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Ammonium oxidation activity promotes stable nitritation and granulation of ammonium oxidizing bacteria

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A B S T R A C T

Two-stage partial nitritation/anammox (PN/AMX) processes have been pointed out as a feasible configuration for achieving mainstream anammox. For two-stage configurations, stable partial nitritation has been reported feasible in granular sludge reactors. This study aimed to explore the operating conditions involved in the development of an autotrophic aerobic granular sludge using floccular sludge as inoculum. The influence of different parameters such as free ammonia concentration, settling time, superficial gas flow velocity and ammonium oxidation rate was investigated. Enhancing ammonium oxidation activity since the early phase of the operation (i.e. using conventional activated sludge as inoculum enriched with a fraction of a floccular nitrifying biomass) promoted a fast development (ca. 30 days) of an autotrophic aerobic granular sludge performing stable nitritation. When the seeded sludge presented a low nitrifying activity (lower than 0.1 g N L^{-1} d^{-1}), the increase of the air-flow rate triggered the formation of an autotrophic aerobic granular sludge since ammonium oxidation activity was promoted. Contrarily, imposing low settling times (10 min) or strong free ammonia inhibitory conditions (FA concentrations higher than 50 mg N L^{-1}) were shown to negatively influence the achievement of high ammonium oxidation rates, hampering the development of an autotrophic aerobic granular sludge. This study demonstrated the importance of ensuring high ammonium oxidation rates (higher than 0.2 g N L^{-1} d^{-1}) for the proper development of an autotrophic partial nitritation granular sludge.

1. Introduction

The partial nitritation/anammox (PN/AMX) process has been proposed as an efficient and cost-effective technology for nitrogen removal in urban wastewater treatment plants (WWTPs) [1]. Under this scenario, two-stage PN/AMX systems have been pointed out as a feasible configuration due to the reported successful results at temperatures as low as 11 °C [2,3]. One of the main challenges of the partial nitritation stage is the repression of nitrite oxidizing bacteria (NOB) during long-term operation, this is currently limiting the full-scale implementation of PN/AMX in the mainstream of WWTPs. However, successful partial nitritation stability has been reported in granular sludge reactors at lab-scale for long-term mainstream conditions [2,3]. Granule morphology allows to stablish oxygen and substrate gradients, which enhances stratification of ammonium oxidizing bacteria (AOB) over NOB [4]. By operating at certain residual ammonium concentrations, AOB increase their oxygen consumption rate, meaning that less oxygen is available for NOB, relegating them to deeper layers [4,5]. Accordingly, a modelling study revealed that AOB presented a better oxygen affinity compared to NOB simply because the position of cell clusters within a stratified granule [6]. Most of lab- and pilot-scale studies that attained stable partial nitritation in granular sludge reactors at mainstream conditions used a pre-formed granular sludge [2,4] or supplied a substratum (i.e. activated carbon) with the inoculum [7,8]. Designing a start-up phase to form a nitrifying granular sludge in which conventional activated sludge (CAS) is used as inoculum seems to be an appealing strategy for the scale-up of this technology. However, seeding CAS as inoculum prevents the initial suppression of nitrite oxidation activity through the same strategy designed for autotrophic aerobic granular sludge (described and applied in [2,7]). The nitrite oxidation can be reduced or even suppressed through inhibition of NOB by free nitrous acid. However, this technology requests the availability of a nitrite concentrated wastewater [31]. An alternative start-up strategy (i.e. in the short term) is to avoid nitrite oxidation by transient inhibition of NOB by free ammonia (FA) within floccular biomass [9,10], and simultaneously develop a granular sludge. To this end, a nitrogen concentrated

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wastewater, such as that coming from the digested sludge dewatering system (i.e. reject water) could be used. Once the granular sludge has been developed, FA inhibition will not be longer required and, thus, operation at mainstream conditions after the start-up phase could be safely imposed by maintaining suitable Dissolved Oxygen/Total Ammonia Nitrogen ([DO]/[TAN]) concentration ratio in the bulk reactor liquid [2,3].

Despite some studies focused on the achievement of an autotrophic aerobic granular sludge performing partial nitritation [11,12], the particular set of operating conditions leading to an effective start-up of this reactor type remains to date not well defined. In contrast, the development of heterotrophic aerobic granular sludge for the simultaneously removal of Chemical Oxygen Demand (COD), nitrogen (nitrification and denitrification) and phosphorus has been widely studied (e.g. NEREDA®) [13]. Heterotrophic aerobic granular sludge has been mostly cultivated from CAS in sequential batch reactor (SBR) operations [14]. The heterotrophic aerobic granulation process is known to be enhanced under short settling times, high shear stress and feast-famine conditions, whereas the exposure to insoluble and particulate COD can comprise the granulation process as filamentous bacteria tend to proliferate [14,15]. Nevertheless, it is unknown whether these conditions could also favour autotrophic aerobic granulation. Indeed, although autotrophic nitrifying granules for nitrification have been developed, the information on the nitrifying bacteria granulation mechanisms remains limited [16,17].

The aim of this study is to investigate the operating conditions that can contribute into the development of an autotrophic aerobic granular sludge achieving stable nitritation. For this purpose, an air-lift reactor seeded with CAS was used by working under a SBR mode. Among the different operational parameters that can influence the formation of a granular biomass performing partial nitritation, this study has focused in the following: (i) FA concentrations in the bulk liquid, (ii) settling time, (iii) shear stress forces (in terms of superficial gas flow velocity) and (iv) ammonium oxidation activity (in terms of ammonium oxidation rates, AORs). Four operational strategies, with different combinations of the above mentioned parameters, have been planned and executed to determine which of these parameters present more influence on the autotrophic granulation.

2. Materials and methods

2.1. Reactor configuration and operation

An air-lift reactor with a working volume of 16.3 L operated in a SBR mode was used (Fig. 1). The reactor presented a downcomer-to-separator diameter ratio of 0.36, a total length-to-downcomer diameter ratio of 11 and a riser height-to-diameter (H_r/D_r) ratio of 19.4. The riser diameter was of 0.05 m and it was located at ca. 10 cm above the reactor bottom. The SBR operation consisted of four phases: a static feeding phase (16 min), an aeration phase (with variable time length) until the total ammonia nitrogen ($TAN = N\text{-}NH_4^+ + N\text{-}NH_3$) decreased to the given TAN set-point. When the aeration was stopped, a settling phase (with a variable time length) occurred followed by the final discharge step (16 min).

Table 1
Experimental conditions for each operational strategy.

	FA (mg N L^{-1}) and pH	Settling time (min)	Superficial gas flow velocity (cm s^{-1})
Strategy-I	7 ± 2 (pH = 8.0 ± 0.2)	30 to 10 in 18 days[c]	0.06 (day 1 to day 18) 0.37 (day 20 to day 40)
Strategy-II	57 (day 0 to 8, pH = 8.5) 17 ± 4 (day 8 to end of operation, pH = 8.0 ± 0.2)	30 to 10 in 44 days[c]	0.06 (day 1 to day 56) 0.23 (day 57 to day 80)
Strategy-III	15 ± 4 (day 0 to 7, pH = 8.1 ± 0.1)[a] 88 (day 8, pH = 8.6)[a] 113 (day 14, pH = 8.6)[b] 47 (day 18, pH = 8.3)[a] 21 ± 7 (day 19 to end of operation, pH = 8.1 ± 0.2)[a]	30 to 10 in 50 days[c]	0.26 ± 0.07[d]
Strategy-IV	14 ± 4 (pH = 8.0 ± 0.1)	30	0.28 (day 1 to day 20) 0.13 ± 0.04 (day 21 to day 60)

[a] Volumetric exchange ratio was maintained at [a]17 and [b]50%, respectively.
[b] Volumetric exchange ratio was maintained at [a]17 and [b]50%, respectively.
[c] The length period refers to the needed time to progressively decrease settling time from 30 to 10 min.
[d] Air and nitrogen gas were supplied in strategy-III. The corresponding air and nitrogen gas contributions into the superficial gas flow velocity are shown in Fig. A.2.

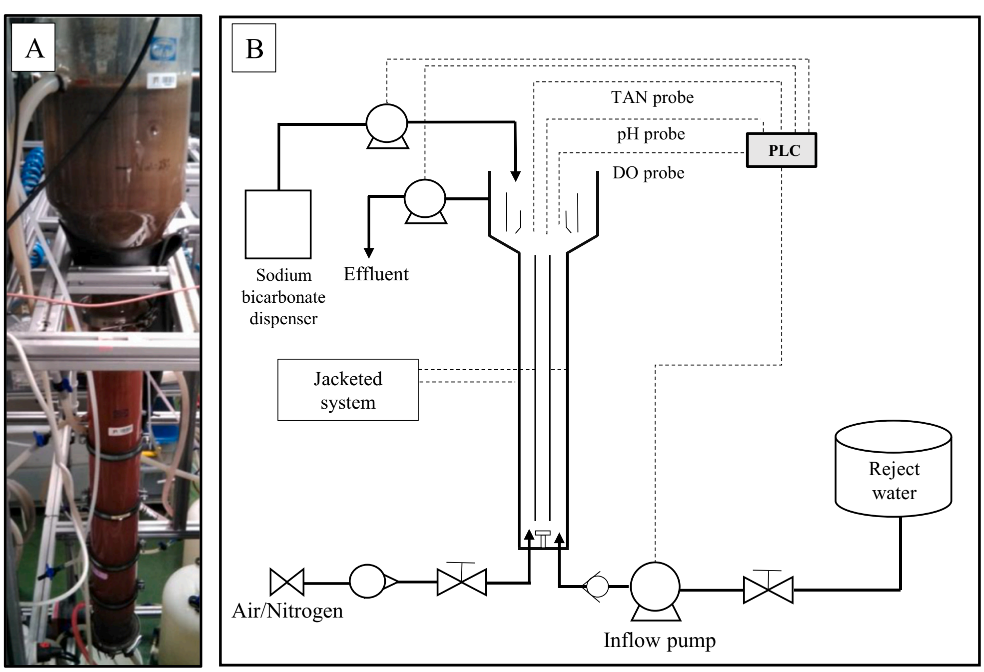

Fig. 1. (A) Picture of the air-lift reactor. (B) Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation. The aeration phase lasted until the total ammonia nitrogen ($TAN = N\text{-}NH_4^+ + N\text{-}NH_3$) decreased to the given TAN set-point. When the aeration was stopped, a settling phase (with a variable time length) occurred followed by the final discharge step (16 min).

and a settling phase (with variable time length) (see Table 1) and a discharge phase (16 min). Cycle length phase was automatically regulated by using the on-line measurement of the total ammonia nitrogen ($TAN = N\text{-}NH_4^+ + N\text{-}NH_3$) concentration in the bulk liquid (Fig. 1). The aeration phase lasted until TAN decreased to the given TAN set-point. Experiments were classified in numbered strategies, as detailed in Section 2.5 (Table 1). Limited research effort has been given to the importance of the volumetric exchange ratio in the development of an autotrophic aerobic granular sludge, as most studies focused on the development of a heterotrophic aerobic granular sludge [18]. Indeed, most of the studies that focused on the development of an aerobic granular sludge performing partial nitration set the volume exchange ratio at 40–50% [11,12]. In this study, the volumetric exchange ratio was set at 40%, except for strategy-III, where it was correspondingly adjusted according to the operational needs (Table 1). The feeding was directly supplied from the bottom of the reactor. During the aeration phase, air (and nitrogen gas, if applicable) were supplied through a porous membrane diffusor located at 30 mm above of the reactor bottom. Air and nitrogen flow rates were controlled by two different rotameters. DO was measured on-line by a means of a DO electrode (DO 60–50, Crison Instruments, Spain). The pH was measured on-line with a pH-electrode (pH 53–33, Crison Instruments, Spain) with a variable set-point by adding dissolved $NaHCO_3$. Ammonium concentrations and temperature in the bulk liquid were measured by using an on-line probe (AISE sc with a CARTRICAL cartridge, Hach Lange, Germany). Temperature was maintained at 20 ± 1 °C by means of a cooling system connected to the reactor jacket. All sensors and actuators were monitored and connected to the PLC system.

2.2. Inoculum and wastewater characteristics

The air-lift reactor was inoculated with CAS from a municipal WWTP in each start-up strategy. For the last experiment (strategy-IV), the air-lift reactor was also inoculated with flocculent sludge coming from a pilot-scale reactor treating a sidestream water successfully performing partial nitritation. The inoculated ratio of volatile suspended solids (VSS)/ total suspended solids (TSS) was 0.8 for all strategies.

The air-lift reactor was fed in all operational strategies with a sidestream water coming from the dewatering of the digested sludge of a municipal WWTP (i.e. reject water). The used reject water was stored in a tank at 13 ± 1 °C. During strategy-I, the reactor was fed with a sidestream water coming from the Manresa WWTP (Catalonia, Spain). The composition of the sidestream presented the following characteristics: TAN: 238–309 mg N L^{-1}; total nitrite nitrogen ($TNN = N\text{-}NO^-_2 + HNO_2$): 1 to 28 mg N L^{-1}; nitrate: 0.5–17 mg N L^{-1}; pH: 7.9 ± 0.1. To work at higher FA concentrations, a sidestream water coming from a different municipal WWTP (Rubí – Valldoreix WWTP, Catalonia, Spain) was used in strategies II, III and IV. The characteristics of this sidestream wastewater were: TAN: 512–963 mg N L^{-1}; TNN: 0–97 mg N L^{-1}; nitrate: 0–21 mg N L^{-1}; pH: 8.1 ± 0.1. The wide range of TAN concentrations depended on the anaerobic digester performance. The variability of TNN and nitrate concentrations were because nitrification occurred within the stored wastewater tank.

2.3. Analytical methods

Influent and effluent samples of the air-lift reactor were regularly analysed to determine TAN, TNN and nitrate concentrations. Samples were previously filtered (0.22 μm) before analysis. TAN concentrations were analysed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany) or by using colorimetric Hach Lange kits (LCK303 or LCK302, Hach Lange, Germany). TNN and nitrate concentrations were analysed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). TSS and VSS concentrations and sludge volume index (SVI) were analysed according to Standard Methods [19]. Average biomass particle size was measured by a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). The percentage of granular sludge was determined as the volumetric fraction of particles with a diameter higher than 200 μm [20].

2.4. Calculations

The nitrogen loading rate (NLR) and ammonium oxidation rate (AOR) were calculated based on Eqs. (1) and (2), respectively. Using nitrite and nitrate concentrations when treating reject water to estimate the AOR it is not very precise since the high concentrations of nitrite (and/or nitrate) together with potential interferences in the analysis would result in a low accuracy measurement. Therefore, AOR was calculated based on influent and effluent TAN concentrations. Additionally, mass balances were periodically checked to evaluate the degree of denitrification, which was minimal as there was no biodegradable COD in the inflow and because no anammox was inoculated. Since TAN, TNN and nitrate concentrations in the effluent were precisely monitored, no specific respirometric assays were performed as they would only provide and indirect measurement of activity and they could affect the granulation process since they should be performed in a mechanical stirred respirometer.

$$NLR = \frac{TAN_{INF}}{HRT} \tag{1}$$

$$AOR = \frac{TAN_{INF} - TAN_{EFF}}{HRT} \tag{2}$$

where

$$HRT = \frac{V_R}{V_R \cdot VER \cdot (\text{number of cycles/day})} \tag{3}$$

and TAN_{INF} corresponds to influent TAN concentrations and TAN_{EFF} to effluent TAN concentrations (g N L^{-1}). HRT, V_R and VER correspond to the hydraulic retention time (day), effective reactor volume (L) and to the volume exchange ratio, respectively.

By using the corresponding TAN concentrations, FA concentrations at the beginning of the cycle (FA_{BC}) and at the end of the cycle (FA_{EFF}) were calculated based on acid-base equilibrium [21],

$$FA = \frac{TAN \cdot 10^{pH}}{e^{\frac{6344}{T+273}} + 10^{pH}} \tag{4}$$

where FA corresponds to the FA concentration (mg N L^{-1}), TAN corresponds to ammonium concentrations at the end of the cycle (TAN_{EFF}) (i.e. effluent) or to the ammonium concentrations at the beginning of the cycle (TAN_{BC}) (mg N L^{-1}). TAN_{BC} concentrations were calculated considering the volumetric exchange ratio and the effective reactor volume. pH and T (°C) correspond to the values of pH and temperature in the air-lift reactor.

The superficial gas flow velocity (u_g, cm s^{-1}) was calculated using the following equation,

$$u_g = \frac{q_{air} + q_{N_2}}{A} \tag{5}$$

where q_{air} and q_{N2} correspond to the air and nitrogen gas flow rates (cm^3 s^{-1}), respectively and A corresponds to the area of the riser (cm^2). The superficial gas flow velocity was used as an indirect form of quantifying the shear stress conditions applied to the biomass of the airlift reactor.

2.5. Operational strategies

The following parameters: (i) FA concentrations in the bulk liquid, (ii) settling time, (iii) shear stress forces (in terms of superficial gas flow velocity), and (iv) ammonium oxidation activity (in terms of AOR) were combined in four different operational start-up strategies to achieve an autotrophic granular sludge performing partial nitritation (Table 1). Each one of the experiments (listed below as strategies) lasted the period of time required to (i) develop a granular sludge and (ii) achieve a stable end product (either nitrite, i.e. partial nitritation or nitrate i.e., nitrification). The NLR, and thus, the HRT, were regulated in each strategy as the cycle length was automatically controlled by the on-line TAN measurement (see Section 2.1 and Fig. 1 for details). Detailed information of each strategy can be found below.

- **Strategy-I.** The aim of strategy-I was to operate at moderate FA concentrations with a fast decrease of settling time and a low initial superficial gas flow velocity. The applied FA concentrations were 7 ± 2 mg N L^{-1} (Table 1), in the range reported for an effectively NOB repression within floccular sludge [22,23]. Settling time was decreased from 30 to 10 min (Table 1). Superficial gas flow velocity was maintained constant in the first part of the experiment by exclusively supplying air. Then, superficial gas velocity was stepwise increased (Table 1) when the granulation process was still not detected after decreasing settling time below 20 min. The reactor was seeded with CAS with an initial biomass concentration of 1 g VSS L^{-1}.
- **Strategy-II.** During strategy-II, high initial FA concentrations were imposed with a progressive decrease of settling time and a low initial superficial gas flow velocity. During the first 8 days of operation, FA concentrations were maintained at 57 mg N L^{-1} (Table 1). From day-8 onwards, FA concentrations were maintained at 17 ± 4 mg N L^{-1} until the end of the operation. Settling time was decreased from 30 to 10 min at slower pace than in strategy-I (Table 1). Superficial gas flow velocity was controlled by exclusively supplying air as in strategy-I (Table 1). The reactor was seeded with CAS with an initial biomass concentration of 2 g VSS L^{-1}.
- **Strategy-III.** The objective of strategy-III was to start the operation at low FA concentrations followed by a sharp increase during a short period to try to knock down NOB, when granule development was still incipient (Table 1). After this initial marked increase, FA concentrations were decreased and maintained at 21 ± 7 mg N L^{-1} until the end of the operation (Table 1). Settling time was decreased as in strategy-II (Table 1). Additionally, nitrogen gas was supplied together with air to operate at higher superficial gas flow velocity (compared to strategies I and II), and thus, to explore the effects of shear stress on granulation (see Fig. SI.2 in Supporting Information for details). The reactor was seeded with CAS with an initial biomass concentration of 1 g VSS L^{-1}.
- **Strategy-IV.** The aim of strategy-IV was to enhance ammonium oxidation activity since the early phase of the operation. The airlift reactor was seeded with CAS (26.1 g VSS) but also with a fraction of floccular biomass performing partial nitritation (12.1 g VSS) (ratio ca. 2:1, respectively). To reduce the influence of other parameters, FA_{BC} concentrations were maintained similar as in strategy-I. Settling time was not decreased but maintained high and roughly constant throughout the operation (Table 1). The superficial gas flow velocity was kept at the same initial value than in strategy-III, though by exclusively supplying air.

3. Results

The time course concentrations of TAN, TNN and nitrate in the effluent as well as TAN influent concentrations for each operational strategy can be found in Fig. 2. Particle size together with settling time and superficial gas flow velocity, as well as AORs have been plotted in Fig. 3. The time course concentrations of FA, nitrate (influent and effluent) and solids can be found in Fig. 4. NLR, together with AOR, and the DO concentrations are depicted in Supporting Information (Figs. SI.1 and SI.3, respectively).

3.1. Strategy-I: operating at moderate FA concentrations with a fast decrease of settling time and low initial superficial gas flow velocity

Initial inoculum size was 93 ± 5 μm and slightly increased to 139 ± 15 μm after 17 days of operation, with a settling time decrease from 30 to 15 min (Fig. 3). Imposing a FA concentration (7 ± 2 mg N L^{-1}, Table 1) in the reactor bulk liquid resulted in a fast achievement of partial nitritation as observed by the gradually nitrate depletion from 28 to 1 ± 1 mg N L^{-1} in 13 days of operation (Fig. 2). Around day-20, particle size increased up to 200 μm (Fig. 3), a threshold usually utilized to identify the achievement of granular sludge. Some days before reaching this milestone, both superficial gas flow velocity and AOR increased. Thereafter, particle size gradually increased, reaching a diameter of 270 ± 21 μm after 38 days of operation (Fig. 3). At the end of the operation (i.e. day 38), 58% of particles were greater than 200 μm with good settleability properties (SVI_5/SVI_{30} = 1), indicating a shift towards granular sludge (Fig. 5). However, from day 22-onwards, partial nitritation was progressively deteriorated, achieving up to a maximum nitrate concentration of 121 mg N L^{-1} in only 11 days (Fig. 2), despite FA concentrations remained stable throughout the operation (Table 1).

In the next experiment (strategy-II), high FA inhibitory concentrations were applied in the early stage of the operation to ensure a proper NOB inhibition. After the initial inhibitory period, FA concentrations were subsequently maintained at higher levels (compared to strategy-I, see Fig. 4A) to strengthen NOB inhibition throughout the operation. Settling time decrease and superficial gas flow velocity followed the same pattern than in strategy-I.

3.2. Strategy-II: operating at initial inhibitory FA concentrations with a progressive decrease of settling time and low initial superficial gas flow velocity

Initial particle size was 98 ± 4 μm and only increased to 149 ± 25 μm in the first 51 days of operation (Fig. 3), despite decreasing settling time from 30 to 20 min. To avoid nitrate production throughout the operation, as occurred in strategy-I, high inhibitory FA conditions were applied (57 mg N L^{-1}, Table 1). However, during the first 8 days of operation, neither nitrite nor nitrate concentrations were detected,

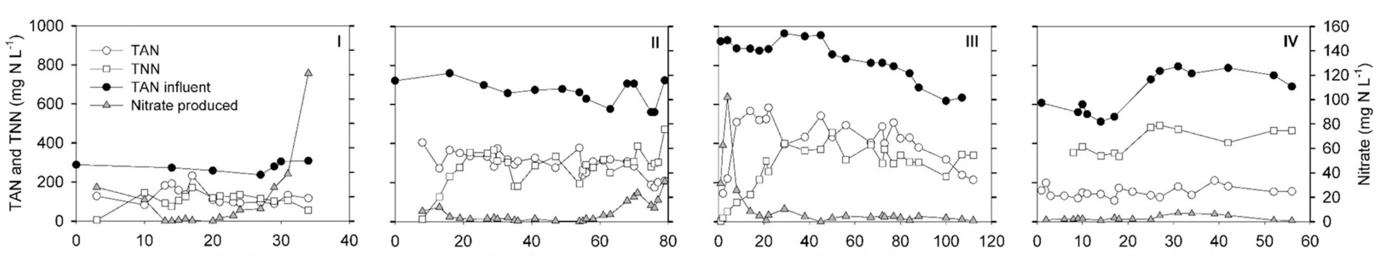

Fig. 2. Operational parameters and reactor performance for the four (I, II, III and IV) start-up strategies.

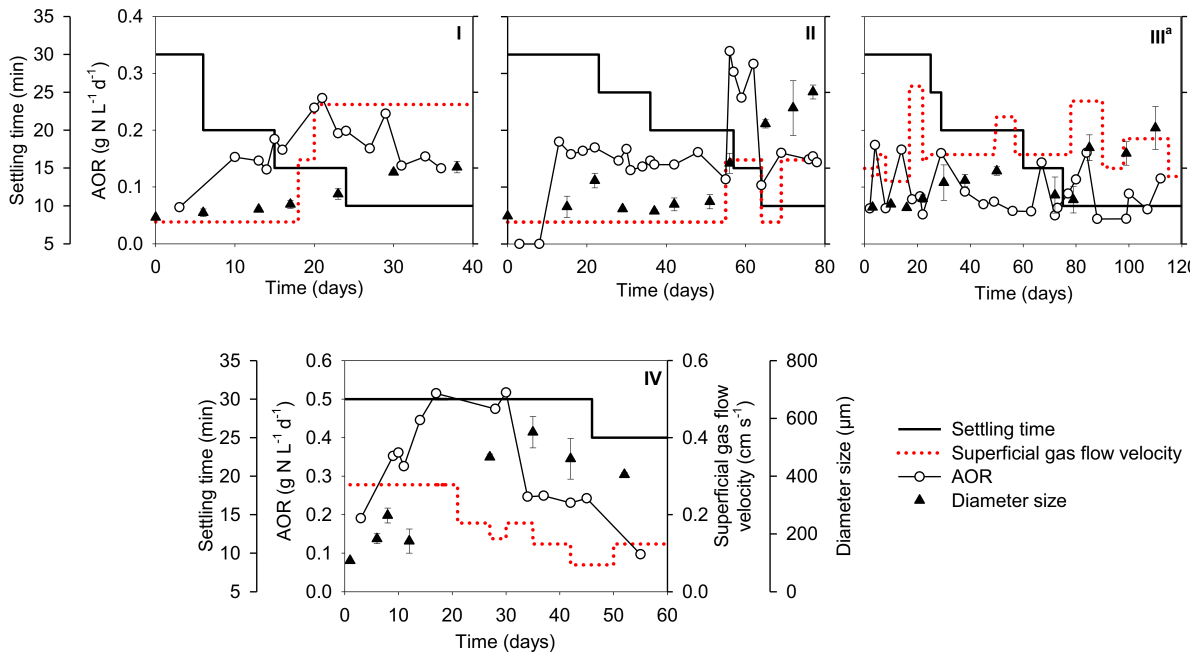

Fig. 3. Time course superficial gas-flow velocity (u_g), ammonium oxidation rates (AORs), diameter size, settling time for the four (I, II, III and IV) start-up strategies. [(a)]The contribution of air and nitrogen flow rates of strategy-III can be found in Table 1 and Fig. SI. 2 in Supporting Information.

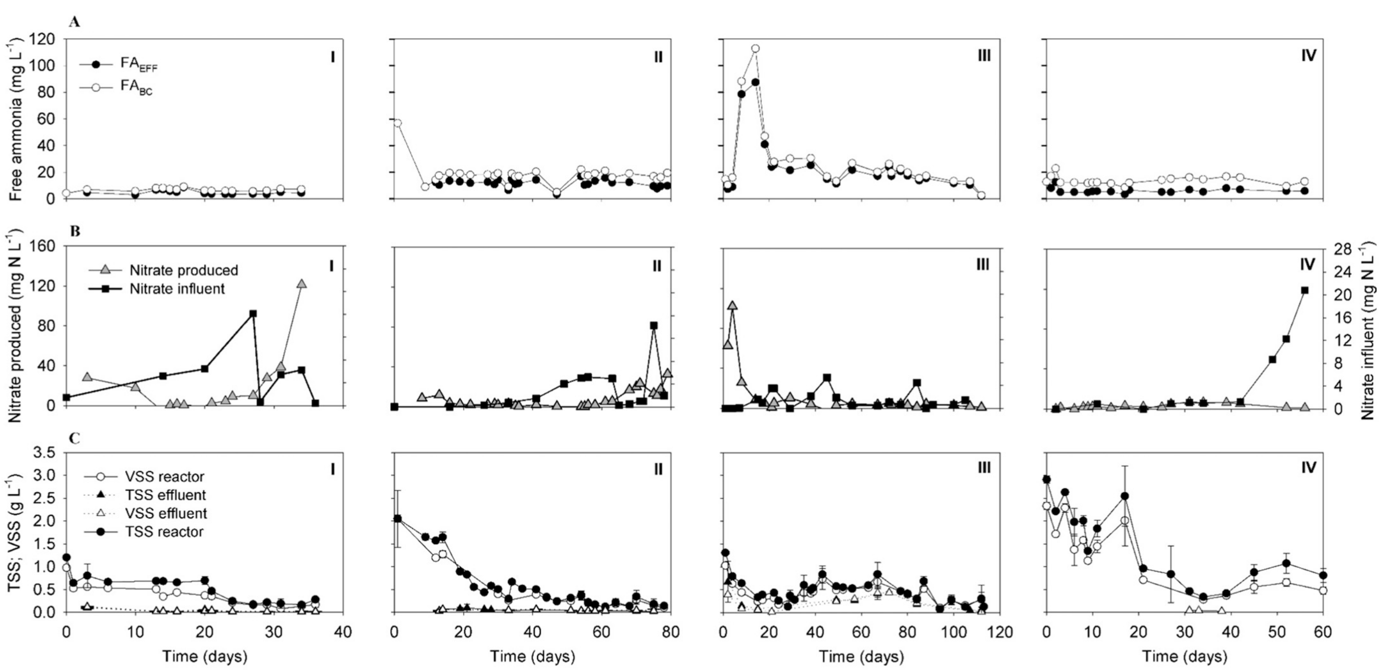

Fig. 4. Operational parameters and reactor performance for the four (I, II, III and IV) start-up strategies. (A) Time course free ammonia concentrations at the end of the cycle (FA_{EFF}) (i.e. effluent) and at the beginning of the cycle (FA_{BC}); (B) Time course of produced nitrate and influent nitrate concentrations and (C) Time course solids concentrations.

indicating an inhibition of both AOB and NOB (Fig. 3). To reduce the FA inhibitory conditions, a cycle was manually performed at day 8 by filling the 40% of the reactor volume with tap water. This operation resulted into a decrease of FA concentrations from 57 to 9 mg N L^{-1}. After alleviating the imposed conditions, FA concentrations were maintained roughly constant throughout the operation (17 ± 4 mg N L^{-1}, Table 1). Consequently, nitrifying activity was detected as observed by the gradual production of TNN and nitrate (up to 12 mg N L^{-1}). Nevertheless, due to the low initial nitrifying activity, the system completed its first cycle after 13 days of operation. Despite the initial nitrate production, partial nitritation was successfully achieved and maintained for 44 days as nitrate concentrations were maintained at 1.9 ± 1.1 mg N L^{-1} (Fig. 2). On day 56, particle size increased up to ca. 300 μm. Just before reaching this goal, the superficial gas flow velocity incremented while the AOR experienced a threefold increase (Fig. 3). A size of 535 ± 25 μm was reached at the end of the operation (Fig. 3). Further, 60% of sludge particles surpassed the 200 μm threshold μm and the SVI_5/SVI_{30} ratio was 1. The difference in size distribution at the initial and at the end of

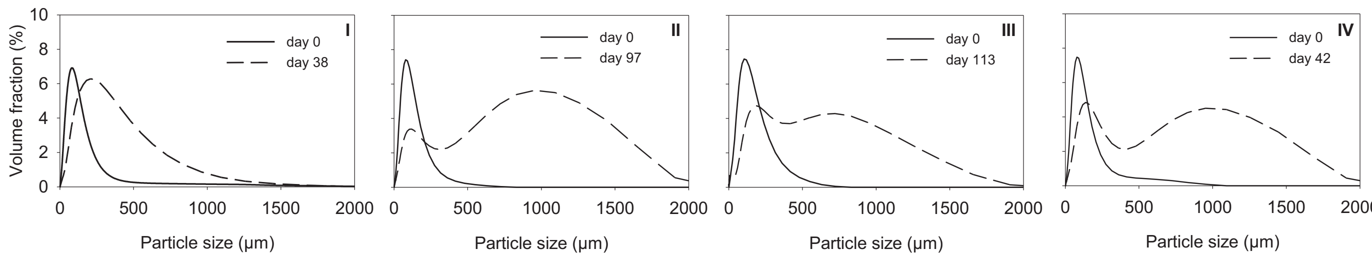

Fig. 5. Size distribution of the sludge for the four (I, II, III and IV) start-up strategies at the initial (solid line) and at the end-phase (dashed line) of the operation.

the operation confirmed the achievement of an autotrophic aerobic granular sludge, more developed than in strategy-I (Fig. 5). However, after attaining a granular sludge, nitrite built-up became compromised after 60 days of operation, as observed for the unceasing nitrate production, resulting into a complete failure of the partial nitritation process.

Based on the obtained results in previous strategies, strategy-III was designed to evaluate the effect of shear stress forces on the development of an autotrophic aerobic granular sludge. On this sense, nitrogen gas was supplied together with air to work at higher initial shear stress conditions than those of previous strategies. Higher air flow rates were required compared to previous strategies to maintain adequate bulk oxygen concentrations (i.e. as oxygen mass transfer rate was reduced because nitrogen gas addition) (Figs. SI.2 and SI.3 in Supporting Information). To reduce the influence of other parameters, settling time was decreased as in strategy-II, while initial FA concentrations were maintained to guarantee an initial ammonium oxidation activity, they were subsequently increased to high inhibitory levels to ensure a proper NOB inhibition and they were finally maintained at those levels of strategy-II to strengthen NOB inhibition throughout the operation (see Fig. 4A).

3.3. *Strategy-III: progressive increase of FA concentration from moderate to high inhibitory concentrations with a progressive decrease of settling time and higher initial superficial gas flow velocity*

The main difference of strategy-III compared to previous strategies was the higher superficial gas flow velocity applied from the beginning of the experiment, as settling time was decreased at the same pace than in strategy-II (Fig. 3). Initial particle size of CAS inoculum was 129 ± 3 μm and progressively increased, reaching an average particle size of 257 ± 15 μm after 50 days of operation (Fig. 3). Nevertheless, it decreased to 155 ± 47 μm from day 50 to 79 (Fig. 3) with a simultaneous biomass loss (Fig. 4C). FA concentrations were initially maintained at 15 ± 4 mg N L^{-1} (Table 1). Full nitrification was detected during the first 4 days of the operation, as observed by the increasing nitrate production. To reverse nitrate production, FA concentrations were gradually increased to avoid AOB inhibition until reaching a maximum of 113 mg N L^{-1} (Table 1). This resulted into a progressive nitrate rate depletion. To avoid negative effects within AOB activity, FA concentrations were progressively decreased and maintained at 21 ± 7 mg N L^{-1} until the end of the operation (Table 1). After this, nitrite built-up took place, while nitrate concentration maintained low (3 ± 2 mg N L^{-1}), resulting into stable partial nitritation (Fig. 2). From day 78-onwards, nitrogen gas supply was almost zero (Fig. SI.2 in Supporting Information). This change affected the oxygen transfer in the bulk liquid, increasing the DO concentration (Fig. SI.3 in Supporting Information) and causing a momentary increase of the AOR (Fig. 3). From then on, granule sludge size increased up to 409 ± 76 μm presenting good settleability properties (SVI_5/SVI_{30} = 1) (Fig. 3). The concentration of granular sludge increased in a 30% within the last 30 days of operation, confirming the achievement of a significant amount of granular sludge at the end of the operation (ca. 60% of particles with size above 200 μm) (Fig. 5). In contrast to previous strategies, stable partial nitritation was successfully maintained until the end of the operation (Fig. 2).

According to the obtained results, a last experiment (i.e. strategy-IV) was planned to work at higher AORs compared to previous experiments and to assess its effects on the development of an autotrophic aerobic granular sludge. The reactor was inoculated with CAS but also with a fraction of an enriched AOB floccular biomass. FA concentrations and settling time were maintained roughly constant throughout the operation. The superficial gas flow velocity was set to maintain the same shear stress forces as in strategy-III but supplying only air.

3.4. *Strategy-IV: enhancing ammonium oxidation activity by working at moderate FA concentrations and constant settling time and superficial gas flow velocity*

Initial particle size was 107 ± 1 μm and rapidly increased up to 465 ± 11 μm in 27 days of operation, crossing the threshold of 200 μm in less than 20 days (Fig. 3). During this period, settling time was maintained high (30 min) while the initial superficial gas flow velocity was in the range of strategy-III (Table 1). Superficial gas flow velocity unintentionally decreased during the operation as reactor diffusor became progressively clogged. The main difference compared to other strategies was that the achieved AOR was, at least, one order of magnitude higher since the beginning of the operation (Fig. 3). Nevertheless, AOR decreased during reactor operation, along with a progressive biomass loss, yet it remained higher than in previous strategies (Figs. 3 and 4C, respectively). Particle size was maintained at 470 ± 61 μm (SVI_5/SVI_{30} = 1), with essentially 50% of aggregates surpassing the 200 μm threshold (Figs. 3 and 5, respectively). Partial nitritation was attained since the early stage of the operation as nitrate concentrations remained low (2 ± 1 mg N L^{-1}) (Fig. 2). It should be stressed that nitrate gradually increased to 7 mg N L^{-1} (from day 27 to 42), though it was properly reversed within next days (Fig. 2).

4. Discussion

4.1. *Effect of free ammonia and settling time decrease in the development of an autotrophic aerobic granular sludge performing partial nitritation*

During the first 13 days of strategy-I, nitrate was produced (Fig. 2) despite working at the reported inhibitory FA concentrations for NOB inhibition within CAS (7 ± 2 mg N L^{-1}) [22,23]. The initial NOB persistence was reduced in strategy-II by applying a higher FA inhibitory concentration (i.e. 57 mg N L^{-1}) (Fig. 4A). However, these FA concentrations severely reduced ammonium oxidation activity (i.e. null AORs) (Fig. 3). Indeed, ammonium oxidation activity was only detected some days after alleviating the applied FA inhibitory conditions (Fig. 3). Regardless of the applied FA concentrations, partial nitritation destabilization occurred at the end of both strategies I and II as observed by the unceasing nitrate production (see Section 4.4 for a detailed explanation). During the initial phase of strategy-III, FA concentrations in the airlift reactor were maintained at values ensuring that the ammonium oxidation activity was not inhibited, in spite of nitrate production (see Fig. 4A and Table 1). These FA concentrations were regulated through the TAN concentration imposed at the beginning of the cycle and the duration of each cycle. Then, FA was progressively increased up to high inhibitory conditions (i.e. 113 mg N L^{-1}), which resulted into a consecutively nitrite built-up (Fig. 2). However, as CAS was not adapted to tolerate high FA concentrations, these were relaxed to avoid negative effects on AOB metabolism [10,24] (Fig. 4A). Along with the suppression of NOB within the seeding sludge, settling time was simultaneously decreased in strategies I, II and III. The reduction of settling time has been described as a mechanism contributing to the achievement of heterotrophic aerobic granulation, mainly due to the selection of slow-growing heterotrophic bacterial species [14]. However, no granule development was observed when settling time was progressively decreased from 30 to 15 min for strategies-I, -II and -III (Fig. 3). Indeed, the reduction of settling time acting as a selection pressure was not beneficial for the obtention of an autotrophic aerobic granular sludge as it resulted into a progressively biomass wash out (ca. 0.5 g VSS L^{-1}, Fig. 4C). This wash out required a long time to be compensated due to the low nitrifying growth rate and, thus, hindered the possibility of attaining high ammonium oxidization activities (Fig. 3).

A fast decrease in settling time negatively influenced the ability of retaining a sufficient concentration of nitrifying bacteria. Also, applying high FA shocks to a non-acclimated sludge showed to present disadvantages in terms of attaining a high ammonium oxidation activity. Consequently, the inhibitory FA concentrations with the concomitant reduction of settling time resulted into low AORs, which were not beneficial for the development of an autotrophic aerobic granular sludge.

4.2. Is the enhancement of superficial gas flow velocity promoting autotrophic aerobic granulation?

Operating at high superficial gas flow velocities allows for higher shear stress conditions, which can promote EPS secretion, contributing to the attachment and self-immobilization process of bacteria [25,26]. This mechanism is well-established for heterotrophic aerobic granular sludge, but it is unclear for autotrophic aerobic biomass. Also, working at high air flow rates grants for greater bulk oxygen concentrations, allowing AOB to increase their activity. The obtained results showed that granule development was achieved in first strategies (I and II) just after increasing the air flow rate, in turn resulting in an increase of the AOR (Fig. 3). To evaluate which mechanisms derived from incrementing the air flow rate triggered the development of an autotrophic aerobic granular sludge (i.e. shear stress conditions vs. ammonium oxidation activity), a third experiment (strategy-III) was performed. In strategy-III, a higher superficial gas flow velocity was applied since the early stage of the operation by supplying both air and nitrogen gas. During this strategy, a faster increase of particle size was detected, from 128 ± 3 μm to 257 ± 15 μm in 50 days, in accordance with the higher superficial gas flow velocity (Fig. 3). However, particle size subsequently decreased from day 50 to 79 (Fig. 3) due to detachment, as a significant increase in the effluent VSS concentration was measured (see Fig. 4.C.III). The high detachment rates declined after day 80, consistent with the interruption of nitrogen gas supply. When only air was supplied (day 78, Fig. SI.2 in Supporting Information), an increase in granule size was measured (from 155 ± 47 μm on day 79 to 339 ± 46 μm on day 85, see Fig. 3). This change also resulted into a marked increase in the ammonium oxidation activity (i.e. AOR) (Fig. 3) and higher bulk DO concentrations (Fig. SI.3 in Supporting Information). Likewise, granular sludge development of previous strategies (I and II) occurred just after incrementing the superficial gas flow velocity and, consequently, observing an increase in the AORs (Figs. 3 and SI.3, respectively). Experiments indicate that the development of an autotrophic aerobic granular sludge was promoted by the higher ammonium oxidation activity derived from boosting air flow rate (i.e. higher DO), whereas the effects of shear stress forces alone were not enough, because they could also trigger significant detachment events. Indeed, high shear stress conditions caused AOB detachment and derived into a failure of the nitritation process in a stratified nitrifying granule structure; the high detachment rates resulted into a thinner AOB layer and into a smaller granular sludge size, incrementing oxygen availability to the inner layers and, thus, allowing nitrite oxidation [27].

4.3. Linking the effects of ammonium oxidation activity to the development of an autotrophic aerobic granular sludge for partial nitritation

The role of ammonium oxidation activity on the development of an autotrophic aerobic granular sludge from CAS was further investigated in strategy-IV. Compared to previous strategies, higher AORs were obtained since the beginning of strategy-IV, as the inoculum was enriched with a fraction of nitrifying biomass (Fig. 3). The high ammonium oxidation activity derived into a high oxygen consumption (i.e. low bulk DO concentrations, Fig. SI. 3 in Supporting Information). Under higher AORs (compared to previous strategies), granular sludge was rapidly developed while partial nitritation was successfully achieved and maintained stable throughout the operation (Figs. 3 and 2, respectively). This is consistent with a study that allowed for a fast start-up of the nitritation process within an aerobic granular sludge by enhancing AOB at high DO concentrations (3–5 mg O_2 L^{-1}), resulting into even higher and more compact granules [28]. As the applied shear stress conditions were maintained similar than in strategy-III and settling time remained high, the development of an autotrophic aerobic granular sludge was shown to be promoted by the enhancement of ammonium oxidation activity in the early phase of the operation.

A period of nitrate production (up to 7 mg N L^{-1}) was detected from day 27 to 42 (Fig. 2) related to an increase in bulk DO concentrations (up to 8 mg O_2 L^{-1}), which could not be monitored due to a failure of the bulk DO monitoring system that lasted for 15 days (Fig. SI.3 in Supporting Information). The higher oxygen availability derived into a transient nitrite oxidation, as nitrate production successfully decreased (≤ 2 mg N L^{-1}, Fig. 2) once bulk DO concentration was lowered to 1.5 mg O_2 L^{-1}, and further maintained at ca. 6 mg O_2 L^{-1} (day 40-onwards, see Fig. SI.3 in Supporting Information). The bulk DO concentration affects the oxygen penetration depth since when bulk DO concentration increases, oxygen can reach deeper layers of the granule. This fast shift from nitrification to partial nitritation when DO is manipulated has been identified as a strong indication of the stratification of nitrifier guilds in granular sludge [4–6].

The first three strategies tested in this study (strategies I, II and III) demonstrated that the reduction of settling time was not promoting the development of an autotrophic granular sludge. Also, the effects of shear stress forces derived from incrementing the superficial gas flow velocity (by adding nitrogen gas) showed to be not fully determinant if a high ammonium oxidation activity was not simultaneously promoted. The obtained results in strategy-IV showed that the high initial ammonium oxidation activity, measured as AOR, accounted for the development of an autotrophic aerobic granular sludge successfully performing partial nitritation. The representative fraction corresponding to nitrifiers in CAS is rather low (i.e. 3%) [12,29]. Thus, promoting high AORs in the early stage of the operation can be challenging. Under these conditions, the positive effects derived from increasing air flow rate were demonstrated to be effective in promoting autotrophic aerobic granular sludge formation as ammonium oxidation activity was enhanced. However, the increase of the air flow rate in a full-scale reactor presents several challenges: (i) the increase in energy demand and (ii) the potential limitation of aeration devices. These challenges would require assessment in future research plus development studies.

4.4. NOB influent inoculation deteriorates partial nitritation stability

Partial nitritation was not successfully maintained in strategies I and II (Fig. 2), even after attaining a significant fraction of granular sludge at the end of reactor operation (ca. 50–60%). In contrast, nitrite oxidation was almost completely suppressed in strategy-III (Fig. 2). The difference among these strategies was not the FA concentrations maintained throughout the operation (Fig. 4A), neither the [DO]/[TAN] ratio (0.014 ± 0.006). Indeed, this last parameter was lower than the reported to maintain a successfully stable nitritation in a granular sludge reactor [7]. The reason that could explain the recovery of nitrite oxidation activity in strategies I and II is the continuous NOB reinoculation with the inflow. The existence of this reinoculation can be inferred from the presence or absence of nitrate influent concentrations (Fig. 4B). The used reject water was stored in a tank several weeks and part of the TAN concentration was progressively oxidized to nitrite, and, further to nitrate (Fig. 4B). Hence, the continuous TAN oxidation clearly indicated the existence of AOB and NOB populations within the storage wastewater and, consequently, both were continuously seeded to the reactor. In contrast, the presence of nitrate in the influent of strategy-III was almost not detected (Fig. 4B). The NOB population developed in the storing tank probably possessed a high tolerance to FA, which resulted in failure of the partial nitritation process [30]. Interestingly, in strategy-IV there was a significant amount of nitrate in the influent from day 40-onwards (Fig. 4B), yet this did not result into nitrate production within the reactor. Compared to previous strategies, the achieved AORs were, at least, one order of magnitude higher (Fig. 3). When NOB inoculation took place in strategy-IV, a significant fraction of granular sludge was already obtained (ca. 50% of particles being greater than 200 μm), which decreased the impact of the NOB seeding, because the number of cycles per day was higher at higher AORs (i.e. shorter HRT and higher washout of the reinoculated NOB). The wastewater storage during the development of a granular sludge performing partial nitritation should be done in conditions to avoid NOB proliferation. For eventual full-scale applications of the partial nitritation process, wastewater storage is not required, therefore this issue would not require particular attention.

5. Conclusions

In this study, a specific operational start-up strategy based on enhancing ammonium oxidation activity was shown to promote the development of an autotrophic partial nitritation granular sludge by using conventional activated sludge as inoculum in a short period of time (ca. 30 days). When the inoculum presented a low activity, the ammonium oxidation activity of the seeding sludge was enhanced by increasing the air flow rate, which allowed for a higher bulk oxygen concentration. Against expectation –and based on the reported conditions for granulation of heterotrophic aerobic sludge– settling time decrease was not linked to autotrophic aerobic granulation. In contrast, high settling times allowed to retain a suitable biomass concentration, beneficial for attaining higher ammonium oxidation activities. Finally, applying strong free ammonia inhibitory conditions in the early start-up phase (i.e. seeding sludge) resulted into low ammonium oxidation rates, which also hindered the development of an autotrophic aerobic granular sludge.

When starting-up the cultivation of aerobic granular sludge for partial nitritation (for two-stage PN/AMX in mainstream) the findings highlighted that it is possible to inoculate activated sludge and use reject water in an SBR configuration by applying the following set of experimental conditions: high aeration flow rates, long settling times and applying a loading rate that avoids FA inhibitory conditions. Once enough biomass concentration is achieved with this experimental start-up strategy, the reactor operation could be switched to continuous mode of operation for partial nitritation of pretreated municipal wastewater.

Declaration of competing interest

The authors of this study declare there is not any conflict of interest regarding this publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2021.102505.

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