, the system tended to evolve to the same stationary state.

3.1.2. Operation at 15 °C and 200 mg NH_4^+-N/L (A-II and A-IV)

The first reduction in temperature to 15 °C in stage A-II provoked a significant decrease of the ammonium and nitrogen removal efficiency to 36% and 16%, respectively (Table 2). The imbalance between the AOB and the anammox bacteria activities provoked the accumulation of nitrite in this stage. Furthermore, the biomass was washed out of the reactor, and the average SRT value decreased to 85 days (Table 3). The main reason for this wash-out was granular disintegration under the operating conditions. The biomass produced did not compensate for the biomass washed out. For this reason, the temperature was restored to 20 °C (Stage A-III) until the recovery of operational stability. Then, the temperature was decreased again to 15 °C (Stage A-IV). The biomass concentration inside the reactor and the average SRT declined again to 9.9 g VSS/L and 62 days, respectively (from 11.5 g VSS/L at 107 days in Stage A-III), but at a slower rate than during the first temperature decrease. However, neither the ammonium nor the nitrogen removal percentages significantly diminished, and the average efficiencies remained at approximately 72 and 57%, respectively (Table 2). In this stage (A-IV), the DO concentration was maintained at a lower value (approximately 1.5 mg O_2/L) than in the previous stages (Table 1). The NOR/AOR ratio indicated that only 10% of the nitrite formed was oxidized to nitrate by NOB.

Although the biomass concentration inside the reactor progressively decreased, the removal efficiency was not affected, and the NOB activity was limited due to the low applied DO level.

3.1.3. Operation at 15 °C and 50–75 mg NH_4^+-N/L (A-V and A-VI)

In the first days of Stage A-V, the ammonium concentration in the feed decreased without a concomitant decrease in the concentration of the supplied DO. An imbalance between the AOB and anammox bacterial activity occurred, which provoked a transient accumulation of nitrite in the bulk liquid. This accumulation can be attributed to the lower anammox activity (caused by the increase of the DO penetration depth inside the granules) and the low NOB activity in the PN-An granular sludge. Later, the newly imposed conditions favoured the progressive development of the NOB, and consequently, an accumulation of nitrate was observed in the system. As a result, the average nitrogen removal during the Stage A-V dropped to approximately 10% (Table 2), while the NOB consumed 57% of nitrite generated by the AOBs. In this stage (A-V) the solids concentration progressively decreased because the biomass growth did not compensate for the biomass loss in the effluent, and the average SRT value was approximately 58 days (Table 3).

The results indicated that decreasing the temperature and the ammonium concentration negatively affected the removal efficiency. Improved biomass retention and suppression of the NOB activity are required to guarantee stable operating conditions. At this point, the operation at a low DO concentration was explored.

In Stage A-VI, significantly low DO concentrations (approximately 0.25 mg O_2/L) were used to suppress the NOB activity (Table 1). The lower air supply to the complete mixture in the

Table 2
Values of nitrogen loading/removal rates and efficiencies during the different operational stages.

	20 °C and 200 mg NH_4^+-N/L		15 °C and 200 mg NH_4^+-N/L		15 °C and 50–75 mg NH_4^+-N/L		
Stage	A-I	A-III	A-II	A-IV	A-V	A-VI	B-I
NLR (g N/L d)	0.90 ± 0.19	0.78 ± 0.12	0.79 ± 0.10	0.76 ± 0.10	0.34 ± 0.08	0.21 ± 0.02	0.11 ± 0.01
AOR (g N/L d)	0.43 ± 0.11	0.33 ± 0.13	0.22 ± 0.8	0.31 ± 0.08	0.07 ± 0.04	0.07 ± 0.03	0.06 ± 0.01
NOR (g N/L d)	0.12 ± 0.10	0.06 ± 0.06	0.07 ± 0.07	0.03 ± 0.04	0.04 ± 0.05	0.02 ± 0.01	0.01 ± 0.01
ANR (g N/L d)	0.50 ± 0.14	0.40 ± 0.18	0.19 ± 0.11	0.44 ± 0.09	0.04 ± 0.02	0.04 ± 0.03	0.08 ± 0.02
NOR/AOR	0.28	0.18	0.32	0.10	0.57	0.29	0.13
AR (%)	78.1 ± 13.8	70.7 ± 26.4	36.3 ± 14.1	71.5 ± 13.5	36.2 ± 21.8	47.0 ± 20.3	89.9 ± 19.4
NR (%)	55.8 ± 14.3	47.4 ± 26.0	16.4 ± 16.3	57.1 ± 9.8	9.9 ± 11.5	17.5 ± 6.0	70.6 ± 19.5

NLR: nitrogen loading rate, AOR: aerobic ammonium oxidation rate, NOR: nitrite oxidation rate, ANR: nitrogen removal rate by anammox, AR: ammonium removal, NR: nitrogen removal.

Table 3
Biomass concentrations and related calculated parameters for the different operational stages.

	20 °C and 200 mg NH_4^+-N/L		15 °C and 200 mg NH_4^+-N/L		15 °C and 50–75 mg NH_4^+-N/L		
Stage	A-I	A-III	A-II	A-IV	A-V	A-VI	B-I
VSS (g/L)	10.7 ± 2.2	11.5 ± 3.9	8.7 ± 2.1	9.9 ± 2.6	2.3 ± 1.1	2.7 ± 1.2	10.5 ± 1.8
Avg. diameter (mm)	3.64 ± 0.17	3.44 ± 0.30	3.50 ± 0.47	2.14 ± 0.59	3.36 ± 0.29	1.34 ± 0.74	2.46 ± 0.19
Density (g VSS/$L_{biomass}$)	68 ± 5	70 ± 22	48 ± 5	57 ± 21	36 ± 6	–	45 ± 30
SRT (d)	103 ± 54	107 ± 61	85 ± 15	62 ± 13	58 ± 29	25 ± 11	283 ± 165
Anammox[a] (g VSS/d)	0.070 ± 0.010	0.055 ± 0.019	0.024 ± 0.018	0.066 ± 0.009	0.005 ± 0.003	0.005 ± 0.005	0.017 ± 0.006
Total[b] (g VSS/d)	0.120 ± 0.050	0.075 ± 0.024	0.069 ± 0.055	0.086 ± 0.011	0.015 ± 0.011	0.011 ± 0.003	0.028 ± 0.010

[a] Anammox produced biomass.
[b] Total produced biomass.

reactor was mitigated by using a mechanical stirrer (at 100 rpm). Because of the mechanical stirring, the specific input power was set to 0.01 kW/m^3 to avoid granular disintegration and the loss of anammox activity, as observed in a study by Arrojo et al. [26] using anammox granular biomass. However, even under these conditions, granular breakage and an almost complete cessation of nitrogen removal activity occurred. This response was attributed to the activity of the anammox bacteria at low temperature, which in combination with the AOB were exposed to the DO present in the bulk liquid. The sedimentation properties of the biomass were poor, and the settling time increased from 1 to 30 min, with a corresponding change in $V_{s,min}$ from 8.1 to 0.3 m/h, which improved the biomass retention. However, the solids concentration inside the reactor progressively dropped due to the granule size reduction, and the the average value of the SRT was 25 days.

Adaptation of the granular biomass did not allow for the operation of the PN-An at low temperature and a low ammonium concentration under stable conditions, even after a reduction of the DO concentration and the enhancement of the biomass retention.

3.2. Non-acclimated biomass: operation at 15 °C and 5***0 m***g NH_4^+-N/L (SBR2, B-I)

SBR2 was inoculated with granular biomass from a reactor that was used to treat 1000 mg NH_4^+-N/L at 30 °C, which was abruptly exposed to new operational conditions (15 °C and 50 mg NH_4^+-N/L). Based on the knowledge gained from the operation of SBR1, biomass retention in SBR2 was enhanced in three ways. First, the height-to-diameter ratio of the reactor was reduced to 1.0 to decrease the shear stress and avoid the disintegration and loss of the granules due to mechanical stirring. Second, the HRT was increased to 0.5 d to reduce the amount of biomass washed out. Third, the settling velocity, which was imposed in the sedimentation phase, was reduced from 0.3 to 0.1 m/h to facilitate the biomass separation before the effluent discharge.

Under these conditions, the biomass concentration remained nearly stable during the entire operational period, with an average value of 10.5 g VSS/L (Table 3). The effluent suspended solid concentration (23 mg VSS/L) was similar to that of the influent. Consequently, even if the biomass growth was low at this temperature, no significant biomass loss from the reactor occurred.

The average ammonium and nitrogen removal efficiency reached 90% and 70%, respectively, in the first part of the experimental period (0–60 days) (Table 2). These values were apparently higher than those obtained in SBR1 operated under similar conditions (stages A-V and A-VI) and analogous to those obtained in stage A-IV with a biomass previously adapted to a low temperature and ammonium concentration. The NOB activity was negligible during this period due to the low amount of NOB present in the inoculated granular sludge, which represented less than 0.5% of the total bacteria. However, the same trend described above was repeated, and the NOB developed slowly, which resulted in an increase in the nitrate concentration in the effluent (Fig. 1a). The NOB activity increased from day 60 on with a consequent decrease of the ANR (Fig. 1b). At this point, a rise in the share of floccular biomass inside the reactor was observed, which was favoured by the high solids retention conditions (with an average SRT value of 283 d).

The contribution of the floccular and granular biomass fractions to the NOB activity of the biomass was evaluated by respirometric tests performed at different temperatures, from 15 to 30 °C (Fig. 2a) and compared to the activities of the entire biomass. At 15 °C, the activity of the floccular fraction was approximately 0.12 g N/g VSS d, remarkably higher than that corresponding to the granular and to the non-separated fractions, which were approximately 0.02 and 0.01 g N/g VSS d, respectively (Fig. 2a). This result indicated that the floccular fraction of the biomass mainly contained NOB. These findings are in agreement with previous research in which the NOB population was found to be more abundant in smaller than in larger granules [27–29].

Taking these results into account, on day 130 of operation, 0.6 g VSS/L of the floccular biomass was manually removed to selectively reduce the NOB activity in the reactor. From day 169 on, the continuous removal of this floccular fraction occurred in the effluent discharge by increasing the settling velocity imposed during the settling phase from 0.1 to 0.3 m/h, with a consequent decrease in the settling time from 28 to 10 min. After these actions were applied, the NOR was satisfactorily reduced and the ANR increased to approximately 0.06 gN/L d (Fig. 1b).

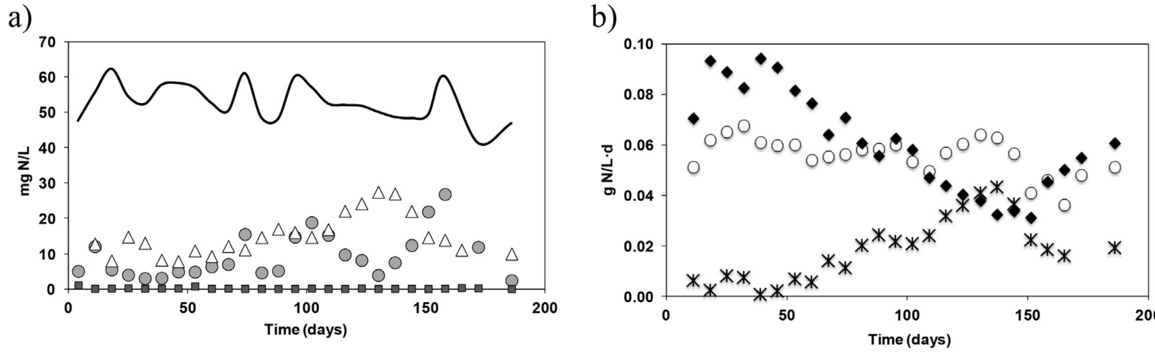

Fig 1. (a) Evolution of the concentrations of NH_4^+-N in the feeding (—) and NH_4^+-N (●), NO_2^--N (■) and NO_3^--N (△) concentrations in the effluent for SBR2. (b) Evolution of ANR (♦), AOR (○) and NOR (✱) for SBR2.

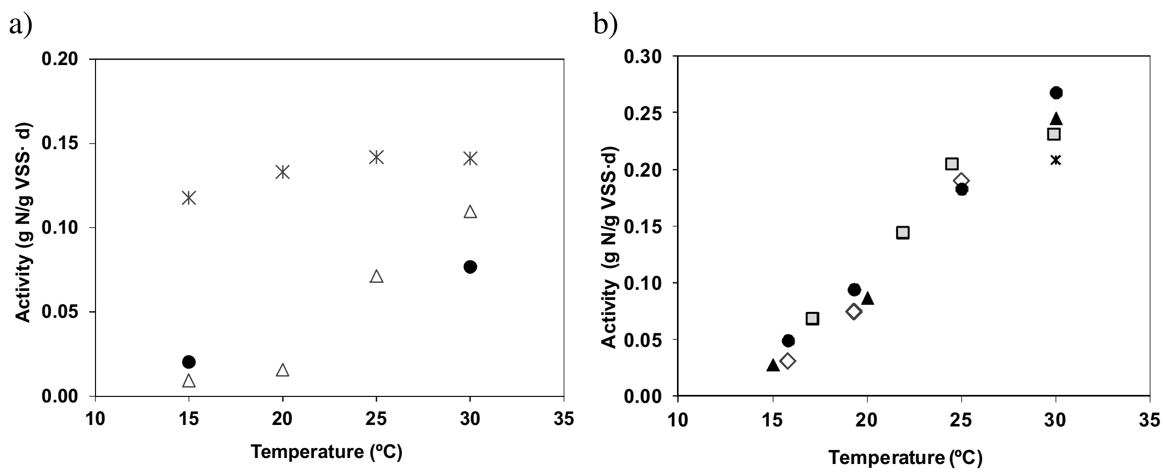

Fig. 2. (a) NOB activity of the biomass from SBR2 on days of operation: 105 (△), 120 for the granular fraction (●) and 120 for the floccular fraction (*). Experiments were performed at temperatures between 15 and 30 °C. (b) Specific Anammox Activity (SAA) of the biomass from SBR2 on days of operation: 1 (●), 69 (■), 91 (◇), 167 (▲) and on day 120 only for the floccular fraction (*).

The results obtained during the operation of SBR2 under low temperature and ammonium concentration conditions indicate that to maintain performance stability, it is necessary not only to operate the system at a low DO level (<0.2 mg O_2/L, Table 1) but also to selectively suppress the NOB activity.

The SAA of the overall biomass was also measured in samples collected on different operational days, with similar corresponding values at all temperatures tested (15–30 °C). These results indicated that the long-term operation (more than 160 days) at a low temperature and ammonium concentration did not cause a detrimental effect on the anammox biomass activity, even at 15 °C with a value of approximately 0.03 g N/g VSS d (Fig. 2b). Furthermore, no significant differences were observed for the SAA corresponding to the floccular fraction compared to the non-separated biomass, measured at 30 °C.

Samples from SBR2 on days 3, 44 and 168 were subjected to a 16S rRNA gene-based amplicon analysis. The results revealed a moderate shift in the microbial population from sidestream to mainstream conditions. The only phylum of NOB detected was *Nitrospirae*, representing less than 0.5% of the total detected population on days 3 and 44, while the proportion on day 168 was 2.42%. The phylum *Planctomycetes* (anammox bacteria) slightly increased with time (5.81% on day 3, 9.28% on day 44 and 7.74% on day 168), which indicates no detrimental effects on the anammox bacteria under mainstream conditions. Two genera were detected, Candidatus *Brocadia* and Candidatus *Scalindua*. The ratio between both (*Scalindua*/*Brocadia*) was of approximately 0.25 for the three samples analysed, which indicates that no specific enrichment occurred, and Candidatus *Brocadia* was predominant.

4. Discussion

4.1. Biomass acclimation

In this study, SBR2 was inoculated with a granular biomass (in operation at 30 °C and 1000 mg NH_4^+-N/L) containing AOB and anammox bacteria and was subjected to low temperature (15 °C) and a low ammonium concentration (50 mg NH_4^+-N/L) without previous acclimation. Under these conditions, the reactor was operated stably for 60 days, with a nitrogen removal efficiency of 70% on average, and NOB activity was not evident. In a previous study, Lotti et al. [8] operated a granular PN-An system at 10 °C and fed with 60 mg NH_4^+-N/L and managed to achieve stable operational conditions for 12 days, with a previous acclimation period of 483 days. When Gilber et al. [2] used an MBBR to perform the PN-An process with an acclimation period of 245 days, stable conditions were maintained for 70 days at 10 °C and 50 mg NH_4^+-N/L. Recently, Laureni et al. [11] reported the stable operation of a MBBR, in which the PN-An process occurred, for 150 days at 15 °C and 21 mg NH_4^+-N/L with an acceptable level of nitrate production over ammonium consumption (16%); however, a previous acclimation period of 250 days was necessary. The comparison of the results of this study with those from other studies, it appears reasonable that a long acclimation period is not required to achieve stable conditions. The start-up time for a PN-An system under mainstream conditions can be significantly shortened in this manner, with biomass produced under sidestream conditions as the inoculum.

Furthermore, the results from the long-term experiments performed in this study with both reactors, with and without an acclimation period, allowed the identification of the main drawbacks to be resolved for successful application of a single-stage PN-An process at a low temperature and ammonium concentration, which were a) overcoming the decrease in the biomass retention capacity, b) maintaining an adequate balance between the AOB and anammox bacterial activities, and c) avoiding the development of NOB activity. Deeper insight into each aspect is provided in the following sections.

4.2. Biomass retention

To maximize the biomass retention in a SBR system, the imposition of a sufficiently long settling time is recommended to limit the wash-out of the biomass. For slow-growing organisms such as the anammox bacteria, guaranteeing biomass retention can help to cope with the decrease of biomass activity associated with the lower temperature (Fig. 2b), as suggested by Hendrickx et al. [30]. In this study, a settling time of only 1 min ($V_{s,min}$ of 8.1 m/h) facilitated biomass retention during operation at 20 °C (Table 3). Under these conditions, biomass production compensated for the biomass wash-out. This was not the case at 15 °C when the estimated biomass production dropped from 0.120 (Stage A-I) to 0.069 g VSS/d (Stage A-II) (Table 3). This value was approximately 10 times lower in the stages A-V and A-VI when the effects of the low temperature and substrate concentrations acted in a synergistic manner.

During the operation of SBR2, the settling velocity was fixed at 0.1 m/h to minimize the biomass wash-out. In this case, the calculated daily biomass growth was approximately 0.028 g VSS/d (B-I, Table 3). This value was higher than the amount of biomass lost in the effluent (0.013 g VSS/d) after subtracting the solids present in the influent (0.168 g VSS/d) and indicated that good biomass retention was achieved inside the reactor. This increase in biomass retention caused the accumulation of floccular biomass that mainly contained NOB, as determined via a batch activity test (Fig. 2a), and needed to be selectively removed by increasing the settling velocity to 0.3 m/h afterwards.

The organic matter content in the feed was low (Table 1), and a maximum of 10 mg TOC/L was removed in the reactor. As a consequence, the growth of a heterotrophic biomass did not significantly influence a microbial population shift that affected the AOB or anammox bacteria.

4.3. Balance between the AOB and the anammox activities

When biomass in a granular form is used for the PN-An process, both the AOB and anammox bacteria operate in a close relationship and are, respectively, located inside the granules in the aerobic and anoxic zone (Fig. S.2, Supplementary material). A reasonable strategy for stable operation relies conditions under which AOB activity is the limiting step. In this manner, a nitrite concentration that inhibits the anammox biomass is never reached, and the development of the NOB is restricted.

This strategy is implemented by controlling the DO concentration in the media to regulate the thickness of the aerobic and anoxic zones inside the granules. This is particularly important when granules developed at a high temperature and ammonium concentration are used at a low temperature and ammonium concentration; i.e., a non-acclimated biomass. This strategy implies a lower the fixed temperature requires a lower oxygen concentration, and therefore lowers the capacity of the system [18]. In fact, in this study, stable operational conditions at 15 °C were only achieved when the DO concentration was maintained at 0.17 mg O_2/L (Stage B-I), while higher DO levels caused system failure (Stages A-II, IV, V and VI). Under these operational conditions the nitrogen removal rate (NRR) achieved was 0.06 g N/L d. A similar NRR was obtained by Laureni et al. [11] (0.02–0.04 g N/L d), who operated a biofilm system at 15 °C and DO levels of 0.15–0.18 mg O_2/L. These results seem to indicate that the maintenance of stable operation at a low temperature and ammonium concentration is possible only when relatively low nitrogen loads are removed. However, considering that the DO concentration in the bulk liquid will determine the removal capacity of the system to a certain extent and that it also depends on biomass consumption, promotion of the AOB in the system could help protect the anammox bacteria from DO inhibitory effect. The improvement of the global removal capacity at a low temperature by inoculating the system with granules with the highest aerobic ammonium oxidation capacity as possible is an option [2], but it must be done carefully to avoid NOB development.

Furthermore, the oxygen penetration depth is affected not only by the temperature and the DO concentration but also by the granular average size (Fig. 3) and density (Fig. 4) [31–33]. If changing the operational conditions causes deterioration of the physical properties of the granules [34] the oxygen penetration inside the granules is different, and the system could become unstable, as occurred in Stages A-IV and A-V. In this case, the size and the density of granules can be monitored with time and the DO concentration inside the reactor can be adjusted to maintain the balance between aerobic ammonium oxidation and anammox activity.

Fig. 3a shows that at a fixed DO concentration, the oxygen penetration depth effect is significant only for small granules (<0.5 μm) or the floccular biomass. However, its influence on the percentage of the aerobic fraction of the granules is relevant for granules smaller than 2.5 mm and consequently affects the activity of the aerobic and anoxic bacterial populations; i.e., the balance between the AOB and anammox activities. For example, the estimated oxygen penetration depth for stage A-I granules varied from 67 μm to 74 μm for the largest and the smallest granules (diameter <0.5 mm), respectively (Fig. 3a). More than 65% of the volume of the smaller granules was penetrated by oxygen; this zone for the large granules was only 6% (Fig. 3b). According to these calculations, the overall volume of biomass subjected to anoxic and aerobic conditions can be estimated as 92% and 8%, respectively.

Regarding the granular density, theoretical calculations for a granule with a diameter of 3.64 mm (Fig. 4) indicated that density variations in the range above 60–70 g VSS/$L_{granule}$ do not cause a significant effect on the DO penetration depth, as was the case for the operational stages at 20 °C (Table 3). On the contrary, changes in the biomass density in the range of the lower values (<40 g VSS/$L_{granule}$) provoke a significant increase in the DO penetration depth. Then, if the biomass density decreases, for example, due to low temperature, the DO concentration must be reduced to avoid inhibiting the anammox process.

The results of this study with a PN-An granular system indicate that the nitrite accumulation in Stage A-VI (see Fig. S.3) was related to the decrease in the average granule diameter, which presumably caused an increase in the overall fraction of the aerobic biomass. Then, if the overall anammox activity was not able to consume the extra amount of nitrite produced, it will accumulate and destabilize the process. In many cases, this effect causes biomass wash-out with the effluent discharge. The balance between the AOB and anammox activities can be restored by reducing the DO concentration. However, once the NOB develop, it is difficult to remove them from the system [1].

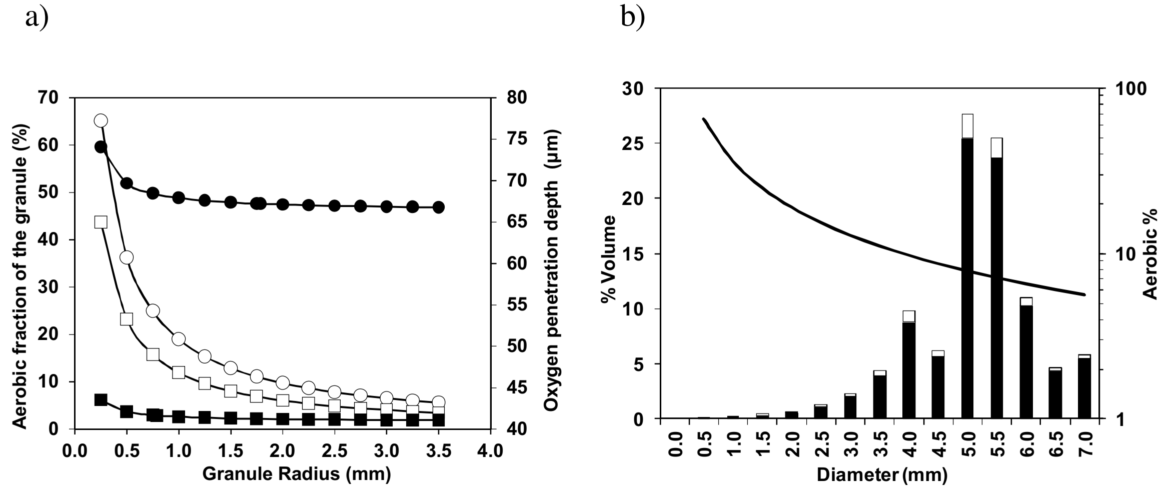

Fig. 3. a) Aerobic fraction of the granules (○) and oxygen penetration depth (●) for a DO concentration of 3.8 mg O_2/L and a temperature of 20 °C; aerobic fraction of the granules (□) and oxygen penetration depth (■) for a DO concentration of 1.35 mg O_2/L and a temperature of 15 °C. b) Size distribution of the granules during stage A-I in percentage of volume of the total distribution when the DO concentration was 4.6 mg O_2/L (day 1–242). In each column, (■) represents the anoxic volume and (□) the aerobic volume. The line (—) represents the fraction of the granule penetrated by oxygen.

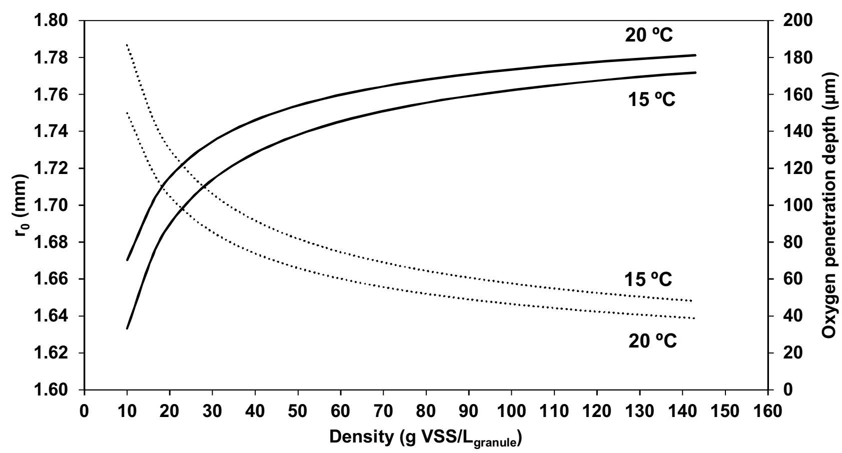

Fig. 4. Anoxic radius (—) in mm and oxygen penetration depth (•••) in µm as a function of the biomass density of the granule (g VSS/L$_{granule}$), calculated for a granule with a diameter of 3.64 mm in the conditions of stage A-I (20 °C) and A-II (15 °C).

4.4. Suppression of NOB

It was previously indicated that the properties of the granular biomass are affected by the operating conditions in the reactor, which are fixed to guarantee the appropriate performance of the biomass. Under these conditions, the development of the NOB is favoured to such an extent that they seriously compete with the anammox bacteria for nitrite and to a lesser extent for DO with the AOB.

For this reason, the continuous repression of the NOB appears essential for a high nitrogen removal efficiency in a PN-An system operated at low temperature and a low ammonium concentration to be maintained. In such a system, the NOB preferably grow in the small granules and can consequently be suppressed by controlling the DO concentration in the bulk liquid to a low level [28], but this is not easy at low temperatures and ammonium concentrations.

Thus, a different strategy is necessary to remove the NOB from the system. An alternative relies on applying a biomass selection that depends on the diameter of the granules. In practice, large granules (containing AOB and anammox bacteria) have a high settling velocity compared to smaller granules that are enriched for NOB. The latter remain on the top part of the sludge bed and can be easily removed by the effluent withdrawal from an SBR. Considering the high density of the granules compared to the suspended biomass, Wett et al. [16] developed a technology based on the use of a hydrocyclone classifier to select for the high-density sludge fraction to retain the anammox enriched granules inside the reactor. Simultaneously, continuous inoculation with a small fraction granular biomass, coming from a reactor operated at a high temperature and ammonium concentration, is performed in the reactor operated at low temperature and ammonium concentration. These authors also suggested operating the reactor under alternate anoxic/anaerobic to aerobic conditions to profit from the longer lag-time of the NOB compared to the AOB. The NOB are removed in this manner, and the AOB remain in the system.

In this study, suppression of the NOB activity was attempted by DO control in SBR1, yielding unsatisfactory results. However in SBR2, an operational strategy based on the selective removal of the floccular fraction by increasing the settling velocity in the reactor from 0.1 to 0.3 m/h successfully washed out the NOB, but a longer operational period was required to improve the stability of the PN-An process with this strategy.

The free ammonia (FA) concentration was low during the entire operational period due to the low temperature and ammonium concentration, the continuous feeding strategy and the performance of the partial nitration and anammox processes that consumed the ammonium in the same unit. In the operational periods corresponding to Stage A-VI (of SBR1) and SBR2, a higher pH (approximately 8.0) resulted in an average FA concentration inside the reactors of 0.33 and 0.17 mg N/L, respectively. According to Chai et al. [35], these values are not sufficient to play a role in suppressing the NOB.

5. Conclusions

- The stable operation (60 days) of an SBR with a non-acclimated granular biomass in which a PN-An process occurred at a low temperature (15 °C) and fed with low ammonium concentration (50 mg NH_4^+-N/L) was achieved with a nitrogen removal efficiency of approximately 70%.
- The subjection of the PN-An granular biomass to progressive adaptation to low temperature and ammonium concentration was proven unnecessary, but it served to identify the crucial factors to control the performance of the process. A high biomass retention was facilitated by an equilibrium between the AOB and anammox activities and the avoidance of NOB development in the biomass.
- The balance between AOB and anammox bacterial activities is related to the proportion of aerobic and anoxic zones inside the granules. The sizes of both regions depend on the DO concentration and the size distribution and density of the granules. Unfortunately, both the size distribution and density of the granules is difficult to control in practice, but they can be monitored throughout the operational period.
- The calculation of the oxygen penetration depth can be used to determine the DO set point necessary to maintain the same aerobic/anoxic ratio when a granular biomass from sidestream conditions is used as the inoculum for a mainstream reactor.
- The development of NOB is hard to avoid or suppress by controlling the DO concentration alone once nearly full biomass retention is achieved in the reactor (SRT >280 d) and the low temperature and ammonium concentration conditions are applied. In that case, the selection of an appropriate settling velocity to wash-out the floccular biomass that mainly contains NOB while guaranteeing the appropriate retention of the granular fraction containing AOB and anammox bacteria appears to be a good strategy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.08.029.

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