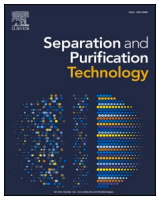
Separation and Purification Technology 317 (2023) 123851

Contents lists available at ScienceDirect

Separation and Purification Technology

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



Rapid start-up and stable maintenance of the mainstream nitritation process based on the accumulation of free nitrous acid in a pilot-scale two-stage nitritation-anammox system

Alba Pedrouso [a,*], Nicolas Morales [b], Belen Rodelas [c], David Correa-Galeote [c], Angeles Val del Rio [a], Jose Luis Campos [d], Jose Vazquez-Padin [b], Anuska Mosquera-Corral [a]

[a] CRETUS Institute, Department of Chemical Engineering, Universidade de Santiago de Compostela, E-15705 Santiago de Compostela, Spain
[b] Aqualia, Guillarei WWTP, Camino de la Veiga s/n, E-36720 Tui, Spain
[c] Department of Microbiology and Institute of Water Research, University of Granada, 18071 Granada, Spain
[d] Facultad de Ingeniería y Ciencias, Universidad Adolfo Ibáñez, Avda. Padre Hurtado 750, 2503500 Viña del Mar, Chile

ARTICLE INFO

Keywords:
Ammonium oxidising bacteria (AOB)
Anammox
Municipal wastewater treatment
Nitrite oxidising bacteria (NOB) suppression
PN/AMX pilot plant

ABSTRACT

Two-stage partial nitritation (PN) and anammox (AMX) systems showed promising results for applying autotrophic nitrogen removal under mainstream conditions. In this study, a pilot-scale (600 L per reactor) two-stage PN/AMX system was installed in a municipal wastewater treatment plant (WWTP) provided with a high-rate activated sludge (HRAS) system for organic carbon removal. The PN/AMX system was operated without temperature control (ranging from 11 to 28 °C) and was subjected to the same variations in wastewater characteristics as the WWTP (22 to 63 mg NH_4^+- N/L). The developed strategy is simple, does not require the addition of chemicals and is characterised by short start-up periods. The PN process was established by applying a high hydraulic load and maintained by *in situ* accumulated free nitrous acid (FNA) of 0.015–0.2 mg HNO_2-N/L. Based on pH value, a controlled aeration strategy was applied to achieve the target nitrite to ammonium ratio in the effluent (1.1 g NO_2^--N/g NH_4^+-N) to feed the AMX reactor. Although NOB were not fully washed out from the system, nitrite accumulation remained (>99 %) stable with no evidence of NOB activity. In the AMX reactor, an overall nitrogen removal efficiency of 80 % was achieved. Regarding effluent quality, 12 ± 3 mg TN/L was obtained, but 5 mg NO_3^--N/L was already in the HRAS effluent. The relative abundance of NOB showed a strong negative correlation with the FNA concentration, providing a good strategy for establishing PN under mainstream conditions.

1. Introduction

Efficient nitrogen removal from wastewater is crucial as wastewater treatment plants (WWTPs) face increasingly stringent effluent quality requirements due to the growing relevance of water reuse [1]. In the search for more energy-efficient WWTPs, systems based on partial nitritation and anammox (PN/AMX) processes have been proposed to autotrophically remove the nitrogen present in the mainstream [2]. Conventional activated sludge systems (CAS) would thus be replaced by an unit for organic matter removal (A-stage) followed by a nitrogen removal unit (B-Stage) [3,4]. Carbon capture is achieved using different approaches, such as high-rate activated sludge systems (HRAS). HRAS consist of a CAS with a shortened solid retention time (SRT) and hydraulic retention time (HRT), in which the organic carbon is adsorbed onto the activated sludge and only 10–30 % of the nitrogen is removed by microbial growth [3]. Stable organic matter removal at this stage is critical for energy recovery and successful implementation of PN/AMX processes. High organic matter content drives the proliferation of heterotrophic bacteria that compete with ammonia-oxidising bacteria (AOB) for dissolved oxygen (DO) and with anammox for nitrite via denitrification [4–6].

In recent years, intensive research on mainstream PN/AMX systems

* Corresponding author.
E-mail addresses: alba.pedrouso@usc.es (A. Pedrouso), nicolas.morales.pereira@fcc.es (N. Morales), mrodelas@ugr.es (B. Rodelas), dcorrea@ugr.es (D. Correa-Galeote), anuska.mosquera@usc.es (A. Val del Rio), jluis.campos@uai.cl (J.L. Campos), jvazquezp@fcc.es (J. Vazquez-Padin), mangeles.val@usc.es (A. Mosquera-Corral).

https://doi.org/10.1016/j.seppur.2023.123851
Received 18 January 2023; Received in revised form 6 April 2023; Accepted 11 April 2023
Available online 14 April 2023
1383-5866/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

has been conducted and promising results have been obtained [7–9]. In addition to low temperatures and nitrogen concentrations, the mainstream exhibits other characteristics, such as high variability in terms of fluctuating temperatures, nitrogen and organic matter loads, and flow rates due to daily and seasonal variations and precipitation periods [10]. The impact of these fluctuations on PN/AMX process performance is often overlooked, but they pose a challenge to PN/AMX stability [7,11–15]. So far, little information is available on the PN/AMX application under fluctuating realistic conditions [4]. In fact, most pilot-scale studies were performed with temperature control [4,16] or have supplemented the feeding with external ammonium when they receive diluted waters to increase the nitrogen loading rate (NLR) and mitigate wastewater composition variability [5,6,15].

Initially, studies on mainstream PN/AMX were focused on the use of single-stage systems because of their excellent results in treating the supernatant of the anaerobic sludge digester in municipal WWTPs [4]. The results from pilot-plant experiences showed that some challenges, such as biomass retention or the proliferation of nitrite oxidizing bacteria (NOB), still hinder the application of PN/AMX processes under mainstream conditions. In fact, the nitrate produced by the NOB limited the nitrogen removal efficiency (NRE) and deteriorate the effluent quality [5,6,11,12]. Hoekstra et al. [5] showed the seasonal effect on the PN/AMX process, as the nitrogen removal rate (NRR) decreased from 223 ± 29 mg TN/(L·d), at 23.2 ± 1.3 °C, to 97 ± 16 mg TN/(L·d) at winter temperatures of 13.4 ± 1.1 °C.

Recently, the potential of the two-stage PN/AMX configuration was demonstrated under mainstream conditions [4] with nitrite accumulation identified as the critical step. However, little pilot-scale experience can be found in the literature. At pilot-scale, controlling the aeration time as a function of ammonia valley produced excellent results with nitrite accumulation ratios (NAR) exceeding 90 % when treating primary settled wastewater (with COD) at low temperatures (12–26 °C) [17,18]. However, Jin et al. [19] that also applied real-time control of aeration length in a two-stage PN/AMX system reported a severe deterioration of NRE due to an imbalance between control parameters caused by frequent variations in feeding composition. These results suggest the need for more straightforward and dynamic control systems to cope with fluctuation in wastewater composition. Kowalski et al. [14] apply a strategy based on the ratio of DO to total ammonium nitrogen ratio in combination with free ammonia (FA) to suppress the NOB activity. However, they found that it was difficult to maintain the stability of the PN process, and 14 % of the nitrogen was oxidised to nitrate.

One of the most commonly reported strategies to achieve the PN process is to exploit the higher sensitivity of NOB to free nitrous acid (FNA) concentrations compared to AOB [4,20] by exposing the sludge from the nitritation reactor to FNA concentrations that inhibit NOB (>0.023 mg HNO_2-N/L). At pilot-scale, Duan et al. [21] observed a 66 % decrease in NOB activity in 10 days when treating a portion of the sludge with FNA in an external unit placed in side-stream, but they begin to recover after 45 days. Although Duan et al. [22] found that the NOB adaptation to relatively high FNA concentrations could be overcome by alternating FNA and FA treatment of biomass, this was not successful at pilot scale [21]. Hausherr et al. [15] have operated a pilot-scale PN reactor in which NOB were successfully inactivated in a plug-flow reactor, but the mechanism that suppresses the NOB activity is unclear. Given this scattered information, it is necessary to investigate the resilience of the two-stage PN/AMX systems at pilot-scale under realistic conditions to shed light on whether the strategies developed at the lab-scale are feasible. In addition, PN/AMX systems are usually inoculated with a relatively high biomass concentration that would be difficult to achieve in the upscaling process. Therefore, the development of strategies that allow rapid start-up and require little inoculation sludge is of great interest.

Hence, this study aims at exploring the feasibility of implementing a pilot-scale two-stage PN/AMX system for the treatment of municipal wastewater at uncontrolled ambient temperature (11 to 28 °C). The robustness of the system to cope with the wastewater fluctuations (22 to 63 mg NH_4^+-N/L and 25–135 mg COD/L) was evaluated as it was implemented *in situ* at the WWTP, with special attention to the achieved effluent quality. The performance of each PN and AMX reactor was evaluated and optimised separately. Microbial community analyses were also performed to evaluate the system performance.

2. Materials and methods

2.1. WWTP configuration and influent composition

The pilot plant was installed in a municipal WWTP operated by FCC Aqualia (Madrid, Spain). The WWTP has a treatment capacity of approximately 260,000 population equivalent and an average wastewater flow of 52,000 m^3/day. The CAS system, equipped with mechanical aeration, was operated with a low HRT (7 ± 1 h) to improve the adsorption of chemical oxygen demand (COD) from the wastewater to the biomass. Due to the low HRT, no nitrogen removal occurred in the CAS unit. Phosphorus is chemically removed in these aeration basins by ferric chloride (approximately 3,500 kg $FeCl_3$/day) before discharge to the secondary clarifiers, which also improves sludge settling capacity. The effluent from the CAS unit had annual average values of 66 ± 11 mg COD/L, 62 ± 11 mg TN/L, 43 ± 10 mg NH_4^+-N/L, 3 ± 1 mg NO_3^--N/L, 0.9 ± 0.1 mg TP/L and 18 ± 4 mg TSS/L. The low COD/N ratio (approximately 1 g COD/g TN) indicated its suitability for feeding the PN/AMX system.

2.2. Pilot-plant set-up and operation

A two-stage PN/AMX pilot plant (Figure S1), with a working volume of 600 L each reactor, was operated without temperature control (11–28 °C). Both reactors were operated as sequencing batch reactors (SBRs) connected to a Supervisory Control and Data Acquisition (SCADA) software to monitor the process and pilot plant operation.

2.2.1. Partial nitritation reactor

The PN reactor (with a useful height to diameter H/D ratio equal to 3.5) was operated as an SBR treating the CAS effluent (operated as HRAS), previously stored in a 220 L buffer tank. The PN reactor was seeded with a mixture of sludge (1.2 g VSS/L in total) from a CAS system of a WWTP, where biological nitrogen removal processes took place, and the flocculent fraction from ELAN® biomass (enriched in AOB but also containing NOB). The addition of the ELAN® biomass, comprising approximately 28 % of the VSS in the inoculum, increased the proportion of nitrifying bacteria compared to the heterotrophic bacteria present in the CAS. The ELAN® biomass come from a single-stage PN/AMX reactor that treated sludge digester supernatant under mesophilic conditions (i.e. a sidestream PN/AMX reactor), located at the WWTP of Guillarei (Tui, NW Spain) [23].

The distribution of the SBR cycle was divided into four phases (Fig. 1. A) comprising: aerated feed, aerobic reaction (with variable length), settling and discharge.

To feed (from the top) and discharge the reactor, two peristaltic pumps were used. Air was supplied through diffusers located at the bottom of the reactor at a rate between 5 and 10 L/min. The air flow rate was manually modified to prevent DO limitations and, especially, to promote the reactor mixing. The DO concentration was monitored online (Hach LDO) but was not actively controlled. The PN reactor was also equipped with online sensors to measure pH (pHD, Hach) and ammonium and nitrate concentrations (AN-ISE, Hach). All probes were connected to a sc100^{TM} controller (Hach) for data acquisition.

The PN reactor was operated for 118 days (April to July) and included three Stages corresponding to changes in the duration of the aerobic phase (Table 1). First, in Stage I, a short aerobic reaction phase of only 10 min (or 100 min if including the feeding phase) was utilized to achieve a high hydraulic loading rate, which promoted the growth of

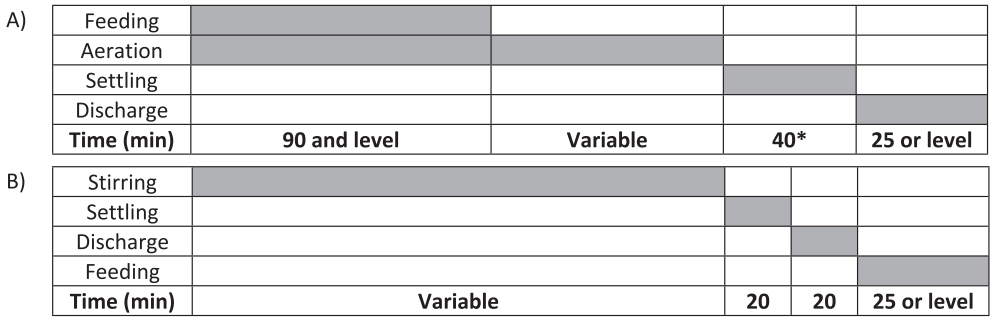


Fig. 1. SBR cycle configurations: A) Partial nitritation (PN) reactor and B) anammox (AMX) reactor. Details of the variable phase length are shown in Table 1. The discharge from the PN reactor directly fed the AMX reactor. *Settling time in the PN reactor was 20 min instead of 40 min from day 0 to 16.

Table 1
Distribution of operational stages for the pilot-scale PN reactor.

Stage (S)	Duration (days)	Cycle (min)	Aeration[a] (min)	Settling (min)	VER (%)	HRT (h)
I	0–16	145	10	20	50	4.8
II	17–49	310 – 215[b]	150–90 (day 21) 60 (day 25)	40	50	7.2–10.2
III	50–118	140 – 275[c]	120 min or pH	40	33	7.5–13.9

[a] In all cases, the previous 90 min were used for the aerated feeding stage.
[b] Cycle length manually adjusted.
[c] Cycle length variable based on pH set point or maximum time.

AOB over NOB. The cycle length was set at 145 min resulting in a low HRT of 4.8 h. During this Stage, the settling time was 20 min and the volume exchange ratio (VER) was 50 %. In Stage II (days 17–50), the duration of the aerobic reaction phase was manually modified to promote the ammonium oxidation and achieve an effluent with a nitrite to ammonium ratio close to the value required for the AMX process (target 1.1 g NO_2^--N/g NH_4^+-N). The aeration phase length was first increased to 150 min, then reduced to 90 min (day 21), and finally to 60 min from day 25 (Table 1). The settling time was increased to 40 min to improve biomass retention. Finally, in Stage III, the VER was reduced to 33 %, and the SBR cycle configuration was changed to a variable duration configuration based on a pH setpoint that terminated the aeration phase. If the setpoint was not reached due to pH prove failure or process instabilities, the cycle terminated with a maximum aeration time of 120 min. The pH setpoint was periodically readjusted based on periodic single-cycle characterisations performed to achieve the target nitrite to ammonium ratio as the composition and temperature of the wastewater fluctuated. As the composition and temperature of the wastewater fluctuated, the pH set point was periodically readjusted according to periodic single-cycle characterisation performed to obtain the target nitrite to ammonium ratio. At all stages, the effluent withdrawal lasted for 25 min or until the required liquid level in the reactor was reached.

2.2.2. Anammox reactor

AMX reactor (Figure S1.C, with a H/D ratio of 0.75) was inoculated with anammox-enriched granular biomass (Figure S2) from the full-scale ELAN® reactor [23] and started up on day 70 of the PN operation. For technical reasons, the discharge pump from the PN reactor directly fed the AMX reactor for 25 min coupling both SBR cycles (Fig. 1). Thus, the reaction time in the AMX reactor was not optimised but was defined by the length of the PN aeration phase. The AMX reactor was an open SBR and it was mechanically stirred for at least 90 min. Once the set-point in the PN reactor was reached, the mechanical stirring in the AMX was stopped (Fig. 1.B). The biomass settled for 20 min and then the effluent was discharged by level. The VER in the AMX reactor was set at 33 %, equal to the PN reactor (Stage III). The AMX reactor was equipped with ammonium, nitrate (AN-ISE, Hach), conductivity and redox sensors connected to a sc100^{TM} controller (Hach).

2.3. Analytical methods

Influent and effluent were periodically sampled. For influent, the 24-hour integrated sample of the full-scale WWTP was used except for cycle characterisation. Individual operating cycles were monitored to evaluate process rates and adjust the pH set point in the PN reactor. After sample filtration (0.45 μm), the COD, ammonium, total nitrogen (TN), nitrite and nitrate concentrations were determined spectrophotometrically using Dr. Lange test kits (Hach Lange, Germany). Alkalinity was determined by titration according to *Standard Methods for Water Examination*, as were concentrations of total and volatile suspended solids (TSS and VSS) and sludge volume index (SVI) [24]. Approximately 2 L of effluent were collected every 5 min during the entire discharge phase to obtain a representative sample for solids concentration determination. Maximum specific nitrifying activities (both for AOB and NOB, SA_{AOB} and SA_{NOB}, respectively) were determined by respirometric tests [25].

2.4. Quantification of microbial populations by quantitative PCR (qPCR)

2.4.1. Biomass sampling and DNA extraction and purification

Biomass samples were taken from the PN reactor after 10, 17, 41, 57, 70, 79, 93, 99 and 113 days of operation, while the AMX reactor was sampled on days 70, 79, 93, 99 and 113 (operational time is counted from the start of the PN rector). To guarantee a homogenous sample, approximately 1 L was obtained at different heights of the PN reactor, mixed and then resampled for DNA extraction. The samples were centrifuged (14,500 × g, 1 min) and the pellets were frozen and stored at −20 °C. DNA was extracted and purified using the FastDNA-2 mL SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, Santa Ana, CA, USA), following the protocols described by Maza-Marquez et al. [26]. A NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Waltham, MA, USA) was used to assess the quality and quantity of DNA samples, stored at −20 °C.

2.4.2. qPCR assays

Absolute quantification of total Bacteria, ammonia oxidising *Betaproteobacteria* (AOB), nitrite oxidising bacteria (NOB, *Nitrospira* spp.), and anammox bacteria (AMX) was performed in the PN and AMX biomass samples by qPCR, using the molecular gene markers and

primers summarised in Table S1A. Triplicate qPCR assays were performed according to MIQE guidelines [27], using True Start Hot Start DNA polymerase (Thermo Scientific, MA USA) and SYBR Green I (Sigma Aldrich, MO, USA) in a total volume of 25 µl with a QuantStudio-3 Real-Time PCR system (Applied Biosystems, CA, USA) as previously described elsewhere [26]. The amplification conditions applied for each molecular marker are listed in Table S1B. To obtain absolute quantifications, standard curves were generated using tenfold dilutions (10^{-1}-10^{-8}) of linearised plasmids carrying inserts of each target gene (16S rRNA gene for total Bacteria and AMX, *amoA* for AOB, and *nxrB* for NOB. All plasmids used as standards were available from previous work [28–30]. The free software "Calculator for determining the number of copies of a template" [31] was used to calculate the copy numbers of the templates.

2.4.3. Statistical analyses

Absolute abundances measured for all target bacterial groups were statistically analysed by the Kruskal-Wallis and Conover-Iman non-parametric tests ($p < 0.05$ significance level), using *IBM* SPSS Statistics v. 21 (SPSS Inc., USA) and R (https://www.r-project.org/). Multivariate statistical analyses (non-metric multidimensional scaling, MDS) were performed with the aid of Primer software (PRIMER-E v. 6.0, Plymouth, UK) following protocols described in detail elsewhere [30].

3. Results and discussion

3.1. Partial nitritation process performance

3.1.1. Nitrite accumulation promotion and maintenance

During the entire operational period, no COD removal was observed in the PN reactor, indicating that the COD present in its feed (approximately 60 mg COD/L) was not readily biodegradable. As the reactor without temperature control, its value ranged from 11 to 28 °C (from spring to summer). The observed decrease in DO concentration was mainly related to the increase in temperature (Fig. 2.A) and/or the evolution of biomass concentration inside the reactor (Figure S3).

Nitrite accumulation was rapidly started up, without additional chemical agents, by applying a relatively high NLR of 248 ± 41 mg TN/(L·d) (Fig. 2.B) which resulted in average sludge nitrogen loading of 1,400 mg N/(g VSS·d). On the first day of operation alone, an ammonium oxidation ratio (AOR) of 72 % was reached, producing 44 mg NO_3^--N/L (Figure S4).

Later, AOR decreased sharply to values below 10 % while the NAR progressively increased up to 99 % (Fig. 2.B). Thus, both AOB and NOB were active in the seeding sludge but did not operate properly under the PN reactor operating conditions, presumably due to the high hydraulic load applied, with an HRT of 4.8 h (Table 1, Fig. 2). However, the low AOR observed limited the nitrite concentration accumulated to 3.9 mg NO_2-N/L (Figure S4.A) which corresponds with an specific ammonium oxidising activity of 62 mg N/(g VSS·d). Moreover, the pH in the effluent was higher than the one in the influent, making the FNA concentration in the system negligible (Fig. 2.C). Therefore, the relatively high hydraulic load applied and the expected AOB abundance higher than that of NOB in the inoculum allowed the selection of the AOB over NOB during this Stage I (with average temperature of 15.4 ± 1.8 °C).

On day 17, the sludge that had been washed-out from the system with the effluent was reintroduced leading to a solid concentration of 1.04 g VSS/L inside the PN reactor. Then, a single cycle with extended aeration (SBR phase 2 = aerobic reaction length of 270 min) was characterised (Fig. 3.A and B). The objective of this cycle was to evaluate the length of the aeration phase required to achieve high nitrite accumulation by promoting the rate of AOB that was limited to values below 30 mg NH_4^+-N/(L·d). The aerobic feeding phase lasted 90 min as both criteria level and time must be achieved (Table 1). However, the actual feeding time was shorter due to the pump flow (7.5 L/min) and took approximately 40 min. During this time, both ammonium and pH increased (Fig. 3). This resulted in a sludge loading rate was 35 mg N/(g VSS·h). During the aerated phase (360 min), the AOB rate doubled the NOB rate, and the pH value decreased from 7.05 to 6.70. Despite FNA concentration increased, it was still well below the inhibitory value reported for NOB metabolism (0.023 mg HNO_2-N/L) [20]. However, Vadivelu et al. [32] showed that FNA already inhibits the NOB anabolism at concentrations of 0.011 mg HNO_2-N/L and completely stops growth at 0.03 mg HNO_2-N/L. Based on the results obtained with this extended aeration cycle characterisation, the total cycle duration on day 17 was extended from 145 min (Stage I) to 310 min (Stage II) to promote the AOB growth but limiting the NOB one.

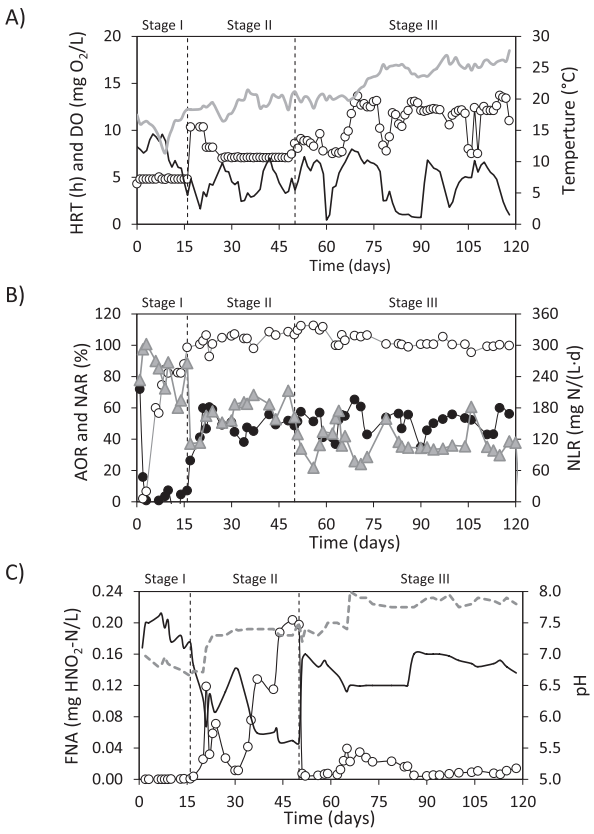


Fig. 2. Evolution of A) average daily hydraulic retention time (HRT, ○), dissolved oxygen (DO) concentration (—) and operational temperature (—); B) ammonium oxidation ratio (AOR, ●), nitrite accumulation ratio (NAR, ○) and nitrogen loading rate (NLR, ▲); C) the free nitrous acid (FNA) concentration (—) and pH values in the influent (···) and effluent (—) of the partial nitritation reactor.

In Stage II (19.2 ± 1.2 °C), the applied NLR was diminished to favour AOR and NAR remained close to 100 % (Fig. 2.B) while nitrite concentration in the effluent progressively increased up to 25 ± 6 mg NO_2^--N/L (Figure S4). Throughout this Stage, the cycle length was manually modified by gradually reducing the aeration phase length (Table 1), decreasing the HRT from 10.2 to 7.2 h (Fig. 2.A) as the AOR increase. Moreover, the aeration flow rate was also increased from 5 to 7 L/min, to ensure that the AOR was not limited by the insufficient DO supplied to the bulk liquid (Fig. 2.A). The obtained nitrite to ammonium ratio in the effluent was close to the stoichiometric value required for the subsequent AMX process (1.15–1.32 g NO_2^--N/g NH_4^+-N) [33,34]. The low operating pH values (6.1 ± 0.1), generated by the alkalinity depletion during the PN process, together with the nitrite accumulated, lead to FNA concentrations up to 0.20 mg HNO_2-N/L (Fig. 2.C) considered the main factor inhibiting the NOB activity. These values are comparable with those observed at the laboratory scale treating primary municipal wastewater [29]. Indeed, the cycle from day 35 is shown in Fig. 3.C and D as an example, and FNA over the NOB inhibitory threshold were

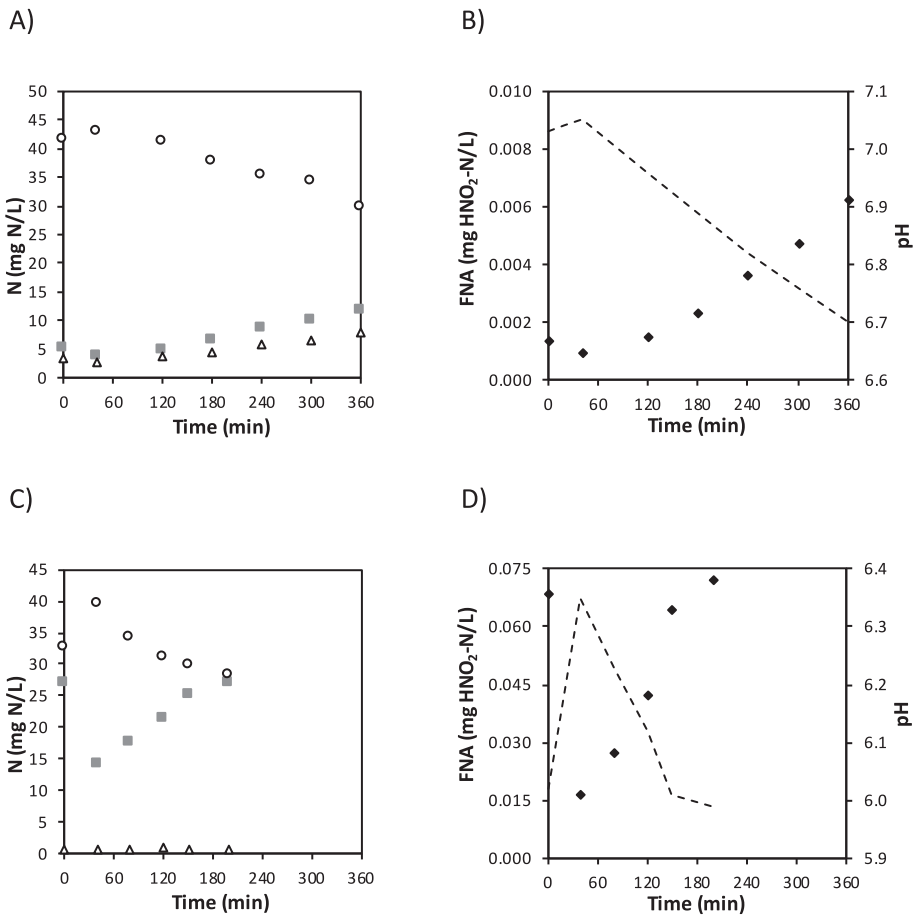


Fig. 3. Cycle characterisations on days 17 (extended aeration) (A and B) and 35 (Stage II) (C and D). A and C) evolution of the ammonium (○), nitrite (■) and nitrate (Δ) concentrations. B and D) evolution of the pH value (–) and FNA concentration (◆). The change in trends at the end of the cycle characterisation on day 35 was due to the beginning of the settling phase.

observed during the entire cycle. The low pH values and FNA do not seem to negatively affect the AOB activity that increased from 58 mg N/(L·d) to 93 mg N/(L·d) during the aerobic phases on day 17 and 35, respectively. This is an advantage of the proposed method compared to those using sludge FNA treatment in an external unit that reported temporally ammonium oxidation collapse after sludge exposure to high FNA concentrations [21]. During the rest of the operational period, significant nitrate production was not detected (Figure 2 and S4); thus, NOB activity was successfully suppressed.

Finally, from day 50 onwards (Stage III), the cycle had variable length. The aeration phase was terminated by a pH set-point (Table 1) to adjust the effluent nitrite to ammonium ratio better while coping with the influent characteristic fluctuations. Excellent correlation between pH and ammonium concentration in the effluent was corroborated with the cycles characterisation (Fig. 3). Therefore, pH was selected as the control parameter since the sensor is more reliable than those used for ammonium determination that required to be calibrated at least once per week as at mainstream conditions with about 50 mg N/L range a low deviation in the measure might incur in significant process instability.

At the beginning of this Stage III, the pH in the effluent sharply increased from 5.6 to 6.6 (Fig. 2.C). This change in the pH value was ascribed to a particular infrastructure event when drinking water with large amounts of sand entered the full-scale WWTP which led to the partial stop of the treatment units and the complete stop of the $FeCl_3$ dosage used for phosphorus removal. As the alkalinity consumed during phosphorous removal (3 mol HCO_3/mol $FeCl_3$) diminished, its concentration in the influent to the nitritation reactor increased from 134 ± 69 to 200 ± 22 mg $CaCO_3$/L. Therefore, the wastewater presented a higher buffering capacity, without ever reaching the pH set-point of 5.6. On day 50, the pH set-point was changed to 6.9 and afterwards, it was reduced progressively to 6.5. The obtained AOR during this stage was in average 51 ± 7 % and the NAR remained close to 100 % (Fig. 2.B). Besides the frequent fluctuations in wastewater characteristics, the WWTPs had also to cope with the particular events that might compromise its operation. In this case, the pilot plant operation remained stable showing its robustness despite the full-scale plant (including HRAS and anaerobic sludge digesters) were stopped for one week to prevent their collapse and allow the sand removal works.

The pH increase caused the decrease of FNA concentration to average values of 0.014 ± 0.001 mg HNO_2-N/L (Fig. 2.C), but it did not affect NOB suppression, and similar nitrate concentrations were observed in the influent and effluent (Figure S4.B). This suggests that another factor (or combination of factors) may have contributed to maintaining the PN process or, the strategy of *in situ* FNA accumulation is robust enough to prevent system destabilisation when inhibitory FNA concentration are not reached temporarily, as shown by Pedrouso et al. [35] who observed a delay of 40 days until nitrate production was observed. However, some studies have reported NOB adaptation to higher FNA concentrations when part of the sludge is treated in an external unit [21,22]. Long-term operation is needed to confirm whether it is possible or not to stably maintain the PN process when treating municipal wastewater under these conditions. Furthermore, during Stage III, the temperature increased to average values of 23 ± 2° C, which may have favoured the growth of AOB over NOB one. Additionally, the relatively low HRT successfully initiated nitrite accumulation in Stage I and may have contributed to maintaining the nitritation process [36].

In addition, batch tests were conducted on days 38 (Stage II) and 79 (Stage III) to measure the SA_{AOB} and SA_{NOB}. The results indicated a value of 195 ± 23 mg NH_4^+-N/(g VSS·d) for SA_{AOB} on day 38, which increased to 360 ± 35 mg NH_4^+-N/(g VSS·d) on day 79. SA_{NOB} was not detected during either batch tests, suggesting that the suppression of NOB was successful. It is worth noting that batch tests were not performed on day, so maximum value of NOB activity is not available but, on day 1, the specific NOB activity was observed to be 228 mg NO_2^--N/(g VSS·d).

3.1.2. Biomass retention capacity

Biomass retention in the PN reactor was a challenge obtaining a relatively high VSS concentration in the effluent (>40 mg VSS/L, Figure S3). This problem is commonly encountered in mainstream PN/AMX systems since, due to the low nitrogen concentrations of this stream, they have to operate with short HRT to obtain a high NLR for substantial biomass growth [8,11]. The low net biomass production did not compensate for the biomass washout provoked by the high flow-rates. Nevertheless, the biomass washout can be seen as a trade-off that helps to promote nitrite accumulation. Li et al. [36] also reported, at lab scale, the rapid establishment of the PN process after simultaneously eliminating both AOB and NOB by applying short SRTs of 10 days. In contrast, Hausherr et al. [15] operated a plug-flow PN reactor with a VER of 90 %, but using longer SRTs ranging from 7 to 40 days, depending solely on the sludge lost in the effluent. They observed an 80-day lag phase in nitrite accumulation during the upscaling from lab to pilot scale.

In the present study, the average SRT in Stage I was 1.5 ± 0.8 days, which helped initiate nitrite accumulation. Later, the SRTs were considerably longer, aiming to retain the biomass, with average values of 11.6 ± 5.0 days in Stage II. However, during Stage I and II, effluent solids were collected and reintroduced into the reactor on a weekly basis, which resulted in longer actual SRTs and no selection based on the SRT was expected. Reintroducing the washed-out biomass allowed for the retention of biomass in the PN reactor, but also led to fluctuating VSS reactor concentrations with an average of 0.5 g VSS/L. Additionally, the high hydraulic stress and the high specific NLR applied (with average values of 1,400 and reaching peaks as high as 2,900 mg N/(g VSS·d), during Stage I led to the formation of aggregates (Figure S5.A). At day 70, biomass reintroduction was stopped since the AMX reactor was started-up, but the nitritation process performance remained stable (Fig. 2). From that time onwards, the SRT was 17.8 ± 8.9 days. The high variation in VSS concentration and SRTs was mainly attributed to determination errors, as biomass stratification was observed in the PN reactor (Figure S5.B), showing that the reactor was not well mixed.

The biomass wash-out could be attributed to the reactor configuration since biomass washing problems were not solved even when the settling time was increased from 20 to 40 min (Table 1) or the VER was reduced from 50 % to 33 % during Stage III. Moreover, biomass stratification inside the reactor (Figure S5.B) could be limiting the potential conversion rates. From day 52 onwards, the aeration flow rate was increased to 10 L/min to improve reactor mixing, but the stratification phenomenon was still present, presumably due to difficulties in resuspending the biomass, as air diffusers are located at 20 cm from the bottom. The implementation of a mechanical stirring would likely promote the achievement of higher AOB rates solving this issue for upscaling purposes and enabling to applied higher NLR.

Regarding the SVI, fluctuating values were obtained again probably due to the fact that the reactor was not properly mixed. The SVI from the seeding sludge was 150 mL/g TSS that was in the range of values commonly found in the CAS systems ranging between 100 and 200 mL/g TSS [10]. During Stages I and II, the SVI remained at average values of 127 ± 14 mL/g TSS and 121 ± 21 mL/g TSS, respectively. Finally, during Stage III the SVI was slightly reduced to values of 95 ± 25 mL/g TSS. The relatively high SVI values (>80 mL/g TSS) also indicated the moderate biomass compaction capacity explaining the observed biomass wash out.

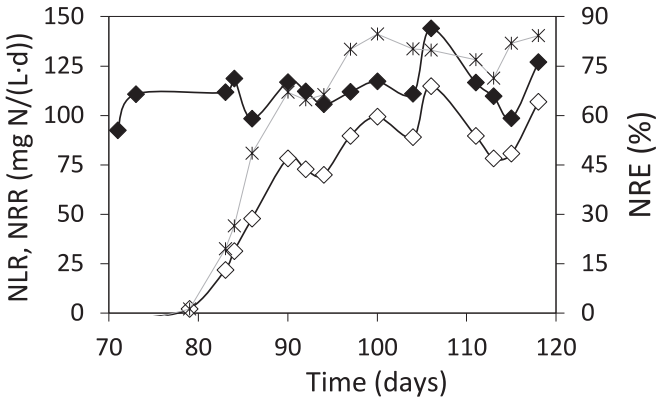


Fig. 4. Evolution of nitrogen loading rate (NLR, ◆), nitrogen removal rate (NRR, ◊), in mg N/(L·d) and the nitrogen removal efficiency (NRE, *) during the anammox reactor operation.

3.2. Anammox process performance

After the stable performance of the PN reactor, from day 70 onwards, its effluent was directly fed to the AMX reactor, which operated at an average NLR of 116 ± 22 mg TN/(L·d) (Fig. 4.A). The HRT was the same as that of the PN reactor, 11.7 ± 1.6 h (Fig. 2.A).

The vigorous mechanical stirring during the first 10 days together with the fact that the AMX reactor is an open-SBR, resulted in high DO concentrations (up to 6 mg O_2/L) in the AMX reactor. Thus, overall nitrogen removal was not observed but ammonium oxidation (Fig. 4 and Figure S6). After installing a variable frequency drive in the mechanical stirring system, the AMX process was quickly established. The reactor experienced transient oxic-anoxic conditions with DO concentrations close to 4 mg O_2/L at the beginning of the cycle due to DO present in the fed stream and mass transfer through the water surface. DO concentration decreased to zero during the first 15 min of the cycle. The NRR exponentially rose to average values of 99 ± 26 mg TN/(L·d) and a NRE of 80 ± 5 % was achieved (Fig. 4). The nitrate produced to ammonium removed ratio was on average 0.15 ± 0.09 g NO_3^--N/g NH_4^+-N and the nitrite to ammonium consumption ratio was of 1.22 ± 0.13 g NO_2^--N/g NH_4^+-N. Both ratios close to those from the theoretical AMX stoichiometry of 0.16–0.26 g NO_3^--N/g NH_4^+-N and 1.15–1.32 g NO_2^--N/g NH_4^+-N [33,34]. The pH of the PN effluent was 6.7 ± 0.3, in the lowest limit of the reported optimal pH range for the anammox bacteria of 6.5 to 8.5 [34], but high NRE of 80 ± 5 % was achieved in the AMX, comparable to previous laboratory results obtained at pH as low as 6.2 and 15 °C [37]. Slight COD removal occurred with average rates of 26 ± 1 mg COD/(L·d), indicating limited denitrification due to a lack of organic matter.

Despite the low initial granular biomass concentration used to inoculate the reactor (0.4 g VSS/L), the AMX process was successfully established with specific AMX rates ranging from 110 to 214 mg N/(g VSS·d). The granular biomass was successfully retained with VSS concentration in the effluent lower than 10 mg VSS/L. Although the too short operational period of this reactor (48 days) that has coincided with spring to summer season (with a temperature of 24.9 ± 1.3 °C), together with the slow growth rate of the anammox bacteria, do not allow to categorically state that the process is able to provide long-term stable performance at mainstream conditions, the results obtained are auspicious.

3.3. Quantification of total Bacteria, AOB, NOB and AMX in the PN-AMX pilot-scale plant and links to operational parameters

Fig. 5 summarises the abundances (number of copies per ng DNA) of the gene markers selected for total Bacteria, AOB, NOB and AMX in the pool of samples from the PN and AMX reactors. Statistically significant differences among samplings were found for all the microbial groups

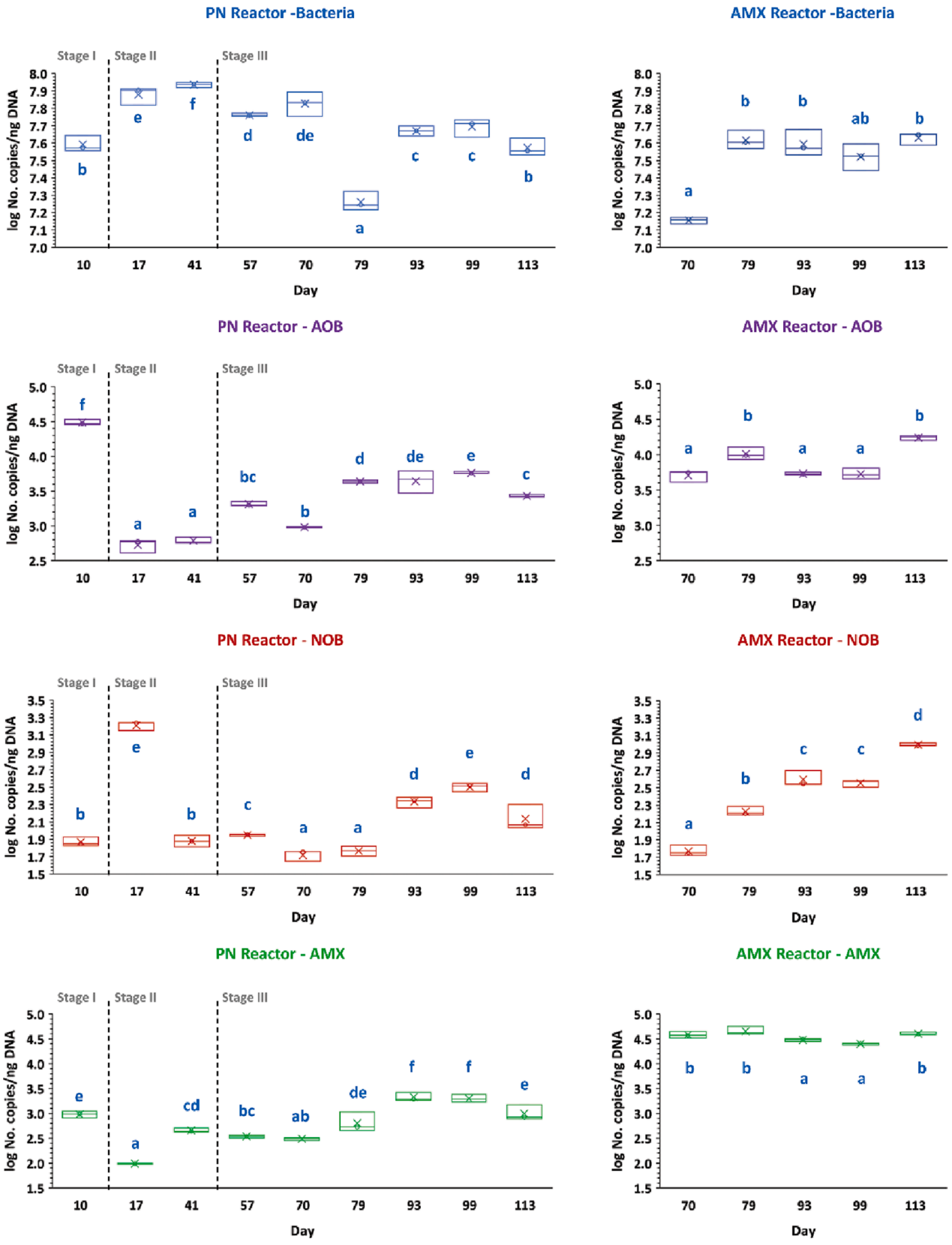


Fig. 5. Notched Box and-Whiskers plots showing the logarithm of the number of copies/ng DNA of the targeted genes markers of total Bacteria, ammonia oxidising bacteria (AOB), nitrite oxidising bacteria (NOB) and annamox bacteria (AMX) in the partial nitrification (PN) and annamox (AMX) reactors, quantified by qPCR. In all the plots, upper and lower bounds of the box denote the 75th and 25th percentiles, upper and lower bounds of bars are the 90th and 10th percentiles. The medians of boxes marked with the same letter in each plot are not significantly different, according to the Conover-Iman test ($p < 0.05$).

quantified. The numbers of total Bacteria were always kept between 10^7-10^8 copies/ng DNA throughout the whole experimental period, in both bioreactors. Quantification of AMX ranged 10^2-10^3 copies/ng DNA in the PN reactor throughout the three operation Stages, and increased their abundance in the AMX reactor over two orders of magnitude (Fig. 5). The abundances of the nitrifiers AOB and NOB were subjected to ample variations, in response to the changes of operational parameters.

In the PN reactor, AOB and NOB average abundances oscillated between $5.4 \pm 1.1 \times 10^2$-$3.1 \pm 0.3 \times 10^4$ and $5.3 \pm 0.7 \times 10^1$-$1.6 \pm 0.2 \times 10^3$ copies/ng DNA, respectively (Fig. 5). During Stage I, biomass sampled on day 10 reached the highest abundance of AOB, and the AOB:NOB ratio achieved under the selected cycling conditions (section 3.1.1) was > 400:1 (Table S2), confirming an effective enrichment of AOB over NOB. In an earlier work by Li et al. [38], achieving an AOB:NOB ratio > 100:1 in the start-up stage was regarded as a key step for the successful long-term operation of PN. At day 17, the reintroduction of the washed-out biomass in the SBR led to a sharp increase of the abundance of NOB, while the AOB numbers fell nearly two logarithmic units; however, after 25 days of further operation under the extended aeration cycling conditions and longer HRT imposed in Stage II (day 41), the NOB numbers decreased to the values of Stage I, and AOB populations dominated again over NOB at 8:1 ratio (Table S2). Accordingly, by the end of Stage II, nitrite concentrations in the effluent increased, while nitrate concentrations were reduced raising the NAR (Fig. 2.B, Figure S4). Further enrichment of AOB over NOB occurred up to a 23:1 ratio after one week of operation under the conditions of Stage III (day 57), being kept near this value in most of the samples analysed throughout this Stage (Table S2). This maintenance might be attributed by the fact that the reintroduction of the biomass washed out was stopped. Similarly, Bao et al. [39] observed AOB:NOB ratios ranging 3:1 to 23:1 during stable operation in a PN system. The molecular characterisation of the dynamics of AOB and NOB populations also agreed with the measures of SA_{AOB} and SA_{NOB} in batch tests in Stages II and III (section 3.1.1).

In the AMX reactor, the abundances of total Bacteria, AOB and AMX populations remained fairly stable during the whole experimental period, mostly oscillating within half a logarithmic unit (Fig. 5). In contrast, NOB populations' abundance increased steadily throughout operation; hence, the AOB:NOB and AMX:NOB ratios decreased accordingly (Table 2). The AMX:NOB ratio was particularly high (>600:1) at the start of operation in the AXM reactor, and was over tenfold reduced (41:1) by the end of the experimental period (Table 2). The good biomass retention in the AMX reactor enable the AMX retention but also other bacteria like the NOB coming with the effluent of the PN reactor that are one of the main reasons of the NOB relative abundance increase. Long-term operation is required to see whether the NOB activity can be controlled in the AMX reactor.

AOB and AMX comprised very small fractions of the total bacterial populations in the PN and AMX reactors, respectively (AOB = 0.007–0.786 ‰ and AMX = 0.745–2.667‰, Table SX1). In previous studies, higher relative abundances of both ammonia-oxidising groups were usually reported. Li et al. [38] measured 10 % AOB by qPCR in a PN-SBR operated with COD:N ratios of 1:1 and 1:3 at 20–25 °C. Massive parallel sequencing methods revealed AOB relative abundances of 0.24 % [40] and 18.3 % [41] in the PN reactors of two-stage PN/AMX plants operated to treating mature landfill leachate and reject water from anaerobic digestion of municipal sewage sludge, respectively. In addition, the relative abundances of AMX in the second unit in the two aforementioned studies were 8.3 % and 1.4 %, respectively [40,41]. However, it must be considered that these PN/AMX two-stage systems bore much higher N-loads (>700 mg/L) and were operated at warmer temperatures (>30 °C) than those used here. In addition, the AMX reactor investigated here was inoculated with a low initial biomass concentration (section 3.2). At mainstream conditions, Hausherr et al. [15] also observed very low relative AOB abundance (0.1 %) while stable PN process was maintained. This might be explained by the increase of other side population that make the relative abundance decrease. Another explanation is the occurrence of a population shift towards heterotrophic nitrification, ammonium oxidation archaea or other nitrifying bacteria like "Candidatus Nitrosoglobus", that can tolerate higher FNA concentrations and low pH values [42].

In the present study, NOB were detected in all samples, indicating that they were not completely washed out. In spite of this, both the PN and AMX processes performed efficiently, in agreement with the reports of several previous studies [5,15,29,38]. However, surviving NOB are reported to exert an important impact in one-stage PN/AMX process performance by limiting the NRE, even if their abundances are 3–4 orders of magnitude lower than that of AMX [43].

MDS and BIO-ENV statistical analyses were conducted aiming to reveal links among the changes of the abundance of the groups quantified by qPCR and the shifts of the operational parameters in the PN/AMX pilot plant, and the results are depicted in Fig. 6 and Table S4. In the PN bioreactor, both AOB and AMX abundances correlated strongly and positively ($r \geq 0.80$) with temperature and concentrations of NH_4^+ in both the influent and effluent, while robust negative correlations were observed ($r \leq -0.80$) with DO and FNA concentrations. Strikingly, a negative correlation was found among AOR and AOB abundance, which was attributed to the low AOR registered in Stage I due to the short aerobic reaction phase imposed (Fig. 2.B), even though the AOB abundance peaked at day 10 (Fig. 5). Higher abundances of NOB were correlated with lower NAR and NO_2^- concentrations in the effluent ($r \geq 0.90$), and conversely favored increased amounts of NO_3^- in the effluent ($r = -0.99$), consistently with the metabolic abilities of this group. Increasing FNA concentrations were also strongly related to lower abundance of NOB ($r = -0.79$), agreeing with its expected inhibitory effect over the growth of nitrite oxidisers [20,32].

In the AMX bioreactor, Bacteria and AOB trends through the ordination fully overlapped, contrary to the behaviour observed in the PN reactor (Fig. 6). The abundances of both groups were strongly and positively correlated ($r \geq 0.80$) with the VSS concentration, while negative correlations were revealed with NH_4^+ concentration in the effluent (r = -0.92). AMX abundances were favored by longer HRT and increasing NO_2^- concentrations in the influent ($r \geq 0.80$), and were negatively correlated with the concentrations of NH_4^+ in the influent and NO_3^- in the effluent ($r \leq 0.70$). It must be taken into consideration that Bacteria, AOB and AMX abundances changed little throughout operation in the AMX reactor, hence the abiotic factors exerted only a fine modulatory effect on their populations' dynamics. NOB abundances displayed links to operational parameters very similar to those observed in the PN reactor, correlating with higher NO_3^- and lower NO_2^- concentrations in the effluent ($r = 0.70$ and $r = -0.82$, respectively) and warmer temperatures ($r = 0.80$), and being disfavored by higher FNA concentrations ($r = -0.85$).

Table 2
Composition of the different streams from the two stage PN/AMX pilot-plant in the last 25 operational days.

Reactor	pH	mg COD/L	mg NH_4^+-N/L	mg NO_2^--N/L	mg NO_3^--N/L	mg VSS/L
HRAS effluent	7.84 ± 0.06	75 ± 8	52.48 ± 1.94	0	4.56 ± 1.44	16 ± 15
PN effluent	6.86 ± 0.09	70 ± 11	25.83 ± 3.08	26.20 ± 3.45	4.50 ± 1.17	26 ± 9
AMX effluent	6.89 ± 0.23	56 ± 12	3.97 ± 2.96	0.99 ± 0.95	7.27 ± 1.34*	11 ± 4

* Note that approximately 62 % comes from the HRAS effluent whereas <3 mg N/L are produced in the AMX reactor.

3.4. Effluent quality and impact of the wastewater composition

The proposed methodology allows for the rapid establishment of the mainstream PN/AMX process without the addition of the chemicals and

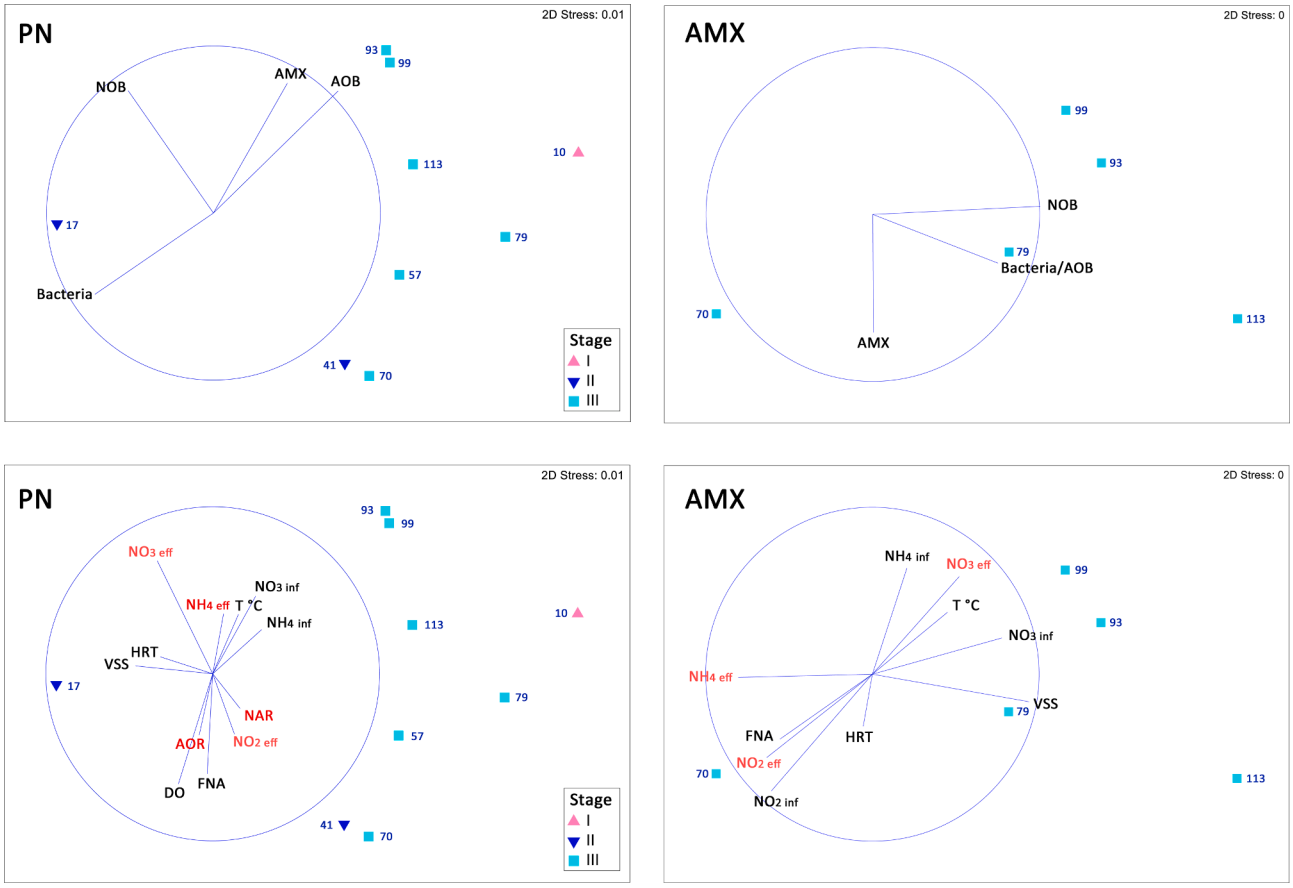


Fig. 6. Non-metric multidimensional scaling (MDS) ordination of the PN and AMX samples, on the basis of the abundance of copies of gene markers of total Bacteria, AOB, NOB and AMX. Vectors on plots represent the trends of the abundance of each marker (upper row) and the strength and directional influence of the operational parameters (lower row) throughout the samples' ordination. AOR and NAR in the PN reactor, and NH_4^+, NO_3^- and NO_2^- concentrations in the effluents of both reactors were also included in the plots to display correlations among the systems' performance and the abundances of the targeted bacterial groups.

with limited amount of biomass used as inoculum. The wastewater composition from the last 25 days of operation of the combined PN/AMX is presented in Table 2. The pH of the AMX effluent was considerably lower than that of the existing HRAS, but it was close to neutral, implying no discharge or reuse restrictions. The effluent solids concentration was not increased, and the effluent COD concentration slightly decreased, improving the effluent quality of the full-scale WWTP.

The TN concentration in the PN/AMX effluent was 11.5 ± 2.6 mg TN/L, which is similar to the results obtained by Kowalski et al. [14] which is over 10 mg TN/L and close to 15 mg TN/L established discharge limits for sensitive areas in the European Union (EU) depending on WWTP size. The TN effluent primarily consisted of nitrate, with an average concentration of 6.9 ± 0.8 mg NO_3^--N/L. Aapproximately 62 % of the nitrate came from the HRAS reactor. The increased in nitrate concentration in the effluent from the HRAS was linked to the temperature increase and impacted the effluent quality and the maximum achievable NRE since barely no nitrate is removed in the PN/AMX system. The performance of the HRAS system needs to be optimised by further reducing the HRT to limit the nitrifying bacteria development. If the nitrate fed to the PN reactor is disregarded when evaluating the effluent quality, the average TN concentration would be 7 ± 3 mg N/L, which is within the EU discharge limits. The NRE achieved (80 ± 5 %) was higher than that obtained by other authors treating municipal wastewater in one-stage PN/AMX pilot scale, and the NRR was comparable [9,11–13]. In a two-stage PN/AMX, Kowalski et al. [14] obtained, at pilot scale, a NRR of 55 mg TN/(L·d) using a moving bed biofilm reactor for PN and organic carbon removal and an integrated fixed-film activated sludge AMX reactor.

The effluent from the present study showed a very low nitrite concentration (<0.8 mg NO_2-N/L) (Figure S6). As nitrite is known to be highly toxic to aquatic environment, it is important to maintain its concentration below 1 mg N/L. It should be noted that the AMX reactor was not fully optimized in the current study but operated coupled with the PN reactor. By extending the reaction phase and/or reducing the amount of oxygen entering into the reactor, which can lead to ammonium oxidation, the presence of nitrite in the effluent can be easily eliminated.

The NRE of the complete system should be enhanced in accordance with the new proposed limits set by the European urban water directive (2022/0345/COD) of 6 mg N/L or a 85 % reduction. As the combination of the PN/AMX processes always convert the 11 % of the nitrogen load into nitrate, further improvement in the effluent quality can be achieved by diverting some raw wastewater (with residual COD) to the AMX systems for removal of produced nitrate and nitrite via heterotrophic denitrification. This was already proved at laboratory scale in a two-stage system [37], but its feasibility at the pilot scale needs to be verified. As the raw water also contains ammonium, the target AOR value should be adjusted to achieve the appropriate nitrite to ammonium ratio for the AMX process. The efficiency of the final polishing stage must be validated at pilot scale, taking into account the varying composition of the wastewater.

The lack of information about the performance of PN/AMX systems treating municipal wastewater under realistic conditions has been pointed out by several authors [4,5,37]. This fact highlights the relevance of the results in the present study, where the feasibility of using the effluent from an HRAS to feed a pilot-scale PN/AMX system was proven.

To sum up, the proposed operational strategy aims to suppress NOB using *in situ* accumulated FNA. The basic principles were outlined by Pedrouso et al. [35] and updated while working with real wastewater. During the start-up phase, the PN reactor is inoculated with sludge, regardless of whether the NOB are active or not. Then, nitrite accumulation is initiated by overloading the system with a low HRT, decoupling AOB and NOB activities. As alkalinity is consumed, the pH drops, and the AOR is adjusted to create the conditions that lead to inhibitory FNA concentrations (shown in Figure S7). To optimize nitrite accumulation, it is desirable to adjust the process control strategy by reaching the maximum achievable AOR that the wastewater alkalinity allowed. As detailed in Pedrouso et al. [35], if ammonium to inorganic carbon (IC) ratio in the incoming wastewater is higher than 0.6 g N/g IC, nitritation maintenance does not required any additional measured. If this ratio is lower than 0.6 g N/g IC, the HRT should be controlled, and if it is higher than 1 g N/g IC, the AOR will be limited by the alkalinity availability.

Then, depending on the AOR required to maintain the PN process, raw wastewater can be bypassed to supplement ammonium to the AMX reactor (if the nitrite to ammonium ratio is higher than 1.2 g N/g N). The amount bypassed depend on the wastewater nitrogen to alkalinity ratio too (g N/g IC). Additionally, to enhance the NRE, raw wastewater can be added to the AMX reactor at the end of the cycle to denitrify the nitrate produced and any possible nitrite present, thereby improving the effluent quality. The nitrogen to alkalinity ratio varied significantly with the geography (as wastewater alkalinity is linked to the type of soil in a location) but it does not fluctuate much over time for a specific facility. To maintain the PN/AMX process at pilot scale while coping with the wastewater fluctuations, ammonium, nitrate and pH would be online measured and flows will be adjusted accordingly.

This operational strategy is simple (without required complex control loops) and flexible being able to cope with fluctuations in wastewater characteristics. Nevertheless, the operational period of the pilot-scale reactors was short, especially for the AMX reactor (48 days), and further research is required to evaluate more extended process stability. Indeed, the operation must also be tested under colder conditions and with more diluted and fluctuating wastewater conditions (winter time). Hausherr et al. [15] obtained a near complete NOB suppression over a year in a plug-flow reactor (sedimentation, bottom-feed, anaerobic mixing and aeration) with a VER of 90 %. Authors recognized difficulties on the operation of these systems (two plug-flow reactors in series) to maintain the flow regime under real-world conditions. More intringly, the mechanism responsible for the NOB inhibition remains uncertain and, according to the authors, this might be resolved before speculating how to combine the PN with the AMX process.

4. Conclusions

A pilot-scale two-stage PN/AMX plant (total 1.2 m^3), fed with wastewater from an HRAS system (22 –63 mg NH_4^+- N/L), was operated successfully under without temperature control and fed wit). The nitritation process (operated for 118 days at 11 – 28 °C) was rapidly achieved by applying a high hydraulic load and sustained by accumulated *in situ* FNA. A variable duration of the aeration phase (controlled by pH) was used to cope with fluctuating wastewater characteristics and to obtain an effluent suitable to be fed to the AMX reactor (1.1 mg NO_2^--N/g NH_4^+-N, NAR 99 %). Although NOB were not entirely washed out from the system, their abundance was negatively correlated with FNA concentration, which suggests that this approach could be employed to better regulate these undesirable populations under the operating conditions applied here.

Despite being inoculated with low biomass concentration (0.4 g VSS/L), the AMX process (operated only during the last 48 days, 24.9 ± 1.3 °C) was quickly established in the open pilot-reactor, achieving a NRE of 80 ± 5 % and a NRR of 94 ± 14 mg TN/(L·d), which was mainly limited by the HRT required for satisfactory biomass retention in the PN reactor. However, the promising results need to be confirmed through long-term operation (including wintertime).

CRediT authorship contribution statement

Alba Pedrouso: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Data curation, Project administration. **Nicolas Morales:** Resources, Writing – review & editing. **Belén Rodelas:** Methodology, Writing – original draft, Writing – review & editing, Visualization, Data curation. **David Correa-Galeote:** Formal analysis, Investigation. **Angeles Val del Rio:** Validation, Funding acquisition, Writing – review & editing, Supervision. **Jose Luis Campos:** Conceptualization, Methodology, Validation. **Jose Vazquez-Padin:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition. **Anuska Mosquera-Corral:** Conceptualization, Validation, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work has been financed by the European Commission (EU) through the LIFE project ZERO WASTE WATER (LIFE19 ENV/ES/000631), the Waterworks 2014 Cofounded Call (Water JPI/Horizon) through the Pioneer_STP (PCIN-2015-022 MINECO (AEI)/ID 199 (UE)) and by the Spanish Government (AEI) through GRANDSEA (CTM2014-55397-JIN), TREASURE (CTQ2017-83225-C2-1-R) and ECOPOLYVER (PID2020-112550RB-C21 and PID2020-112550RB-C22) projects. Alba Pedrouso also acknowledges the Xunta de Galicia (Spain) for her post-doctoral fellowship (ED481B-2021-041). Authors from the USC belong to the Galician Competitive Research Group (GRC D431C-2021/37). Authors gratefully acknowledge the staff of the WWTP for their support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2023.123851.

References

[1] European Comission, Proposal for a DIRECTIVE OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL concerning urban wastewater treatment (recast), (2022). https://environment.ec.europa.eu/system/files/2022-10/Proposal%20for%20a%20Directive%20concerning%20urban%20wastewater%20treatment%20%28recast%29.pdf.

[2] B. Kartal, J.G. Kuenen, M.C.M. van Loosdrecht, Sewage Treatment with Anammox, Science 328 (2010) 702–703. https://doi.org/10.1126/science.1185941.

[3] H. Guven, R.K. Dereli, H. Ozgun, M.E. Ersahin, I. Ozturk, Towards sustainable and energy efficient municipal wastewater treatment by up-concentration of organics, Prog. Energy Combust. Sci. 70 (2019) 145–168. https://doi.org/10.1016/j.pecs.2018.10.002.

[4] L. Wang, W. Gu, Y. Liu, P. Liang, X. Zhang, X. Huang, Challenges, solutions and prospects of mainstream anammox-based process for municipal wastewater treatment, Sci. Total Environ. 820 (2022) 153351. https://doi.org/10.1016/j.scitotenv.2022.153351.

[5] M. Hoekstra, S.P. Geilvoet, T.L.G. Hendrickx, C.S. van Erp Taalman Kip, R. Kleerebezem, M.C.M. van Loosdrecht, Towards mainstream anammox: lessons learned from pilot-scale research at WWTP Dokhaven, Environmental Technology. 40 (2019) 1721–1733. https://doi.org/10.1080/09593330.2018.1470204.

[6] D. Seuntjens, B.L.M. Bundervoet, H. Mollen, C. De Mulder, E. Wypkema, A. Verliefde, I. Nopens, J.G.M. Colsen, S.E. Vlaeminck, Energy efficient treatment of

A-stage effluent: pilot-scale experiences with shortcut nitrogen removal, Water Sci. Technol. 73 (2016) 2150–2158,https://doi.org/10.2166/wst.2016.005.
[7] S.M. Podmirseg, M. Gómez-Brandón, M. Muik, B. Stres, M. Hell, T. Pümpel, S. Murthy, K. Chandran, H. Park, H. Insam, B. Wett, Microbial response on the first full-scale DEMON® biomass transfer for mainstream deammonification, Water Res 218 (2022) 118517. https://doi.org/10.1016/j.watres.2022.118517.
[8] H. De Clippeleir, S.E. Vlaeminck, F. De Wilde, K. Daeninck, M. Mosquera, P. Boeckx, W. Verstraete, N. Boon, One-stage partial nitritation/anammox at 15 °C on pretreated sewage: feasibility demonstration at lab-scale, Appl. Microbiol. Biotechnol. 97 (2013) 10199–10210. https://doi.org/10.1007/s00253-013-4744-x.
[9] M. Laureni, D.G. Weissbrodt, K. Villez, O. Robin, N. de Jonge, A. Rosenthal, G. Wells, J.L. Nielsen, E. Morgenroth, A. Joss, Biomass segregation between biofilm and flocs improves the control of nitrite-oxidizing bacteria in mainstream partial nitritation and anammox processes, Water Res 154 (2019) 104–116. https://doi.org/10.1016/j.watres.2018.12.051.
[10] G. Tchobanoglous, H.D. Stensel, R. Tsuchihashi, F.L. Burton, M. Abu-Orf, G. Bowden, W. Pfrang, Wastewater engineering: treatment and resource recovery, Fifth edition, McGraw-Hill Education, New York, NY, 2014 http://catdir.loc.gov/catdir/enhancements/fy1501/2014415848-t.html (accessed January 16, 2023.
[11] A. Pedrouso, I. Aiartza, N. Morales, J.R. Vázquez-Padín, F. Rogalla, J.L. Campos, A. Mosquera-Corral, A. Val del Rio, Pilot-scale ELAN® process applied to treat primary settled urban wastewater at low temperature via partial nitritation-anammox processes, Sep. Purif. Technol. 200 (2018) 94–101. https://doi.org/10.1016/j.seppur.2018.02.017.
[12] M. Han, S.E. Vlaeminck, A. Al-Omari, B. Wett, C. Bott, S. Murthy, H. De Clippeleir, Uncoupling the solids retention times of flocs and granules in mainstream deammonification: A screen as effective out-selection tool for nitrite oxidizing bacteria, Bioresour. Technol. 221 (2016) 195–204. https://doi.org/10.1016/j.biortech.2016.08.115.
[13] D.J.I. Gustavsson, C. Suarez, B.-M. Wilén, M. Hermansson, F. Persson, Long-term stability of partial nitritation-anammox for treatment of municipal wastewater in a moving bed biofilm reactor pilot system, Sci. Total Environ. 714 (2020) 136342. https://doi.org/10.1016/j.scitotenv.2019.136342.
[14] M.S. Kowalski, T.R. Devlin, A. di Biase, J.A. Oleszkiewicz, Effective nitrogen removal in a two-stage partial nitritation-anammox reactor treating municipal wastewater – Piloting PN-MBBR/AMX-IFAS configuration, Bioresour. Technol. 289 (2019) 121742. https://doi.org/10.1016/j.biortech.2019.121742.
[15] D. Hausherr, R. Niederdorfer, H. Bürgmann, M.F. Lehmann, P. Magyar, J. Mohn, E. Morgenroth, A. Joss, Successful mainstream nitritation through NOB inactivation, Sci. Total Environ. 822 (2022) 153546. https://doi.org/10.1016/j.scitotenv.2022.153546.
[16] C. Rong, T. Wang, Z. Luo, Y. Guo, Z. Kong, J. Wu, Y. Qin, T. Hanaoka, S. Sakemi, M. Ito, S. Kobayashi, M. Kobayashi, Y.-Y. Li, Seasonal temperatures impact on the mass flows in the innovative integrated process of anaerobic membrane bioreactor and one-stage partial nitritation-anammox for the treatment of municipal wastewater, Bioresour. Technol. 349 (2022) 126864. https://doi.org/10.1016/j.biortech.2022.126864.
[17] S. Gu, S. Wang, Q. Yang, P. Yang, Y. Peng, Start up partial nitrification at low temperature with a real-time control strategy based on blower frequency and pH, Bioresour. Technol. 112 (2012) 34–41. https://doi.org/10.1016/j.biortech.2011.12.028.
[18] Q. Yang, Y. Peng, X. Liu, W. Zeng, T. Mino, H. Satoh, Nitrogen Removal via Nitrite from Municipal Wastewater at Low Temperatures using Real-Time Control to Optimize Nitrifying Communities, Environ. Sci. Technol. 41 (2007) 8159–8164. https://doi.org/10.1021/es070850f.
[19] P. Jin, B. Li, D. Mu, X. Li, Y. Peng, High-efficient nitrogen removal from municipal wastewater via two-stage nitritation/anammox process: Long-term stability assessment and mechanism analysis, Bioresour. Technol. 271 (2019) 150–158, https://doi.org/10.1016/j.biortech.2018.09.097.
[20] Y. Zhou, A. Oehmen, M. Lim, V. Vadivelu, W.J. Ng, The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants, Water Res 45 (2011) 4672–4682, https://doi.org/10.1016/j.watres.2011.06.025.
[21] H. Duan, S. Watts, M. Zheng, Z. Wang, J. Zhao, H. Li, P. Liu, J. Dwyer, P. McPhee, M. Rattier, E. Larsen, Z. Yuan, S. Hu, Achieving robust mainstream nitrite shunt at pilot-scale with integrated sidestream sludge treatment and step-feed, Water Res 223 (2022) 119034, https://doi.org/10.1016/j.watres.2022.119034.
[22] H. Duan, L. Ye, X. Lu, Z. Yuan, Overcoming Nitrite Oxidizing Bacteria Adaptation through Alternating Sludge Treatment with Free Nitrous Acid and Free Ammonia, Environ. Sci. Technol. 53 (2019) 1937–1946, https://doi.org/10.1021/acs.est.8b06148.
[23] N. Morales, A. Pedrouso, A. Val del Rio, A. Mosquera-Corral, Anuska, J.L. Campos, J.R. Vázquez-Padín, Jose Ramón, Start-up of the first full scale reactor based on the granular ELAN® technology to remove nitrogen from the co-digester supernatant, in: Delft (The Netherlands), 2018.
[24] E.W. Rice, L. Bridgewater, A.P.H. Association, A.W.W. Association, W. E. Federation, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, 2012.
[25] J. López-Fiuza, B. Buys, A. Mosquera-Corral, F. Omil, R. Méndez, Toxic effects exerted on methanogenic, nitrifying and denitrifying bacteria by chemicals used in a milk analysis laboratory, Enzyme Microb. Technol. 31 (2002) 976–985, https://doi.org/10.1016/S0141-0229(02)00210-7.
[26] P. Maza-Márquez, R. Vílchez-Vargas, A. González-Martínez, J. González-López, B. Rodelas, Assessing the abundance of fungal populations in a full-scale membrane bioreactor (MBR) treating urban wastewater by using quantitative PCR (qPCR), J. Environ. Manage. 223 (2018) 1–8, https://doi.org/10.1016/j.jenvman.2018.05.093.
[27] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clin. Chem. 55 (2009) 611–622, https://doi.org/10.1373/clinchem.2008.112797.
[28] C.-G. David, M. Diana E., T. Germán, B. David, P. Laurent, B. Eulogio J., Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments, FEMS Microbiology Ecology. 83 (2013) 340–351. https://doi.org/10.1111/j.1574-6941.2012.01479.x.
[29] A. Pedrouso, D. Correa-Galeote, P. Maza-Márquez, B. Juárez-Jimenez, J. González-López, B. Rodelas, J.L. Campos, A. Mosquera-Corral, A. Val del Rio, Understanding the microbial trends in a nitritation reactor fed with primary settled municipal wastewater, Sep. Purif. Technol. 256 (2021) 117828. https://doi.org/10.1016/j.seppur.2020.117828.
[30] P. Maza-Márquez, R. Vílchez-Vargas, N. Boon, J. González-López, M.V. Martínez-Toledo, B. Rodelas, The ratio of metabolically active versus total Mycolata populations triggers foaming in a membrane bioreactor, Water Res 92 (2016) 208–217. https://doi.org/10.1016/j.watres.2015.12.057.
[31] G.A. Jackson, R.S. Livingston, L.K. Riley, B.A. Livingston, C.L. Franklin, Development of a PCR Assay for the Detection of Spironucleus muris, J. Am. Assoc. Lab. Anim. Sci. 52 (2013) 165–170.
[32] V.M. Vadivelu, Z. Yuan, C. Fux, J. Keller, The Inhibitory Effects of Free Nitrous Acid on the Energy Generation and Growth Processes of an Enriched Nitrobacter Culture, Environ. Sci. Technol. 40 (2006) 4442–4448. https://doi.org/10.1021/es051694k.
[33] T. Lotti, R. Kleerebezem, C. Lubello, M.C.M. van Loosdrecht, Physiological and kinetic characterization of a suspended cell anammox culture, Water Res 60 (2014) 1–14. https://doi.org/10.1016/j.watres.2014.04.017.
[34] M. Strous, J.G. Kuenen, M.S.M. Jetten. Key Physiology of Anaerobic Ammonium Oxidation | Applied and Environmental Microbiology, 1999. https://doi.org/10.1128/AEM.65.7.3248-3250.1999.
[35] A. Pedrouso, Á. Val del Río, N. Morales, J.R. Vázquez-Padín, J.L. Campos, R. Méndez, A. Mosquera-Corral, Nitrite oxidizing bacteria suppression based on in-situ free nitrous acid production at mainstream conditions, Sep. Purif. Technol. 186 (2017) 55–62. https://doi.org/10.1016/j.seppur.2017.05.043.
[36] S. Li, J. Li, S. Yang, Q. Zhang, X. Li, L. Zhang, Y. Peng, Rapid achieving partial nitrification in domestic wastewater: Controlling aeration time to selectively enrich ammonium oxidizing bacteria (AOB) after simultaneously eliminating AOB and nitrite oxidizing bacteria (NOB), Bioresour. Technol. 328 (2021) 124810. https://doi.org/10.1016/j.biortech.2021.124810.
[37] A. Pedrouso, A. Val del Rio, N. Morales, J.R. Vazquez-Padin, J.L. Campos, A. Mosquera-Corral, Mainstream anammox reactor performance treating municipal wastewater and batch study of temperature, pH and organic matter concentration cross-effects, Process Saf. Environ. Prot. 145 (2021) 195–202. https://doi.org/10.1016/j.psep.2020.07.052.
[38] J. Li, D. Elliott, M. Nielsen, M.G. Healy, X. Zhan, Long-term partial nitrification in an intermittently aerated sequencing batch reactor (SBR) treating ammonium-rich wastewater under controlled oxygen-limited conditions, Biochem. Eng. J. 55 (2011) 215–222. https://doi.org/10.1016/j.bej.2011.05.002.
[39] P. Bao, S. Wang, B. Ma, Q. Zhang, Y. Peng, Achieving partial nitrification by inhibiting the activity of Nitrospira-like bacteria under high-DO conditions in an intermittent aeration reactor, J. Environ. Sci. 56 (2017) 71–78. https://doi.org/10.1016/j.jes.2016.09.004.
[40] H. Li, S. Zhou, W. Ma, P. Huang, G. Huang, Y. Qin, B. Xu, H. Ouyang, Long-term performance and microbial ecology of a two-stage PN–ANAMMOX process treating mature landfill leachate, Bioresour. Technol. 159 (2014) 404–411. https://doi.org/10.1016/j.biortech.2014.02.054.
[41] J. Dosta, J. Vila, I. Sancho, N. Basset, M. Grifoll, J. Mata-Álvarez, Two-step partial nitritation/Anammox process in granulation reactors: Start-up operation and microbial characterization, J. Environ. Manage. 164 (2015) 196–205. https://doi.org/10.1016/j.jenvman.2015.08.023.
[42] Z. Wang, M. Zheng, J. Meng, Z. Hu, G. Ni, A. Guerrero Calderon, H. Li, H. De Clippeleir, A. Al-Omari, S. Hu, Z. Yuan, Robust Nitritation Sustained by Acid-Tolerant Ammonia-Oxidizing Bacteria, Environ. Sci. Technol. 55 (2021) 2048–2056. https://doi.org/10.1021/acs.est.0c05181.
[43] F. Persson, C. Suarez, M. Hermansson, E. Plaza, R. Sultana, B.-M. Wilén, Community structure of partial nitritation-anammox biofilms at decreasing substrate concentrations and low temperature, J. Microbial. Biotechnol. 10 (2017) 761–772. https://doi.org/10.1111/1751-7915.12435.