

Nitrite oxidizing bacteria suppression based on in-situ free nitrous acid production at mainstream conditions

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Abstract

The application of autotrophic nitrogen removal processes in the main line of wastewater treatment plants will contribute to achieve its self-energy-sufficiency. However, the effective suppression of nitrite oxidizing bacteria (NOB) activity at the conditions of low temperature and low ammonium concentration (mainstream conditions) was identified as one of the main bottlenecks. In this study, stable partial nitrification at 16 °C and 50 mg

NH_4^+ -N/L was achieved maintaining inside the reactor free nitrous acid (FNA) concentrations inhibitory for NOB ($> 0.02 \text{ mg HNO}_2\text{-N/L}$), without dissolved oxygen concentration control. The FNA inhibitory concentration was generated by the partial nitrification process, and its stimulation was studied with two different inhibitors: sodium azide and nitrite. The microbiological analysis revealed that, throughout the operational period with inhibitory FNA levels, the NOB populations (dominated by *Nitrospira*) were effectively washed out from the reactor. This is an advantage that allowed maintaining a good stability of the process, even when the FNA concentration was not enough to inhibit the NOB, taking about 40 days to develop significant activity. The observed delay on the NOB development is expected to enable the establishment of corrective actions to avoid the partial nitrification destabilization. The use of the FNA to achieve a stable partial nitrification process is recommended to profit from the natural pH decrease associated to the nitrification process and from its favoured accumulation at low temperatures as those from the mainstream. In this research study an analysis about the influence of ammonium and alkalinity concentrations was also performed to know in which scenarios the FNA inhibitory concentration can be achieved.

Keywords: Ammonium Oxidizing Bacteria; flocculent sludge; Free Nitrous Acid; mainstream; Nitrite Oxidizing Bacteria; partial nitrification.

1. Introduction

In the last years a new perspective to understand the wastewater treatment plants (WWTPs) arose, which considers their function not only to treat the wastewater, to meet more stringent disposal limits, but also to act as a pool of resources [1]. The energy

contained in the chemical compounds (i.e. organic matter) of the municipal wastewater is one of the most interesting resources. For this reason, the nitrogen removal via the nitrite pathway, based on the inhibition of the nitrification process, has gained much attention compared to the conventional nitrification-denitrification processes. If the nitrite route is applied, approximately 40% less organic matter is consumed for heterotrophic denitrification [2] or, preferably, no organic matter is needed if it is autotrophically removed via the anammox process. Furthermore, 25% less energy is required for aeration [3]. Thus, multiple efforts are focused on the nitrification process consisting on the ammonium oxidation to nitrite by the ammonium oxidizing bacteria (AOB).

However, the nitrite oxidizing bacteria (NOB) suppression is still one of the main bottlenecks to extend the application of the combined partial nitrification-anammox processes to the mainstream conditions. For example, Lotti et al. [4] operated an one-stage partial nitrification-anammox reactor at 19 °C and measured higher nitrate produced to ammonium consumed ratios (≥ 0.35 g N/ g N) than the stoichiometric one (0.11 g N/g N). Laurenzi et al. [5] obtained in a partial nitrification-anammox moving bed biofilm reactor (MBBR), operated at 15 °C, a nitrate produced to ammonium consumed ratio of 0.16 g N/g N, close to the expected value. In their study, the reached nitrogen removal efficiency was of 70%, while the nitrogen removal rates achieved were of 20-40 mg N/(L·d). The low removal rates were caused by the limiting dissolved oxygen concentrations that were applied to avoid the NOB activity development (indeed the relative abundances of AOB and NOB were similar).

To operate a partial nitrification based system in stable conditions multiple strategies have been proposed to induce the NOB depletion, for example the control of parameters such as: sludge retention time (SRT); pH value; free ammonia (FA) and free nitrous acid (FNA) concentrations; real time effluent concentrations of ammonium, nitrite and nitrate;

dissolved oxygen (DO) concentration; aeration length; or applying transient anoxic and aerobic periods [6, 7]. But scarce information is available about research studies focused on the NOB suppression in single partial nitrification units at mainstream conditions. Reino et al. [8] obtained stable partial nitrification at 70 mg $\text{NH}_4^+\text{-N/L}$ and 10 °C, in a granular biomass system, for more than 250 days by means of a control strategy based on maintaining a very low value of the dissolved oxygen to total ammonia nitrogen concentration ratio. Although, this strategy requires the maintenance of high pH values, controlled to 8.0, and ammonium in excess in the bulk liquid [8]. Wang et al. [9, 10] have already shown the feasibility to achieve and maintain the partial nitrification at mainstream conditions (40 - 57 mg $\text{NH}_4^+\text{-N/L}$ and 22 °C) throughout a FNA based strategy. They proposed to expose a fraction of the partial nitrifying sludge, present inside the partial nitrification reactor, to the FNA produced in an additional unit placed in the sidestream line, to inhibit the NOB activity. This strategy is based on the fact that AOB are significantly less sensitive to FNA than NOB.

The FNA concentration required to inhibit the 50% of AOB activity has been found to be in the range 0.42-1.72 mg $\text{HNO}_2\text{-N/L}$ [11]. This value is much higher than the 0.02 mg $\text{HNO}_2\text{-N/L}$ needed for the observed complete inhibition of NOB activity [12]. Therefore, the use of FNA appears as a suitable alternative to inhibit the NOB at mainstream conditions, which need to be further explored to establish those conditions that guarantee its production inside the partial nitrification unit. This becomes more complicated when the inoculated sludge has already a significant NOB activity, which makes the initial accumulation of required nitrite concentrations difficult. In this case the use of an inhibiting compound, such as sulphide, hydrazine, hydroxylamine or azide, which removes the NOB activity can be adequate [6].

When sodium azide is chosen, the difference on the inhibition thresholds over AOB and NOB (IC_{50} values of 40 mg NaN_3/L and 0.025 mg NaN_3/L , respectively [13]) can be exploited to inhibit selectively the NOB activity with no or minimal effect over AOB. Actually, this compound is frequently used to perform AOB batch activity tests [14]. However, the long-term effect of sodium azide on NOB, to research its inhibitory effect or the possible acclimation of the biomass to this compound, has not been studied yet.

Therefore, the aim of this study was to evaluate the operation of a partial nitrification unit with flocculent biomass, at 16 °C and fed with 50 mg NH_4^+-N/L , by producing in-situ FNA at inhibitory concentrations to suppress the NOB activity. Furthermore, the addition of nitrite and a chemical inhibitor (sodium azide) were evaluated as a strategy to initiate the nitrite accumulation necessary to achieve the desired FNA concentration.

2. Materials and Methods

2.1. Reactor setup and operation

A glass laboratory sequencing batch reactor (SBR), with a working volume of 2 L (useful H/D ratio of 0.38), was used to perform the partial nitrification process. It was provided with a set of two peristaltic pumps to introduce the feeding solution, through the top, and to discharge the effluent, at a medium height in the column reactor. The volume exchange ratio (VER) was of approximately 50%. Air was supplied through an air diffuser at the bottom of the reactor to guarantee the complete mixture and to supply the oxygen required for the biological reactions. Variable air flow rates, ranging from 0.3-1.5 L/min, were supplied to maintain the dissolved oxygen (DO) concentration in the range from 0.2 to 8.0 mg O_2/L . The pH values were not controlled and varied from 8.0 to 6.3. The reactor temperature was maintained at 16 ± 1 °C.

The SBR operated in 3-hour cycles comprising: 158 min of continuous feeding and aeration, 20 min of settling and 2 min of effluent withdrawal. The different periods of the operational cycle were controlled by a programmable logic controller (Siemens S7-224CPU).

The inoculum consisted of 9.1 g VSS/L of sludge from a one-stage partial nitrification-anammox laboratory reactor operated at 16 °C and fed with 50 mg $\text{NH}_4^+\text{-N/L}$ [15]. At the moment of the collection the measured maximum specific AOB and NOB activities were significant: 0.050 ± 0.006 g $\text{NH}_4^+\text{-N/(g VSS}\cdot\text{d)}$, and 0.072 ± 0.012 g $\text{NO}_2^-\text{-N/(g VSS}\cdot\text{d)}$, respectively.

The feeding media composition contained 50 ± 7 mg $\text{NH}_4^+\text{-N/L}$ supplemented with 0.4-0.8 NaHCO_3 , 0.040 K_2HPO_4 , 0.015 KH_2PO_4 , 0.040 MgSO_4 in g/L and 0.2 mL/L of a trace solution [16]. The feeding media was prepared with tap water with average concentrations of inorganic carbon (IC) and nitrate of 2.5 ± 0.1 mg IC/L and 1.8 ± 0.6 mg $\text{NO}_3^-\text{-N/L}$, respectively.

The reactor operated during 690 days in seven operational stages (I-VII) depending on the feeding alkalinity (IC concentration) and the added concentration of inhibitor (sodium azide or nitrite) (Table 1). The Stage I (0-64 days) corresponded to the establishment of a fully nitrifying process. In Stage II (65-110 days) the feeding was supplemented with 5 mg/L of sodium azide (NaN_3), as NOB inhibitor. In both stages the inorganic carbon concentration was of 123 ± 11 mg IC/L. Then in Stage III (111-210 days) this concentration was reduced to 57 ± 4 mg IC/L. This condition remained during Stage IV (211-384 days) while the NaN_3 addition was stopped. In Stage V (385-440 days) the IC concentration was increased to 99 ± 6 mg IC/L to restore the conditions for complete nitrification. Then, in Stage VI (441-489 days) the IC was reduced to 52 ± 3 mg IC/L to analyse the influence of the alkalinity limitation. Finally, in Stage VII (490-690 days)

nitrite as NaNO_2 was supplemented to the feeding with a concentration of $78 \pm 5 \text{ mg NO}_2^-$ -N/L to restore the inhibitory levels of FNA. The supplied nitrite concentration was calculated according to the maximum NOB activity obtained by batch test providing nitrite in excess to assure that NOB activity in the system (0.351 g NO_2^- -N/(g VSS·d)) was not enough to fully oxidize it to nitrate.

Table 1. Operational conditions and influent characteristics of the different operational stages.

Stage	Days	NH_4^+ -N/IC (g N/ g IC)	Inhibitor addition	Objective
I	0-64	0.39 ± 0.07	No	Total nitrification
II	65-110	0.38 ± 0.05	NaN_3 (5 mg/L)	Nitrite accumulation
III	111-210	0.88 ± 0.09	NaN_3 (5 mg/L)	Partial nitrification
IV	211-384	0.90 ± 0.07	No	FNA inhibitory levels
V	385-440	0.52 ± 0.04	No	Alkalinity increase
VI	441-489	1.01 ± 0.06	No	Alkalinity limitation
VII	490-690	0.93 ± 0.04	NaNO_2 (80 mg N/L)	FNA inhibitory levels restoration

2.2. Batch activity tests

Sludge samples were collected periodically from the reactor to determine the maximum specific ammonium and nitrite oxidizing activities. Both activities were determined by respirometric assays using a Bench model Oxygen Meter (YSI 5300) with oxygen selective probes (YSI 5331) according to the methodology described by López-Fiuza et al. [13].

2.3. Analytical methods

The DO concentration and temperature in the bulk liquid were measured on-line using a luminescent DO probe (LDO, Hach Lange). The pH value was measured using an electrode connected to a measurer (Crison 506). Liquid samples, collected from the reactor (influent and effluent) three times a week, were filtered through $0.45 \mu\text{m}$ pore size prior to analysis. Spectrophotometric methods were applied to determine the ammonium

[17], nitrite and nitrate [18] concentrations. Total organic and inorganic carbon concentrations (TOC, IC) were measured with a Shimadzu analyzer (TOC-5000).

Samples from the reactor and the effluent were collected to determine the biomass concentration as total suspended solids (TSS) and volatile suspended solids (VSS), according to standard methods [18]. The sludge volume index (SVI) of the biomass was determined according to standard methods [18].

Bacterial community present in the biomass samples was identified by the Fluorescence *in situ* Hybridization (FISH) technique and 16S rRNA gene-based amplicon analysis (Base Space, Illumina®) according to the procedure described by Fra-Vazquez et al. [19]. The FISH probes used were: EUB338mix for all *Bacteria*, Ntspa712 for *Nitrospira* and Nit3 for *Nitrobacter spp.*

2.4. Calculations

The FNA concentration was calculated according to Anthonisen et al. [20] at the operational temperature and pH values in the bulk liquid, and considering the nitrite and ammonium concentrations, respectively. The solids retention time (SRT) was estimated as the volatile suspended solids concentration in the reactor multiplied by the hydraulic retention time (HRT) and divided by the volatile suspended solids concentration in the effluent.

A sensitivity analysis was carried out, using Matlab® software, to determine the impact of the inlet ammonium concentration (from 10 to 100 mg $\text{NH}_4^+\text{-N/L}$) and the inlet ammonium to inorganic carbon concentration ratio (from 0.5 to 1.5 g $\text{NH}_4^+\text{-N/g IC}$) on the FNA accumulation. These ranges were set considering the ammonium and inorganic carbon concentrations normally found in municipal wastewater produced in areas with low-moderate alkaline freshwater. It was considered that the oxidized ammonium is only converted to nitrite consuming 2 mol of inorganic carbon per 1 mol of ammonium

oxidized. If the IC supplied is in excess then a full conversion of ammonium to nitrite is achieved and some IC is left. If the IC is not sufficient a fraction of ammonium is not oxidized. In both cases the pH is estimated from the remaining concentrations of ammonium and IC, and the respective equilibriums CO₂-carbonate-bicarbonate and ammonium-ammonia. With the obtained pH values and the nitrite concentrations (produced from the ammonia oxidation) the FNA concentration was estimated.

3. Results

3.1. Reactor operation

The SBR was operated for 690 days (Figure 1). During the first 64 days of operation (Stage I), the nitrification process was established. AOB and NOB measured activities were approximately of 0.05 g NH₄⁺-N/(g VSS·d) and 0.072 g NO₂⁻-N/(g VSS·d), respectively (Table 2). Ammonium concentration in the influent ranged from 35 to 60 mg NH₄⁺-N/L, and was completely oxidized to nitrate after 20 days (Figure 1.a). Nitrite in the effluent, as intermediate of the nitrification process, was not detected during this stage. The DO concentration inside the reactor oscillated from 0.3 to 3.0 mg O₂/L (Figure S.1, Supplementary Material).

In Stage II, immediately after the addition of 5 mg/L of sodium azide an inhibitory effect on the previously developed NOB activity was observed. Despite the average DO concentration was increased (Figure S.1), the accumulation of nitrite experienced a progressive increase up to around 35 mg NO₂⁻-N/L, while nitrate concentration proportionally decreased. Approximately 45 days of sodium azide supply were required to eliminate most of the NOB activity. The inlet ammonium to inorganic carbon concentration ratio (NH₄⁺-N/IC) was approximately of 0.38 g N/g IC in Stages I and II, which is low enough to accomplish the complete nitrification.

In Stage III the supplied bicarbonate concentration was decreased (0.88 g N/g IC) to limit the AOB activity efficiency to 50% (Figure 1.a). Although, the NaN_3 acted as inhibitor of the NOB activity, in this stage, the decrease of pH value (due to alkalinity limitation) promoted the accumulation of FNA up to 0.01 mg $\text{HNO}_2\text{-N/L}$ (Figure 1.b). The inorganic carbon concentration in the effluent decreased from 66 ± 8 to 7 ± 2 mg IC/L. The average values of the nitrogen compounds in the effluent were 18.4 ± 5.7 mg $\text{NH}_4^+\text{-N/L}$, 25.9 ± 5.6 mg $\text{NO}_2^-\text{-N/L}$ and 2.6 ± 1.0 mg $\text{NO}_3^-\text{-N/L}$. Most fraction of the nitrate concentration measured was already present in the tap water used for the feeding media preparation, and therefore its production by NOB was considered negligible. This observation was confirmed by the undetectable NOB activity measured in the performed respirometric assays (Table 2).

Table 2. Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) activities estimated by respirometric assays performed at 16 °C.

Sample collection		AOB activity	NOB activity
Stage	Day	g $\text{NH}_4^+\text{-N}/(\text{g VSS}\cdot\text{d})$	g $\text{NO}_2^-\text{-N}/(\text{g VSS}\cdot\text{d})$
I (0-64)	0	0.050 ± 0.006	0.072 ± 0.012
III (111-210)	203	0.068 ± 0.028	ND
IV (211-384)	238	0.103 ± 0.039	ND
	288	0.220 ± 0.011	ND
V (385-440)	400	0.195 ± 0.052	ND
	412	0.178 ± 0.002	0.149 ± 0.004
VI (441-489)	457	0.416 ± 0.065	0.291 ± 0.013
	483	0.393 ± 0.036	0.351 ± 0.022
VII (490-690)	512	0.398 ± 0.026	0.186 ± 0.008

598	0.382 ± 0.014	0.093 ± 0.006
640	0.388 ± 0.021	0.052 ± 0.012

ND: Not detected

In Stage IV the sodium azide supply was stopped. The NOB activity repression due to the sole action of the FNA was studied at concentrations from 0.01 (first 40 days of the stage) to approximately 0.06 mg $\text{HNO}_2\text{-N/L}$ (days 250-384) (Figure 1.b). The interruption of the sodium azide addition in the feeding provoked the pH decrease (increasing the FNA concentrations) since azide in aqueous solution is known to release hydroxyl ions ($\text{pK}_a = 4.6$) [21]. During the whole stage the NOB activity in the reactor was negligible (again the nitrate present came from the tap water) and it was not detected from the respirometric experiments either (Table 2). Although the AOB activity increased up to $0.220 \text{ g NH}_4^+\text{-N/(g VSS}\cdot\text{d)}$. The composition of the effluent in stage IV was $21.3 \pm 3.6 \text{ mg NH}_4^+\text{-N/L}$, $27.9 \pm 2.4 \text{ mg NO}_2^-\text{-N/L}$ (average $\text{NO}_2^-/\text{NH}_4^+$ molar ratio of 1.3), $1.9 \pm 0.4 \text{ mg NO}_3^-\text{-N/L}$ and $5.8 \pm 3.6 \text{ mg IC/L}$. The length of this stage (174 days) together with the applied hydraulic residence time (0.25 days) provided a long enough experimental period to discard any residual effect of NaN_3 on the observed inhibition.

Therefore, the results obtained throughout Stage IV indicated that, at the conditions of low ammonium concentration and temperature (mainstream conditions), is possible to achieve a stable partial nitrification, without NOB activity, thanks to the inhibitory effect of the FNA formed in the bulk liquid itself. This inhibitory FNA concentration was achieved due to the synergist effect of the nitrite accumulated in the system and the pH value.

To confirm this finding, in Stage V (385-440 days) the IC concentration in the feeding was doubled reaching values high enough to achieve the complete nitrification. At the beginning of this stage all the ammonium was oxidized to nitrite, but as the pH value rose up the FNA concentration in the bulk liquid decreased (Figure 1.b) to values under those identified as inhibitory for NOB of 0.02 mg HNO₂-N/L [12, 22]. However, the development of significant NOB activity took approximately 40 days and nitrate production was observed in the reactor only after day 424 of operation (Figure 1.a). Throughout this period, the average effluent composition was 4.8 ± 1.1 mg NH₄⁺-N/L, 42.0 ± 1.3 mg NO₂⁻-N/L, 2.1 ± 0.5 mg NO₃⁻-N/L and 35.9 ± 4.0 mg IC/L. The absence of NOB activity during the first days of Stage V was also confirmed by respirometric assays, being on day 400 (16 days after IC change) still negligible (Table 2). However, a few days later (on day 412) the measured NOB activity was of 0.149 ± 0.004 g NO₂⁻-N/(g VSS·d) (Table 2), despite it became significant inside the reactor only after day 424 (Figure 1.b), being of approximately 0.06 g NO₂⁻-N/(g VSS·d). Once the nitrate production in the reactor initially appeared it increased exponentially.

This observation is very interesting since a control strategy based on the FNA concentration will allow anticipating the NOB appearance with enough time to avoid problems of destabilization of the partial nitrification process.

Later, in Stage VI (441-489) the inorganic carbon in the influent was again decreased to see if the alkalinity limitation by itself could lead to the partial nitrification establishment. Despite obtaining the lowest pH values (6.23 ± 0.27), FNA inhibitory levels were not achieved as nitrite was not present in the effluent (1.3 ± 1.1 mg NO₂⁻-N/L). Thus, ammonium was oxidized to nitrate. During this stage, the average effluent composition was 19.9 ± 2.9 mg NH₄⁺-N/L, 32.9 ± 5.3 mg NO₃⁻-N/L and 2.0 ± 1.5 mg IC/L. Therefore, only with alkalinity limitation the NOB suppression was not possible. During this stage

both AOB and NOB specific activities were similar and greatly increased up to $0.393 \text{ g NH}_4^+\text{-N}/(\text{g VSS}\cdot\text{d})$ and $0.351 \text{ g NO}_2^-\text{-N}/(\text{g VSS}\cdot\text{d})$, respectively (Table 2).

Finally, during Stage VII (490-690) sodium nitrite was supplied in the SBR feeding ($78 \pm 5 \text{ mg NO}_2^-\text{-N/L}$). Consequently, nitrate production immediately decreased and nitrite started to accumulate inside the reactor from 0.1 to $8 \text{ mg NO}_2^-\text{-N/L}$ in 3 days. Despite the almost immediate accumulation of the FNA concentration in the system, from day 560 the nitrite concentration in the effluent was approximately $20 \text{ mg NO}_2^-\text{-N/L}$ and no complete nitrate depletion was reached. The pH value was constant during the whole stage, thus FNA increased progressively following the same pattern as the nitrite accumulation and it was stabilized at $0.02 \pm 0.002 \text{ mg HNO}_2\text{-N/L}$. The average effluent composition in this reached steady state was: $18.9 \pm 4.3 \text{ mg NH}_4^+\text{-N/L}$, $20.3 \pm 7.3 \text{ mg NO}_2^-\text{-N/L}$, $15.8 \pm 9.1 \text{ mg NO}_3^-\text{-N/L}$ and $4.1 \pm 3.0 \text{ mg IC/L}$. From the total amount of oxidized ammonium the 70% was accumulated as nitrite and the 30% as nitrate. Different reasons might be considered to explain this fact, as the long SRT of the biomass (higher than 100 days). The suspended solids in the effluent of Stage VII are comparable to the previous stage (Figure 1.c), making difficult a complete and efficient NOB washout. However, the most probable reason can be the relatively low FNA levels achieved. The maximum FNA concentration during this Stage VII was $0.024 \text{ mg HNO}_2\text{-N/L}$, whereas during stage IV the FNA reached values up to $0.06 \text{ mg HNO}_2\text{-N/L}$ (Figure 1.b). The nitrite concentration added was calculated at the beginning of the Stage VII, but probably more nitrite should be added to obtain higher FNA levels. Presumably, higher FNA concentration would speed up the nitrate production depletion by inhibiting the NOB activity, which could be achieved by the addition of higher nitrite concentration in the influent.

3.2. Long-term effect of sodium azide on AOB

Sodium azide is known to inhibit both, AOB and NOB activities, with IC_{50} values of 40 and 0.025 mg NaN_3/L , respectively [13]. Furthermore, this compound is reported to inhibit the NOB anabolism meaning that when it is present these bacteria cannot survive [23]. Due to its so different inhibitory effect over both populations, NaN_3 at low concentrations (1.56 mg NaN_3/L) is commonly used to remove the NOB activity from a nitrifying sludge when the maximum specific ammonium oxidizing activity is determined by batch tests [14].

The effect of the sodium azide concentration, used in this study (5 mg NaN_3/L), over the AOB activity was previously tested by batch respirometric assays and no detrimental effect was observed (data not shown). However, its long-term effect on the AOB activity needs to be verified directly in the conditions of the experiment performed in this study. For this purpose the AOB specific activity of the biomass sample collected from the reactor at the end of Stage III (during the addition of 5 mg NaN_3/L) did not diminished compared to that of the inoculum, and was of 0.068 g $NH_4^+-N/(g\ VSS \cdot d)$ (Table 2).

The conversion efficiencies of ammonium oxidation measured inside the reactor (Figure 1.a) varied due to the imposed operational conditions, mainly due to the different alkalinity values present in the medium, and presumably cannot be attributed to any negative effect of NaN_3 .

3.3. Biomass characteristics

The SBR was inoculated with 9.1 g VSS/L of flocculent biomass (Figure 1.c). During Stages I and II till day 140 of Stage III, a fast decrease of the inoculated biomass concentration occurred to values of 2.8 g VSS/L. Then, the rate of biomass loss decreased, and the concentration of solids at the end of Stage III was around 2.0 g VSS/L. Throughout the rest of the reactor operation, the biomass concentration inside the reactor

was almost constant and approximately of 1.4 ± 0.2 g VSS/L (Figure 1.c) without significant morphological changes (see Figure S.2). The average solids concentration in the effluent was of 0.005 ± 0.002 g VSS/L. The sludge volume index (SVI) was between 20 and 30 mL/g TSS, showing the good settleability properties of the flocculent sludge. The estimated average solids retention time (SRT) was higher than 100 days.

The initial sharp decrease of biomass concentration was probably due to the wash out of microbial populations present in the inoculum but not retained inside the reactor due to the imposed operational conditions. In Stage I, probably the anammox bacteria were mainly removed and in Stages II and III the NOB populations.

To study the evolution of NOB populations inside the reactor the 16S rRNA gene-based amplicon analysis was applied to biomass samples collected in different operational days. Detailed results can be looked up in the Supplementary Material (Figure S.3). The values obtained, in terms of abundance percentage, for the genus *Nitrospira* sp. (the most abundant NOB genus in the present study) were: 2.0% (day 0), 27.9% (day 44), 3.5% (day 248), 3.5% (day 303), 0.4% (day 336) and 0.01% (day 372). These results indicated that the NOB were developed during Stage I (sample day 44) being an abundant population when the full nitrification process took place. Then, with the addition of the sodium azide in Stages II and III, the NOB abundance was significantly reduced, proved also by the absence of positive results by FISH analysis. However, it was in Stage IV (samples 248, 303, 336 and 372) when their abundance was drastically reduced. These results indicate that the strategy followed in Stage IV, maintaining FNA concentrations up to 0.02 mg HNO₂-N/L inside the reactor, was successful to reduce the NOB abundance. By the FISH technique *Nitrospira* population was analyzed in samples of Stage I (day 0), Stage IV (day 248), Stage V (day 414), Stage VI (days 458 and 469) and Stage VII (days 518, 602, 668 and 690). The microscopy visualization showed a positive

result in the inoculum sample (Figure S.4) and no positive signal on day 248, indicating that *Nitrospira* were active in Stage I and inactive in Stage IV. The samples visualized from Stage V to Stage VII, when the NOB activity was again observed inside the reactor, showed again presence of *Nitrospira*. Regarding *Nitrobacter spp.*, identified by 16S rRNA gene-based amplicon analysis, the abundance obtained in the analyzed days was lower than 0.015%, while after applying the FISH probe specific for *Nitrobacter spp.*, a negative signal was obtained for the samples analysed throughout the complete operational period. These observations indicate that the developed NOB belong mainly to *Nitrospira* genus outcompeting *Nitrobacter* genus.

4. Discussion

4.1. FNA accumulation as strategy to obtain stable partial nitrification

Among the bacteria involved in the nitrification process AOB are significantly less sensitive to FNA than NOB[23]. In the present study, inhibitory FNA concentration levels for NOB were reached, during the operation of the SBR, due to the synergist effect of the nitrite accumulated in the system and the remained alkalinity.

The concentration of FNA depends on the values of temperature, nitrite concentration and pH (Figure S.5). The pH is the variable with the exponential effect on the concentration of FNA [20]. The temperature is the parameter that affects FNA concentration to a lesser extent. The lower the temperature the higher the FNA concentration achieved, contrary to what occurs with the free ammonia (FA) concentration. In this way, the low temperature of the mainstream (between 5 - 20 °C) plays in favor of the FNA instead of the FA accumulation.

The FNA concentration is linearly dependent on the nitrite concentration, thus to maximize the nitrite concentration inside the partial nitrification unit can be of interest. For this reason, a fraction between the 50 and 100% of the flow of the stream containing ammonium can be nitrified. The fraction of the stream treated in the partial nitrification reactor (containing nitrite or a variable mixture of nitrite and ammonium) will be mixed with the untreated stream (containing only ammonium) to achieve the adequate $\text{NO}_2^-/\text{NH}_4^+$ ratio (1.3 mol/mol) for the subsequent anammox process.

Therefore, once the nitrite is accumulated inside the partial nitrification unit, the remained alkalinity, which determines the pH value, is the key parameter to maintain an appropriate FNA concentration inhibitory for NOB but not for AOB activity (values between 0.02 and 0.42 mg $\text{HNO}_2\text{-N/L}$). Similarly to the case of the temperature, and according to the equations proposed by Anthonisen et al. [20], the lower the pH the higher the FNA concentration, contrary to the response corresponding to FA concentration. As the nitrification process consumes alkalinity, the natural trend of the process is to decrease the pH respect to the feeding. Therefore, to control the partial nitrification process based on the FNA concentration instead of on the FA one can be an advantage.

However, the possible acclimation of NOB to increasing FNA and FA concentrations and the reversibility of the inhibitory effect of both compounds is a drawback to take into account [6]. In the present study, once the partial nitrification was obtained by FNA accumulation its long-term influence was assessed and no acclimation of NOB was detected. These results are in good agreement with Wang et al. [10] who maintained the partial nitrification at the conditions of 22 °C and 57 mg $\text{NH}_4^+\text{-N/L}$ with the inhibition of NOB by FNA for 280 days. Their strategy was based on the generation of FNA inhibitory concentrations in an external unit. As these authors stated, this alternative implies an

optimization of: 1) the sludge fraction treated from the partial nitrification; 2) the contact time with FNA in the external unit; and 3) the frequency of the sludge treatment.

There is no agreement about the synergist effect of inorganic carbon limitation and inhibition caused by free nitrous acid on AOB activity. As an example, Torá et al. [24] found, by respirometric assays performed at 30 °C, that inorganic carbon limitation enhanced the inhibitory effect of FNA on an AOB enriched culture whereas Vadivelu et al. [25] stated the opposite. In the present study, no inhibitory effect on AOB activity was observed. The FNA values obtained (Figure 1.b) were sufficiently high to affect NOB but too low to provoke AOB inhibition, as the maximum FNA level reached was of 0.06 mg HNO₂-N/L. Contrary, Wang et al. [10] applied a FNA (concentrations up to 1.82 mg HNO₂-N/L) based strategy to maintain the partial nitrification process and they observed an inhibition of 32% of the AOB activity. Producing the FNA in the mainstream reactor it is difficult to achieve concentrations as high as those inhibitory for AOB that would limit the nitrification reactor capacity.

The novelty of the strategy proposed in the present research work is the in-situ production of the inhibitory FNA concentration taking the advantage of the natural pH decrease due to the ammonium oxidation process. In this way, two units of the system could be avoided compared with the strategy of the external FNA production proposed by Wang et al. [10].

4.2. Influence of the inlet ammonium to inorganic carbon ratio (NH₄⁺-N/IC)

Contrary to most of the mainstream partial nitrification studies published up to date [8, 10, 26], the strategy proposed in this research work to suppress the NOB activity based on the FNA concentration does not always require a direct pH control. It depends as well on the inlet ammonium to inorganic carbon ratio (NH₄⁺-N/IC). Recently, Wang et al. [10]

also pointed out the importance of the wastewater alkalinity on the effectiveness of the FNA to inhibit the NOB.

Durán et al. [27] also observed that the FNA caused the suppression of NOB from a reactor where the partial nitrification took place at 20 °C and 400 mg $\text{NH}_4^+\text{-N/L}$. They based the operation of this system on the control of the $\text{NH}_4^+\text{-N/IC}$ ratio, which was maintained at values of approximately 0.82 g N/g C, close to the values used in the present study. However, in this case both, temperature and ammonium concentration, were larger than those found in mainstream.

With this in mind four possible scenarios can be described as a function of the $\text{NH}_4^+\text{-N/IC}$ ratio (Table 3), considering that the stoichiometric $\text{NH}_4^+\text{-N/IC}$ inlet ratios needed to oxidize the 100% and 50% of the total incoming ammonium concentration to nitrite are 0.6 and 0.8 g N/g C, respectively.

Table 3. Treatment strategies in the partial nitrification (PN) unit depending on the influent $\text{NH}_4^+\text{-N/IC}$ ratio.

Case	$\text{NH}_4^+\text{-N/IC}$ ratio (g N/g IC)	Stream to PN unit (%)	Ammonium oxidized to nitrite (%)	Action required
A	<0.6	50	100	Bypass and pH control
B	0.6 - 0.8	50 – 100	50 - 100	Bypass to anammox unit
C	0.8 - 1.0	100	50	None
D	> 1.0	100	50	Alkalinity supply

If the $\text{NH}_4^+\text{-N/IC}$ ratio is lower than 0.6 g N/g IC (case A: Stages I, II and V) there is an excess of alkalinity for the partial nitrification process and the 100% of the inlet ammonium content can be oxidized to nitrite. The pH decrease associated to the partial nitrification process is not enough to reach FNA inhibitory levels. In this case, a pH control inside the

reactor is required to maintain the partial nitrification process stability, but it is performed in only half of the total flow. The untreated 50% of the flow is bypassed directly to the subsequent anammox reactor.

Isanta et al. [26] and Reino et al. [8] achieved and maintained a stable partial nitrification at 70 mg $\text{NH}_4^+\text{-N/L}$ and with a ratio of 0.62 g N/g IC for more than 400 days (at 12.5 °C) and for more 250 days (at 10°C), respectively. In both studies the pH was controlled at a value of 8 and they applied the same strategy based on the control of dissolved oxygen/total ammonium nitrogen (DO/TAN) concentrations ratio. This strategy appears as more appropriated to treat effluents with large alkalinity contents as pH is high enough to favour the FA accumulation.

In the case of $\text{NH}_4^+\text{-N/IC}$ ratios between 0.6 and 0.8 g N/g IC (case B) the inlet ammonium oxidized to nitrite can vary between the 100% and 50%, respectively. The percentage of ammonium oxidized will determine the fraction of the flow stream treated in the partial nitrification unit and the fraction bypassed to the anammox reactor. Wang et al. [10] applied a FNA-based strategy using synthetic wastewater characterized by a $\text{NH}_4^+\text{-N/IC}$ ratio of 0.62 g N/g IC, and they observed the oxidation of the total incoming ammonium to nitrite. Later, these authors limit the ammonium oxidation to 50% by reducing the DO level and controlling the aeration duration as alternative to bypassed part of the inflow stream.

If the $\text{NH}_4^+\text{-N/IC}$ ratio is between 0.8 and 1.0 g N/g C (case C: Stages III, IV, VI and VII), the alkalinity value is adequate to partially oxidize the ammonium to nitrite and to obtain an effluent with an $\text{NH}_4^+/\text{NO}_2^-$ molar ratio of 1.3, suitable to be treated in subsequent anammox unit. In both cases, the 100% of the stream flow enters the partial nitrification unit, and the 50% of the ammonium content is oxidized to nitrite.

Finally, when the $\text{NH}_4^+\text{-N/IC}$ ratio is higher than 1.0 g N/g C there is not enough alkalinity for the partial nitrification process, and external alkalinity supply is required to reach a ratio around 1 g N/g C.

Additionally, the sensitivity analysis (Figure S.6) indicated that at $\text{NH}_4^+\text{-N/IC}$ ratio of 0.6 g N/g IC the maximum FNA concentration is achieved. At values lower than this ratio the remaining excess of alkalinity causes the increase of pH (up to 8.4) making the FNA concentration negligible. At values higher than 0.6 g N/g IC, the alkalinity is limited and the estimated pH value is approximately 5.6, which allowed the obtaining of inhibitory FNA concentrations over 0.02 mg $\text{HNO}_2\text{-N/L}$ (Figure S.6). The lower $\text{NH}_4^+\text{-N/IC}$ ratio (limit to 0.6 g N/g IC) and the higher ammonium concentrations, the higher FNA concentrations. This FNA concentrations can achieved values higher than 0.42 mg/L that might inhibit the AOB activity [23] when the ratio is next to 0.6 g N/g IC and the ammonium concentration is higher than 90 mg $\text{NH}_4^+\text{-N/L}$. At a $\text{NH}_4^+\text{-N/IC}$ ratio of 1.17 g N/ g IC the 50% of the ammonium is oxidized whereas when the $\text{NH}_4^+\text{-N/IC}$ ratio is 1.5 the ammonium oxidation is limited to the 39%.

4.3. Nitrite accumulation activation

To define the appropriate procedure required to start up a partial nitrification system is essential for a feasible application of the proposed FNA strategy.

If the sludge used as inoculum for the partial nitrification unit contains low or negligible NOB activity, during the start-up of the process is possible to accumulate enough nitrite concentration inside the reactor to have FNA inhibitory levels. The direct use of sludge from a partial nitrification unit operated in sidestream conditions or the use of an AOB enriched sludge, without or with low NOB presence, as inoculum would lead to the nitrite accumulation. Then, if the $\text{NH}_4^+\text{-N/IC}$ ratio is adequate the FNA concentrations inhibitory

for NOB can be reached. In a similar way, another strategy relies on starting up the unit at temperatures over 20 °C where AOB activity is favoured and then decrease the temperature. This strategy was used by Isanta et al. [26] who achieved the partial nitrification after 35 days of operation treating 70 mg $\text{NH}_4^+\text{-N/L}$ at temperatures higher than 20 °C and then they moved to mainstream conditions decreasing the temperature.

However, to have available an AOB enriched sludge is not always possible and commonly activated sludge is used as inoculum. In this case, the use of an “activator” of the nitrite accumulation can be a solution, like a specific NOB inhibitor [6]. Wang et al. [28] restored a nitrate build-up in one-stage partial nitrification-anammox reactor by adding hydroxylamine as a NOB specific chemical inhibitor. However, they observed that this effect was reversible when the addition of the inhibitor was stopped.

In the present study, performed at laboratory scale, a NOB inhibitor (sodium azide) acted as “activator” allowing the establishment of the inhibitory FNA concentrations for NOB activity suppression. The sodium azide effects on the anammox activity have been previously assessed in batch experiments providing different results depending on the state of aggregation of the biomass [29]. The batch tests indicated that a concentration of 5 mg $\text{NaN}_3\text{/L}$ (used in the present study) caused a 50% inhibition on the flocculent anammox biomass activity and only a 15% on the granular one. Moreover, this inhibition was reversible in both cases [29]. Although, the application of sodium azide at laboratory scale allowed enhancing the FNA inhibition mechanism, another strategy must be applied to promote the nitrification process at full scale, to avoid the use of sodium azide (due to its toxicity). For example, during Stage VII the nitrite was successfully tested as activator for FNA accumulation with an immediate effect over the NOB activity.

4.4. Effective NOB washout

The microbiological analysis showed that throughout Stages II to IV the NOB (dominated by *Nitrospira* sp.) practically disappeared, which indicates an effective wash out of these bacteria and not only their inhibition by the presence of the FNA. This selective removal of the nitrite oxidizing bacteria provides with some advantages: (1) feasibility to operate the process at a relatively high DO concentration, which is advantageous to achieve a high nitrification rate; and (2) robustness of the stability of the partial nitrification process even operating at conditions favorable for the NOB activity development, because these bacteria are not present inside the reactor (or they are in a minimal concentration). If the operational conditions become adequate for the NOB activity development, for example, a seasonal increase of the wastewater alkalinity, several days will be required to reach a level of NOB activity high enough to destabilize the partial nitrification process. Meanwhile, a corrective action can be implemented to avoid this negative evolution. As an example, in the present study such a destabilization needed approximately 40 days in Stage V when the FNA concentration was decreased. This is also an advantage if a punctual incident occurs during the reactor operation, e.g., an increase of the DO concentration. In this study, despite the DO concentration reached values as high as 6 - 7 mg O₂/L in some operational days of Stage IV (Figure S.1), the nitrite oxidation rate to nitrate was maintained negligible. Isanta et al. [26] found, operating a partial nitrification reactor at mainstream conditions (12.5 °C and 70 mg NH₄⁺-N/L), that an event of high DO concentration (8 mg O₂/L) maintained during few hours, was immediately reflected in the appearance of NOB activity. This was probably because these bacteria were present in enough number and sufficiently active in the reactor to take quick advantage of the favorable operational conditions.

Furthermore, Isanta et al. [26] used an inoculum with *Nitrobacter* and hypothesized that their strategy is not adequate to suppress *Nitrospira*. Despite *Nitrobacter* are considered

the key NOB in nitrifying wastewater units at high nitrogen concentrations [30], the predominant NOB specie in full-scale plants is *Nitrospira* [31, 32]. *Nitrospira* like-NOB was the population suppressed in the present study by FNA. These findings are in agreement with Wang et al. [10] who claimed that FNA inhibition was the main factor responsible for the elimination of *Nitrospira* while DO limitation led to the *Nitrobacter* washout. This fact may be explained as *Nitrobacter* are r-strategists and they are well adapted to high nitrite concentrations and have lower oxygen affinity compared to *Nitrospira* (k-strategists) [32]. *Nitrobacter* were present in minimal abundance from the start-up of the reactor but they were outcompeted by *Nitrospira* (Figure S.3).

Conclusions

- Long term partial nitrification at mainstream conditions, low temperature and low nitrogen concentration (16 °C and 50 mg $\text{NH}_4^+\text{-N/L}$), was established and maintained, in a suspended biomass SBR without dissolved oxygen concentration control.
- The promotion of FNA concentrations higher than 0.02 mg $\text{HNO}_2\text{-N/L}$ inside the reactor allowed maintaining the partial nitrification process stable and washing out effectively the NOB (*Nitrospira*). Therefore, if the operational conditions became the adequate for NOB development, they will last several weeks in to have significant activity inside the reactor. However, if the operational conditions favour the NOB activity more than 40 days are required to detect significant nitrate concentrations.
- The addition of a specific NOB inhibitor (sodium azide at 5 mg/L) or nitrite enables the start-up of the nitrite accumulation to produce FNA in-situ, without affecting the AOB activity. However, the use of sodium azide at full scale is not feasible.

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