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Understanding the microbial trends in a nitritation reactor fed with primary settled municipal wastewater

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Abstract

Partial nitritation was pointed out as the key step to implement the autotrophic nitrogen removal processes at low temperature. This study investigated the initiation and maintenance of a nitritation process with simultaneous COD removal in a sequencing batch reactor (SBR) run at 15 °C and fed with primary settled urban wastewater characterized by 42 ± 10 mg DOC/L and 45 ± 4 mg NH_4^+-N/L. A nitrite accumulation ratio of nearly 100 % was observed and the long-term (354 days) process stability was successfully maintained despite the municipal wastewater composition fluctuations. The absence of nitrite oxidizing bacteria (NOB) activity was attributed to the free nitrous acid (FNA) *in-situ* accumulated at high levels (0.02 – 0.20 mg $HNO_{2\ A}$-N/L). Despite nitrate production was not observed, the quantification of bacterial groups indicated that NOB were present in the SBR sludge throughout the entire operational period. Ammonium oxidizing bacteria (AOB) abundance and community structure were significantly influenced by the organic matter present in the feeding. Average organic matter removal efficiencies of 80 % were obtained without observing any detrimental effect over the nitritation process performance, due to the functional redundancy within both the chemoheterotrophic and AOB communities.

Keywords: autotrophic nitrogen removal; free nitrous acid; low temperature; mainstream; nitrite oxidation inhibition.

1. Introduction

The combination of the partial nitritation (PN) and anammox (AMX) processes is one of the most promising alternatives for the fully autotrophic removal of nitrogen from wastewater [1, 2]. Until now, PN/AMX technologies have been successfully implemented for the treatment of the reject waters of anaerobic sludge digesters in wastewater treatment plants (WWTPs), reducing the nitrogen load recirculated back to the mainstream. Driven by the outstanding economic and environmental associate benefits of the PN/AMX implementation, multiple efforts are focused on its application to the mainstream of WWTPs. When PN/AMX processes are applied for municipal wastewater treatment, the concentrations of COD which are commonly present (7 - 12 g COD/g N after primary settling) represent a shortcoming [2, 3]. COD supports the development of aerobic heterotrophic bacteria, which compete with nitrifying bacteria for dissolved oxygen (DO), hindering the establishment of a steady-state autotrophic process. Moreover, nitrite oxidizing bacteria (NOB) activity suppression has been widely reported as one of the weakest points to achieve the nitritation process stability treating municipal wastewater (< 100 mg N/L) at low temperature (< 25 °C).

One of the most widely applied strategies to develop the nitritation process in a single unit relies on controlling the duration of the aerobic reaction phase based on the ammonia valley. In sequencing batch reactors (SBRs), as soon as ammonium oxidation was complete, an inflexion point (ammonia valley) in the pH profile was observed, since the oxidation of the nitrite to nitrate consumes less alkalinity [4]. The change from negative to positive in the value of dpH/dt was used to terminate the aeration. This strategy was successfully applied at low temperature, achieving a nitrite accumulation ratio (NAR) higher than 95 % [5] even at pilot-scale [4, 6]. However, if the alkalinity is limited to oxidize all the ammonium (i.e. nitrogen to inorganic carbon (N/IC) ratios higher than 0.6 g N/g IC) the pH will only decrease, and no ammonia valley will take place.

Among other strategies to implement the nitritation process under mainstream conditions, the feasibility of those based on the higher sensitivity of NOB to free nitrous acid (FNA) concentrations compared to ammonia oxidizing bacteria (AOB) stands out. NOB are completely inhibited at FNA concentrations of 0.023 mg HNO_2-N/L, whereas 0.4 mg HNO_2-N/L results in 50 % suppression of the AOB activity [7]. This approach was explored by exposing the sludge from the nitritation reactor to inhibitory FNA concentrations in an external unit [8-10], or producing it *in-situ* inside the nitritation reactor [11, 12]. As an advantage, the *in-situ* production of the FNA does not require pH adjustment nor sludge pumping to an external unit, reducing the sludge mechanical stress. Moreover, more extreme conditions are applied when the external unit is used that might also cause AOB activity reduction. However, these strategies based on the biocidal effect of FNA over NOB have been only tested treating mineral medium mimicking municipal wastewater [11, 12].

The success of the strategy proposed by Pedrouso, et al. [11] relies on the limitation of the N/IC ratio. This ratio must be theoretically higher than 0.6 g N/g IC in the wastewater fed to the nitritation reactor to maintain long-term process stability. The wastewater alkalinity depends mostly on the local source of freshwater. In this sense, it is necessary to corroborate if this strategy is adequate to be applied to municipal wastewater treatment.

Hence, the present study aims at demonstrating the feasibility of achieving and maintaining the long-term stability of a PN process by producing *in-situ* FNA in the presence of organic matter oxidation, in order to treat primary-settled municipal wastewater at low temperature (15 °C). The reactor performance and specific bacterial activities were monitored to evaluate the process stability. Furthermore, microbial community analyses were performed to understand how the changes of the operational parameters influenced bacterial community

structure and population dynamics as well as to corroborate if the operational strategy fosters AOB predominance over NOB.

2. Materials and Methods

2.1. Reactor setup and operational conditions

A 2-L nitritation reactor was operated as an SBR at 15 ± 1 °C and the volume exchange ratio was fixed at 50 %. Air was supplied, and manually regulated, to the SBR through an air pump (KNF Labport) coupled to a coarse bubble diffuser to guarantee the complete mixture and oxygen supply for the biological reactions. Neither DO concentration nor pH value were controlled and ranged from 0.5 – 7.5 mg O_2/L and 5.4 – 7.5, respectively (See Figure S1 in Supporting Material).

The operational period lasted 354 days divided into four Stages. In Stage I, synthetic medium was fed to the reactor containing 50 mg NH_4^+-N [11]. Afterwards, primary settled municipal wastewater was fed during Stages II - IV (Table 1). At the end of Stage II, the sludge from the SBR was anoxically stored in the reactor medium at 4 °C for 25 days, and it was used in Stage III to restart the nitritation unit. Finally, in Stage IV, the applied organic matter and nitrogen loads decreased compared to previous stages, due to the decrease of those component concentrations in the used municipal wastewater as it was collected during rainy periods (winter time).

The SBR was operated using three different cycle distributions (See details in Figure S2, Supporting Material) controlled by a programmable logic controller (Siemens S7-224CPU). From day 0 to 142, the 3-hour cycle was distributed as 158 min of feeding and aeration, 20 min of settling and 2 min of withdrawal. On day 143, the cycle configuration was modified, and the aerated feeding lasted for 60 min, while 98 min were used just for aeration to decouple organic matter oxidation from the nitritation process. Finally, on day 334, the cycle length was

reduced from 180 min (8 cycles/day) to 144 min (10 cycles/day), shortening the hydraulic retention time (HRT) from 6 h to 4.8 h.

Table 1. Feeding characteristics during the different operational stages.

Stage	Days	TN (mg N/L)	pH	IC (g IC/L)	NH_4^+-N/IC (g N/g IC)	TOC (mg/L)
S-I	0 – 137	50 ± 3	7.70 ± 0.10	55 ± 6	0.89 ± 0.02	-
S-II	138 – 182	33 ± 2	6.92 ± 0.09	41 ± 7	0.80 ± 0.05	40 ± 4
S-III	207 - 310	45 ± 10	7.20 ± 0.25	67 ± 8	0.68 ± 0.08	45 ± 9
S- IV	311- 354	20 ± 1	7.01 ± 0.09	26 ± 2	0.61 ± 0.02	22 ± 3

IC: Inorganic carbon; TN: total nitrogen; TOC: total organic carbon.

The SBR was seeded with 2.2 g VSS/L of suspended sludge from a PN/AMX reactor that treated industrial saline wastewater (ca. 18 g NaCl/L) at 30 °C [13]. The measured maximum specific activity (SA), at 15 °C, of AOB (SA_{AOB}) was 11 ± 2 mg NH_4^+-N/(g VSS·d), whereas NOB one (SA_{NOB}) was not detected.

2.2. Analytical methods

DO concentration and temperature in the bulk liquid were continuously measured (LDO HQ40d, Hach Lange). Influent and effluent samples were periodically taken from the SBR. pH values were determined with a Hach Sension$^+$ meter, and then, samples were filtered through 0.45 µm pore size filters before analysis. Spectrophotometric methods were used to determine the ammonium [14], nitrite and nitrate [15] concentrations. Total organic and inorganic carbon (TOC and IC) concentrations were measured with a Shimadzu analyzer (TOC-L CSN, Shimadzu). Total nitrogen (TN) concentration was also measured in the TOC-L analyzer coupled with a TNM-L Unit. The concentrations of total and volatile suspended solids (TSS and VSS) in samples

collected from the reactor and its effluent were determined according to Standard Methods [15]. Single operational cycles were also monitored in specific operational days to evaluate the evolution of the concentrations of the different compounds inside the reactor. Respirometric batch tests were periodically carried out to assess the SA following the methodology described by Lopez-Fiuza, et al. [16] and using a biological oxygen monitor (BOM, YSI Inc. model 5300). SA_{AOB} and SA_{NOB}, as well as the aerobic heterotrophic activity (SA_{aerHET}) when organic matter was fed to the SBR, were determined, in triplicates, at 15 °C. Equations for the FNA, NAR and ammonium oxidation ratio (AOR) calculations are included in Supporting Material.

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

2.3.1. DNA extraction and purification of sludge samples for qPCR assays

Samples were retrieved from the SBR after 138, 147, 153, 161, 166, 171, 231 and 266 days of operation (Stages II and III) for microbiological analyses. The samples were centrifuged (14,500 × g, 1 min), and the sediments were frozen and kept at -20 °C. DNA was extracted from the sedimented samples and purified using the FastDNA-2 ml SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO), following already described methods [17]. The quality and concentration of the DNA were measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Waltham). DNA samples were stored at -20°C.

2.3.2. qPCR assays

Total bacteria, ammonia oxidizing *Betaproteobacteria* (AOB), NOB (*Nitrospira* spp.) and AMX bacteria were quantified in the SBR samples by qPCR, using the molecular gene markers and primers summarized in Table S1A. Quantitative amplifications were performed in accordance with the MIQE guidelines [18], using True Start Hot Start DNA polymerase (Thermo Scientific) and SYBR Green I (Sigma Aldrich) in a total volume of 25 µl, with the aid of a QuantStudio-3 Real-Time PCR system (Applied Biosystems). Previously described protocols were followed

156 [19]. The particular amplification conditions used for each reaction are summarized in Table
157 S1B. For the absolute quantification of the target microbial groups, standard curves were
158 constructed using a series of tenfold dilutions (10^{-1}-10^{-8}) of linearized plasmids carrying inserts
159 of the target genes. Details on the plasmid standards used are provided in Supporting Material.
160 All quantitative amplifications were performed in triplicates.

161 **2.4. Illumina Miseq sequencing, DNA data processing and analysis**

162 Amplification of the V3-V4 region of the 16S rRNA gene was performed using the prokaryotic
163 specific-primers Pro341F/Pro805R [20]. Raw data from Illumina sequencing process were
164 analyzed using the Mothur MiSeq-pipeline and software mothur v1.43.0 [21]. The
165 methodologies followed for alignment, OTU clustering and taxonomic classification are fully
166 detailed in Supporting Material. The BLASTn suite (http://blast.ncbi.nlm.nih.gov) was used for
167 manual sequence similarity analysis of AOB and NOB-related OTUs. Phylogenetic and
168 molecular evolutionary analyses were conducted using MEGA version X [22]. A p-distance-
169 based evolutionary tree was inferred from the partial 16S rDNA gene sequences using the
170 Neighbor-Joining algorithm. A bootstrap test was conducted to infer the reliability of branch
171 order, with a round of 1000 reassemblings. The OTUs sequences were deposited in the NCBI
172 database under the accession number
173 (https://submit.ncbi.nlm.nih.gov/subs/genbank/SUB7677265).

174 **2.5. Statistical analysis**

175 The changes of the absolute abundances of the four targeted microbial groups throughout the
176 sampling period were statistically analyzed using *IBM* SPSS Statistics v. 21 (SPSS Inc., USA) and
177 R (R studio team, 2016; http://www.r-project.org/). The Kruskal-Wallis and Conover-Iman non-
178 parametric tests ($p<0.05$ significance level) were used to search for differences among the
179 groups of samples since the Shapiro-Wilk test indicated that the majority of the data sets did

not fit the normal distribution. The Primer software (PRIMER-E v. 6.0, Plymouth, UK) was used for the multivariate statistical analyses, following the protocols fully described elsewhere [17], and detailed in Supporting material.

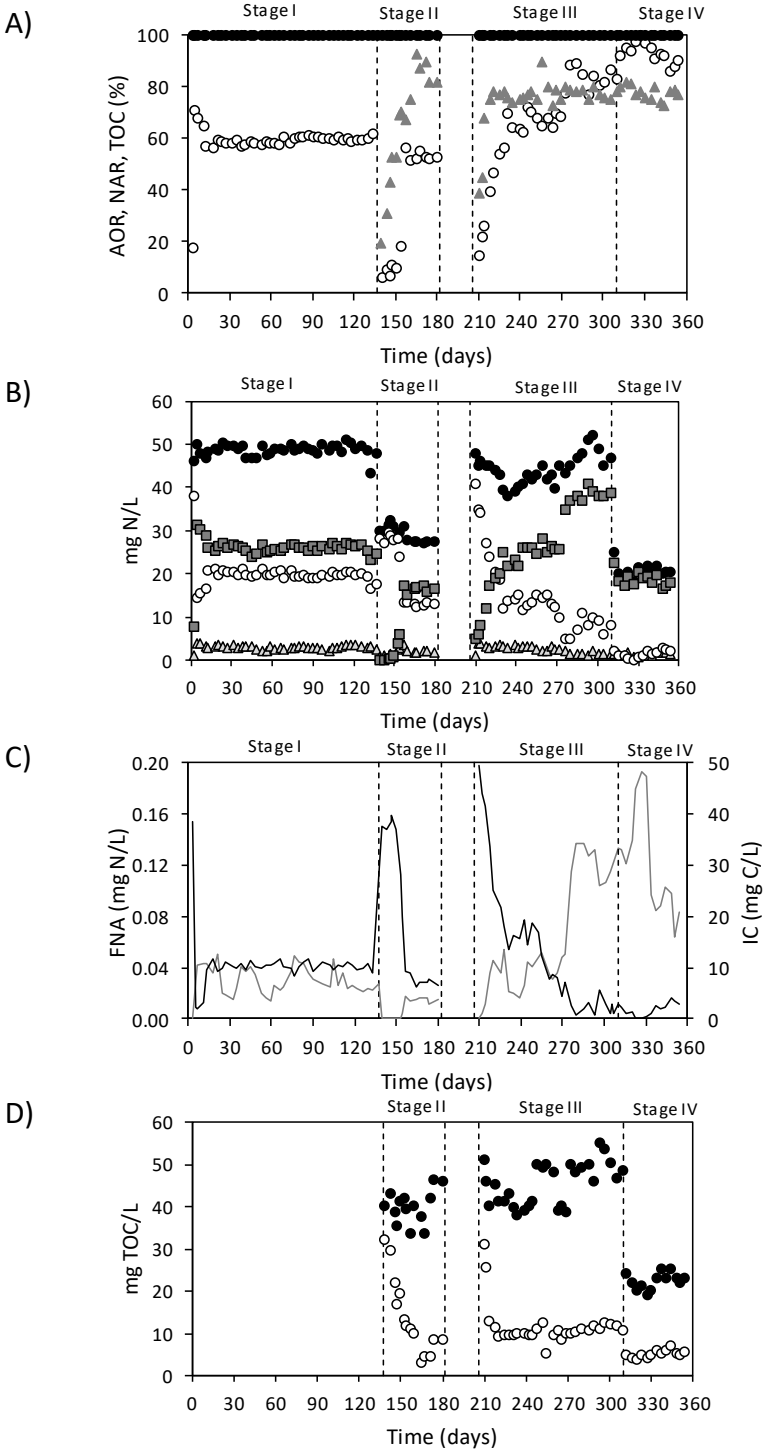
3. Results and discussion

3.1. Nitritation establishment by natural *in-situ* FNA accumulation

The SBR operated for 354 days fed with low ammonium concentrations (< 50 mg NH_4^+-N/L) and at 15 °C (Figure 1). In Stage I, despite the initial low SA_{AOB} of the inoculated biomass (Figure 2), the nitritation process was established fast. The AOR was already 20 % on day 3 and reached 60 % after 5 days of operation (Figure 1.A). Then, the nitritation process was successfully maintained with negligible nitrate production leading to a NAR of 100 % (Figure 1). Nitrate concentration in the effluent was already present in the influent. Despite IC in the effluent was not depleted (10 ± 2 mg IC/L, Figure 1.C) it was close to the alkalinity affinity constant value of 6 mg IC/L, commonly considered in the activated sludge modelling [23]. Therefore, AOR could be either partially limited by the alkalinity concentration, by the DO concentration (Figure S1) and/or the cycle duration.

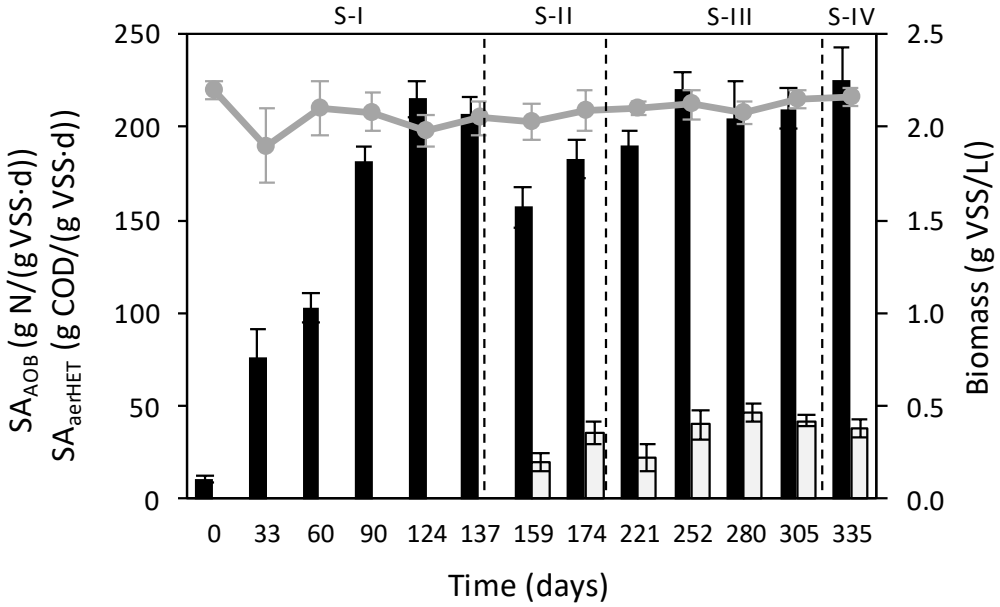
In this study, the nitritation process was successfully started-up using an inoculum with negligible SA_{NOB} without requiring any additional action. Although the inhibition caused on the SA_{NOB} by salt concentrations above 4 - 8 g NaCl/L has been reported to be reversible [24, 25], NOB activity remained suppressed in the present nitritation reactor due to the *in-situ* FNA accumulation at concentrations ranging from 0.02 to 0.08 mg HNO_2-N/L (Figure 1.C).

The progressive increase of the AOB activity during Stage I was confirmed by batch tests measuring SA_{AOB} up to 207 ± 9 mg NH_4^+-N/(g VSS·d), while SA_{NOB} was never detected (Figure 2).



203 Figure 1. Evolution of: A) Nitrite accumulation ratio (NAR, ●), ammonium oxidation ratio (AOR, ○) and
204 total organic carbon (TOC) oxidation ratio (▲), expressed in percentages; B) Concentrations of NH_4^+ in
205 the influent (●) and NH_4^+ (○), NO_2^- (■) and NO_3^- (Δ) in the effluent, expressed in mg N/L; C) Free nitrous
206 acid (FNA) concentration (–), in mg HNO_2-N/L, and inorganic carbon (IC) concentration (–), expressed as
207 mg IC/L; D) TOC concentration in the influent (●) and effluent (○) in mg TOC/L..

208 Pedrouso, et al. [11] already demonstrated the NOB suppression by *in-situ* FNA production
209 using a seeding sludge with relatively high SA_{NOB} and an initial NOB chemical inhibitor. These
210 authors hypothesized that when SA_{AOB} is higher than SA_{NOB}, it is possible to maintain the
211 nitritation process without chemicals addition as it was proven in the present study. In these
212 conditions, nitrite accumulates in the system, which leads to FNA inhibitory concentrations (if
213 the ratio of N/IC ranged from 0.6 to 1.0 g N/ g IC), simplifying the start-up of the nitritation
214 process.



215
216 Figure 2. Evolution of the maximum specific ammonium oxidizing bacteria activity (SA_{AOB}, ■) and specific
217 aerobic heterotrophic activity (SA_{aerHET}, □) in batch tests, and biomass concentration inside the
218 nitritation reactor (●) during the different Stages (S). Note that SA_{NOB} was also determined but it was
219 under the detection level of the measurement method during the whole operational period.

220 **3.2. Performance of the nitritation and organic matter oxidation in the same unit**

221 Primary settled municipal wastewater was fed to the SBR in Stage II (Table 1). This change in
222 the feeding provoked that, initially, the DO concentration raised from average values of 2.5 mg
223 O_2/L to 6 mg O_2/L (Figure S1) as it was not consumed by AOB (no ammonium oxidation
224 occurred) either by heterotrophic bacteria due to their limited presence. On day 143, the SBR

operational cycle configuration was modified shortening the feeding phase to first promote the organic matter oxidation and once TOC concentration is low enable the ammonium oxidation (Figure S2). 15 days later, over 70 % of the incoming TOC was removed and approximately 50 % of the ammonium was oxidized to nitrite (Figure 1). The lower IC consumption led to higher pH values in the effluent compared to Stage I (Figure 1 and S1). The higher pH value and lower nitrite production caused the FNA concentration decrease close to the reported NOB inhibition values of 0.02 mg HNO_2-N/L (Figure 1.C).

Despite the SBR operated for 44 days at FNA concentrations under the inhibitory values, NAR remained at 100 % (Figure 1.A) and SA_{NOB} was not detected (Figure 2). These results confirm the robustness of the *in-situ* FNA strategy and are in good agreement with Pedrouso, et al. [11] that also observed a delay of more than 40 days to detect SA_{NOB} when FNA concentrations fell below inhibitory values. Contrary, Duan, et al. [9] with an *ex-situ* FNA sludge treatment observed that NOB activity recovered as soon as the FNA treatment was stopped and despite the sludge treatment was restarted, the NAR recovery was limited to 30 %. One possible explanation is the fact that in the *ex-situ* treatment strategy, only a sludge fraction is exposed to the FNA in a limited period of time, while with the *in-situ* approach, sludge is continuously in contact with the FNA produced.

At the beginning of Stage II, when the organic matter started to be fed, the SA_{AOB} decreased from 207 ± 9 to 157 ± 11 mg NH_4^+-N/(g VSS·d) and later it was partially recovered (Figure 2). In the meanwhile, the SA_{aerHET} reached values of 35 ± 6 mg COD/(g VSS·d) (Figure 2). Moreover, the biomass concentration inside the SBR remained at values of approximately 2 g VSS/L (Figure 2), while the effluent one slightly increased but it remained under 15 mg VSS/L (it was 6 mg VSS/L in Stage I) resulting in solid retention times (SRT) decrease from 80 -100 days to 20 - 30 days.

3.3. Recovery of oxidation processes after anoxic starvation

In Stage III, the SBR was restarted after 25 days stopped and it took approximately 12 days to recover the TOC oxidation of 70 % (Figure 1.A). Once TOC removal was re-established, the AOR rose exponentially and stabilized at 60 % after 6 days (day 225) without nitrate production (Figure 1.A and B). Nan, et al. [26] observed, from batch tests, that starvation periods (of 5 days before or 12 hours after the FNA sludge treatment) enhanced the resistance of NOB to FNA. However, the starvation period before Stage III did not seem to affect the NOB suppression in the present study negatively.

The incoming N/IC ratio decreased from Stage II (0.80 ± 0.05 g N/g IC) to Stage III (0.68 ± 0.08 g N/g IC) (Table 1) and average pH values decreased to 6.1 ± 0.2 (reaching minimum values of 5.8, Figure S1). The lower pH value, combined with the higher nitrite concentration reached in the effluent (Figure 1.B), caused the increase of the FNA concentration to average values of 0.06 ± 0.04 mg HNO_2-N/L and up to 0.13 mg HNO_2-N/L (Figure 1.C), much higher than the NOB inhibitory threshold [7]. The high FNA values did not apparently affect the AOB activity and the SA_{AOB} average values increased to 210 mg NH_4^+-N/(g VSS·d) (Figure 2).

During Stage III, the AOR further increased from the average 67 ± 7 % (days 225 - 276) to 85 ± 3 %. On days 225 - 276, the AOR could be limited by the reaction fixed time and/or by the operational conditions imposed, such as the aeration flow rate. At the end of this Stage, the average N/IC ratio of 0.68 g N/g IC (Table 1) enables the oxidation of approximately 85 % (close to the obtained values). Therefore, the AOR was limited by the almost fully depleted IC concentration (Figure 1.C).

To deeply evaluate the nitrogen conversion in the reactor, on day 266 one SBR operational cycle was monitored (Figure 3.A and B). Once the feeding phase ended, pH, ammonium and IC concentrations dropped following a similar pattern. Consequently, nitrite concentration rose

from 14 to 30 mg NO_2^--N/L leading to the *in-situ* FNA accumulation. The FNA concentrations were higher than 0.02 mg HNO_2-N/L only in the last 40 min of the cycle (Figure 3.B). Even in these conditions, nitrate production was not detected. The IC was almost entirely consumed with 2 mg IC/L (Figure 3.A) at the end of the cycle.

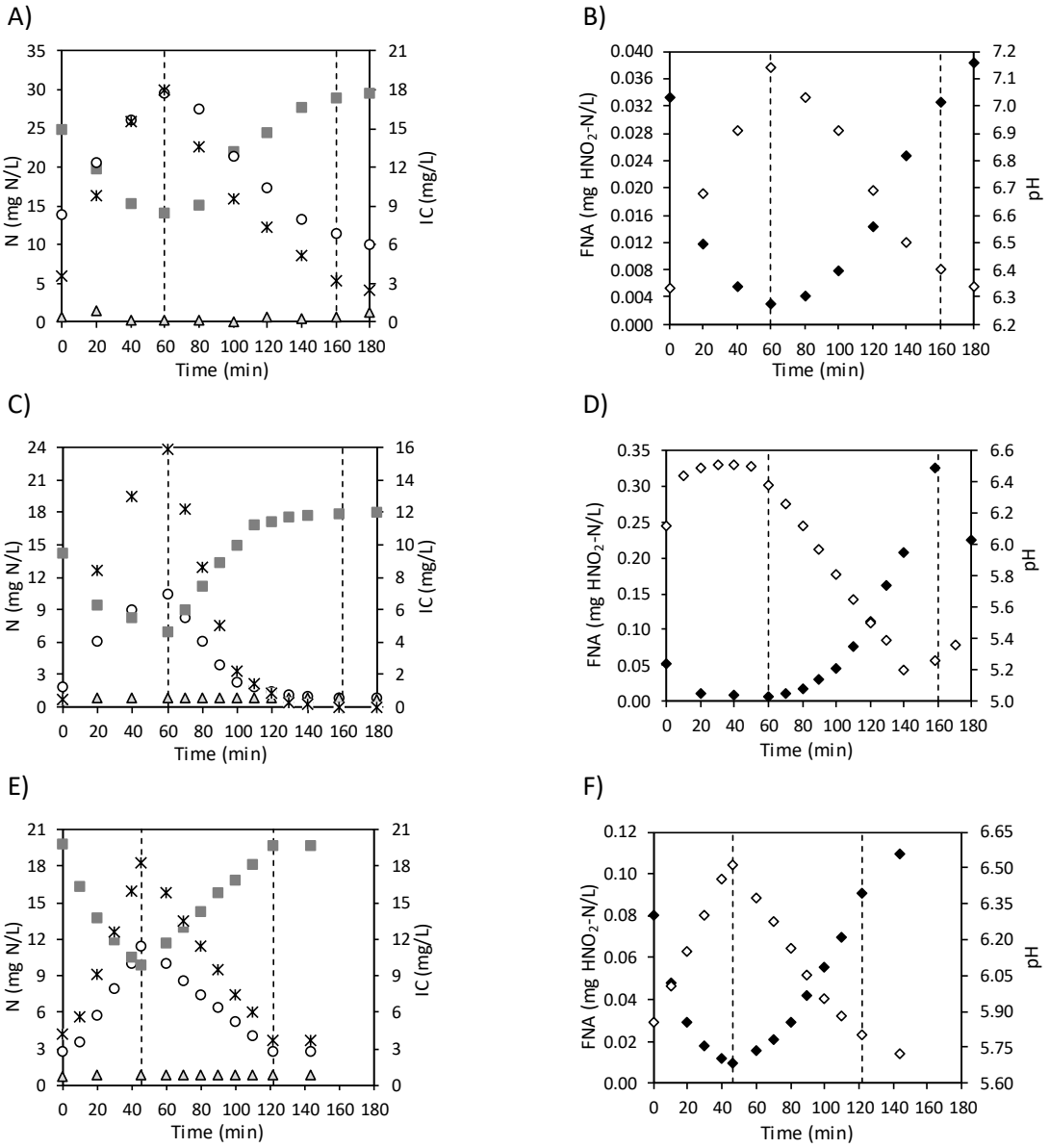


Figure 3. SBR cycle characterization on day 266 (Stage III) (A, B) and on day 331 (C, D) and day 345 (E, F) (Stage IV). A, C, E) Evolution of ammonium (○), nitrite (■) and nitrate (Δ) concentrations expressed as mg N/L and inorganic carbon (IC) concentration as mg IC/L (*). B, D, F) Evolution of the pH value (◊) and FNA concentration (♦) expressed as mg HNO_2 -N/L.

3.4. Optimization of the operational cycle to treat very low nitrogen concentrations

In Stage IV, diluted wastewater was fed to the system characterized by a N/IC ratio was 0.61 ± 0.02 g N/g IC (Table 1), close to the stoichiometric ratio of 0.59 g N/g IC required for the complete oxidation of ammonium [11]. Thus, both ammonium and IC were almost entirely consumed (Figure 1). Due to the lack of enough alkalinity to buffer the system, pH values as low as 5.3 were obtained and in turn, the FNA concentration rose up to 0.20 mg HNO_2-N/L.

The cycle monitored on day 331 (Figure 3.C and D) proved that ammonium and IC were consumed before the end of the reaction period. As IC concentration decreased, the pH value sharply dropped. Thus, the FNA concentration augmented to values higher than 0.30 mg HNO_2-N/L that might partially inhibit the AOB activity. According to Zhou, et al. [7], 0.40 mg HNO_2-N/L resulted in a 50 % suppression of the AOB activity, whereas Jiang, et al. [27] observed the 57 % of the AOB activity was loss by exposing the sludge to FNA concentrations of 1.2 mg HNO_2-N/L. Although, as far as alkalinity is available, full ammonium abatement is not recommended and concentrations of 2 - 5 mg NH_4^+-N/L should remain to avoid limiting the AOB growth rate [28]. Contrary to the observed continuous reactors, in SBR systems, this effect should not be relevant since, from the major part of the cycle, higher ammonium concentrations were present (Figure 3.C).

To avoid part of the cycle that is inactive (ammonium is already oxidized), on day 334, the cycle length was shortened to 144 min (Figure S2). By doing this, the average pH values slightly increased (from 5.56 ± 0.10 to 5.79 ± 0.06; Figure S1) and the AOR decreased, obtaining ammonium concentrations around 2 - 3 mg NH_4^+-N/L in the effluent. This indicated that the reaction phase adjusted to the duration of the cycle (Figure 1 and 3) confirmed by the cycle characterization performed on day 345 that leads to a similar pattern to that observed in Stage III (Figure 3 E, F) with FNA concentration up to 0.11 mg HNO_2-N/L.

The average biomass concentration inside the reactor remained at 2 g VSS/L (Figure 2). The SA_{AOB} was over 200 mg NH_4^+-N/(g VSS·d) (Figure 2) which indicated that, despite the presence of organic matter, the ammonium oxidation capacity inside the reactor was 400 mg N/(L·d). Considering these results, the HRT might be further reduced, increasing the applied nitrogen load that was approximately 150 ± 42 mg N/(L·d).

3.5. Quantification of microbial groups by quantitative PCR (qPCR) and links to operational parameters in the SBR

The absolute abundances of gene markers of total Bacteria, AOB, NOB and AMX in samples retrieved from the SBR are displayed in Figure 4. The Kruskal-Wallis and Conover-Iman tests showed that there were significant differences among the samplings ($p < 0.05$) for all the microbial groups quantified.

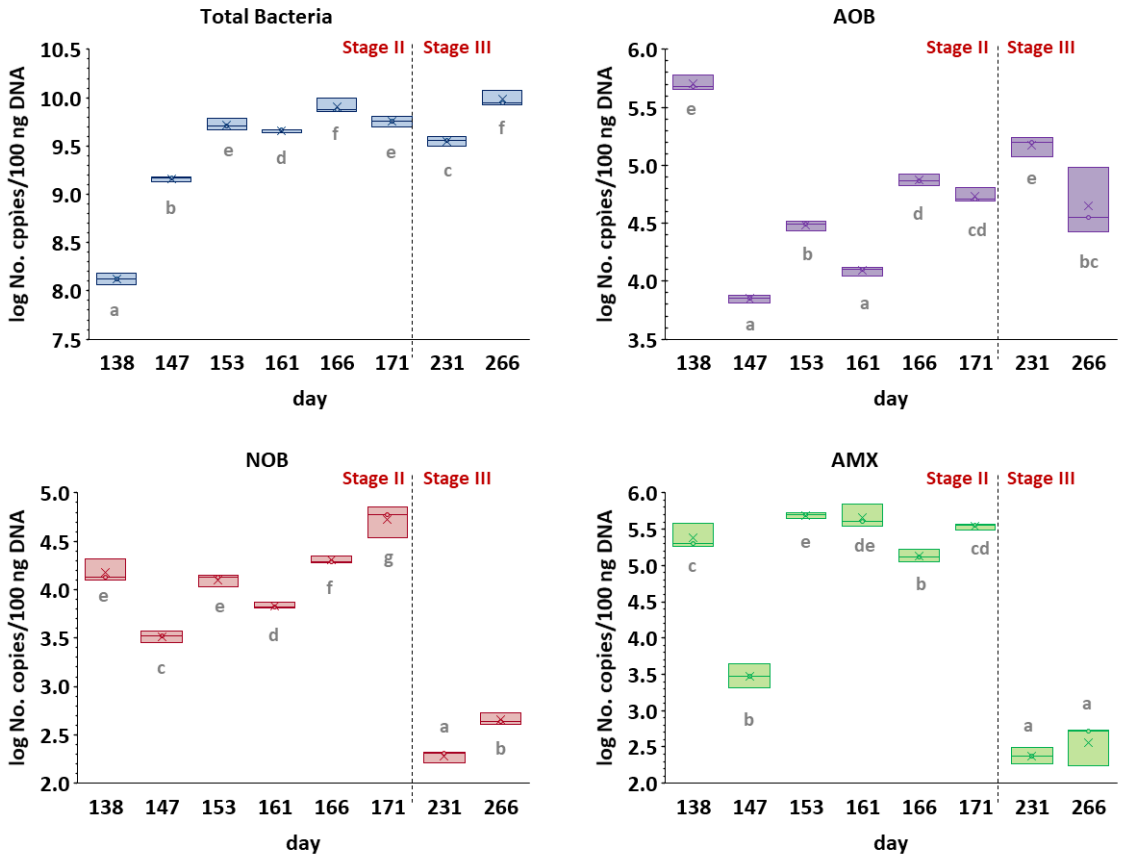
At day 138, when the SBR started being fed with primary settled municipal wastewater, the average abundance of gene markers of total Bacteria was $1.33\ 10^8 \pm 1.95\ 10^7$ copies/100 ng DNA, which increased steadily for the next 15 days reaching $5.31\ 10^9 \pm 7.87\ 10^8$ copies/100 ng DNA on day 153, and then remained without differences until the end of Stage II (Figure 4). Simultaneously, the average abundance of copies of the *amoA* gene abruptly decreased between days 138 and 147 (*ca.* 2 orders of magnitude), then recovered progressively up to day 166, maintaining a similar abundance by the end of Stage II (day 171) (Figure 4).

The shifts of the abundance of both total Bacteria and AOB were in accordance with the observed changes in the SBR system performance. Following the change of the feeding on day 138, ammonia oxidation halted, leading to a severe reduction of the size of the AOB community, but the subsequent adjustment of the operational cycle configuration on day 143 allowed for the successful adaptation of the community to achieve organic matter and ammonia oxidation in the same unit (section 3.2, Figure 1A). The observed patterns were

329 consistent with the previous knowledge on the response of nitrifiers to organic matter in
330 WWTPs [29].

331 Regarding the samples analyzed during Stage III (days 231 and 266), the abundance of both
332 Bacteria and AOB were similar or slightly higher than those measured at the end of Stage II
333 (Figure 4), in agreement with the performance of organic carbon removal and AOR (76 - 79 %
334 and 69 - 79 %, respectively, Figure 1A), and indicating the full recovery of both functional
335 communities 20 days after the anoxic starvation step. AOB display a variety of physiological
336 traits and molecular mechanisms advantageous for their survival under the limitation of
337 ammonia or DO, which compensate for their lower growth rates compared to most
338 chemoorganoheterotrophic bacteria [30], enabling them to regain activity in a short time after
339 starvation periods. The abundance of gene markers of NOB was maintained in the range of 10^3
340 - 10^4 copies/100 ng DNA throughout Stage II (Figure 4), but their activity was negligible in the
341 SBR operation (Figure 1) and according to the respirometric assays (Figure 2). In contrast with
342 the results observed for total Bacteria and AOB, the quantifications of NOB in the two samples
343 of Stage III rendered values close to the limit of detection of the qPCR method. Several studies
344 have previously reported that NOB are more sensitive than AOB to starvation and take longer
345 to recover their metabolic activity after addition of substrates [31, 32]. The difference of the
346 activity recovery rates among AOB and NOB has been related with a delayed transcriptional
347 response of the *nxrB* genes to the addition of substrates, which require longer periods than
348 the *amoA* genes, leading to the subsequent dominance of AOB over NOB populations [33].
349 Nonetheless, nitrification gene markers were quantified in the SBR until day 266, 59 days after
350 the restart of the system, which are nearly twofold the recovery periods reported previously
351 for NOB [31]. In this sense, the FNA concentrations measured in this period, falling within the
352 range reported to 100 % inhibit nitrite oxidation, could induce the wash-out of NOB in
353 nitritation systems [7].

A



B

day	Total bacteria	AOB	NOB	AMX
138	$1.33\ 10^{8} \pm 1.95\ 10^{7}$	$5.12\ 10^{5} \pm 8.30\ 10^{4}$	$1.55\ 10^{4} \pm 4.45\ 10^{3}$	$2.55\ 10^{5} \pm 1.09\ 10^{5}$
147	$1.44\ 10^{9} \pm 8.92\ 10^{7}$	$7.09\ 10^{3} \pm 5.36\ 10^{2}$	$3.29\ 10^{3} \pm 4.39\ 10^{2}$	$3.13\ 10^{3} \pm 1.16\ 10^{3}$
153	$5.31\ 10^{9} \pm 7.87\ 10^{8}$	$3.05\ 10^{4} \pm 2.85\ 10^{3}$	$1.28\ 10^{4} \pm 1.86\ 10^{3}$	$4.89\ 10^{5} \pm 3.98\ 10^{4}$
161	$4.54\ 10^{9} \pm 1.81\ 10^{8}$	$1.24\ 10^{4} \pm 1.10\ 10^{3}$	$6.82\ 10^{3} \pm 5.55\ 10^{2}$	$4.85\ 10^{5} \pm 1.84\ 10^{5}$
166	$8.20\ 10^{9} \pm 1.46\ 10^{9}$	$7.47\ 10^{4} \pm 9.10\ 10^{3}$	$2.02\ 10^{4} \pm 1.61\ 10^{3}$	$1.36\ 10^{5} \pm 2.83\ 10^{4}$
171	$5.71\ 10^{9} \pm 7.43\ 10^{8}$	$5.50\ 10^{4} \pm 8.40\ 10^{3}$	$5.54\ 10^{4} \pm 1.92\ 10^{4}$	$3.44\ 10^{5} \pm 3.41\ 10^{4}$
231	$3.56\ 10^{9} \pm 4.12\ 10^{8}$	$1.51\ 10^{5} \pm 2.90\ 10^{4}$	$1.91\ 10^{2} \pm 2.67\ 10^{2}$	$2.42\ 10^{2} \pm 6.30\ 10^{1}$
266	$9.69\ 10^{9} \pm 1.90\ 10^{9}$	$5.30\ 10^{4} \pm 3.83\ 10^{4}$	$4.58\ 10^{2} \pm 6.72\ 10^{2}$	$4.10\ 10^{2} \pm 2.05\ 10^{2}$

Figure 4. A. Box-plots showing the logarithm of the number of copies/100 ng DNA of the targeted genes markers of total Bacteria, AOB, NOB, and AMX, quantified by qPCR in the SBR. **B**. Average ± standard deviation of the number of copies of the targeted gene markers per 100 ng DNA in the SBR. The medians of boxes marked with the same letter in each plot are not significantly different, according to the Conover-Iman test ($p<0.05$).

363 Except on day 177, the *amoA*/*nxrB* ratio (calculated from data shown in Figure 4B) was always
364 > 1, indicating the dominance of AOB within the nitrifying community, and in agreement with
365 the higher activity of AOB over NOB measured throughout both Stages II and III. These results
366 indicated that the *in-situ* FNA strategy succeeded to effectively inhibit NOB in the SBR (Figure
367 1) even when populations of this functional group were not wholly absent in the sludge. In this
368 sense, an earlier study also reported the occurrence of NOB in short-cut biological N removal
369 treatment systems, even when high nitritation rates (> 96 %) were being achieved [34].

370 Although the aim of this study was to develop only the nitritation process, AMX were present
371 in the SBR sludge, as they were already present in the inoculum coming from a PN/AMX [13].
372 The abundance of AMX gene markers followed similar patterns to that of AOB during Stage II
373 (Figure 4), despite the operating conditions being unfavourable for AMX (Figure S1, Figure 1).
374 Results are in agreement with those of previous studies detecting AMX in all compartments of
375 full-scale municipal WWTPS. AMX often reach similar abundances than AOB, and can even
376 grow under high COD/N ratios and DO concentrations >2 mg/L [35, 36]. Then, the AMX
377 abundance in the SBR was drastically reduced in Stage III, indicating a poorer recovery capacity
378 after anoxic starvation. Although AMX can survive through starvation periods, cell death is
379 reported to account for >73 % of AMX decreased activity during anoxic starvation [37].

380 MDS and BIO-ENV statistical analyses were conducted in search of links among the shifts in the
381 abundance of the four bacterial groups quantified by qPCR and the changes of the operational
382 parameters and performance of the SBR (Figure 5, Table S2). AOB abundance displayed the
383 strongest correlations with most of the abiotic variables included in the analysis (Figure 5,
384 Table S2). In terms of SBR performance, strong positive correlations were observed with %
385 AOR and % IC removal and the concentrations of NO_2^- detected in the effluent ($r > 0.800$). The
386 number of copies of *amoA* genes were higher at increasing DO and NH_4^+ concentrations and at

lower amounts of TOC in the influent (r > 0.900), all factors widely reported to benefit nitrification [29]. In addition, AOB were the only bacterial group whose abundance was significantly linked with increased acidity (r = 0.998) and the generation of FNA (r = 0.956).

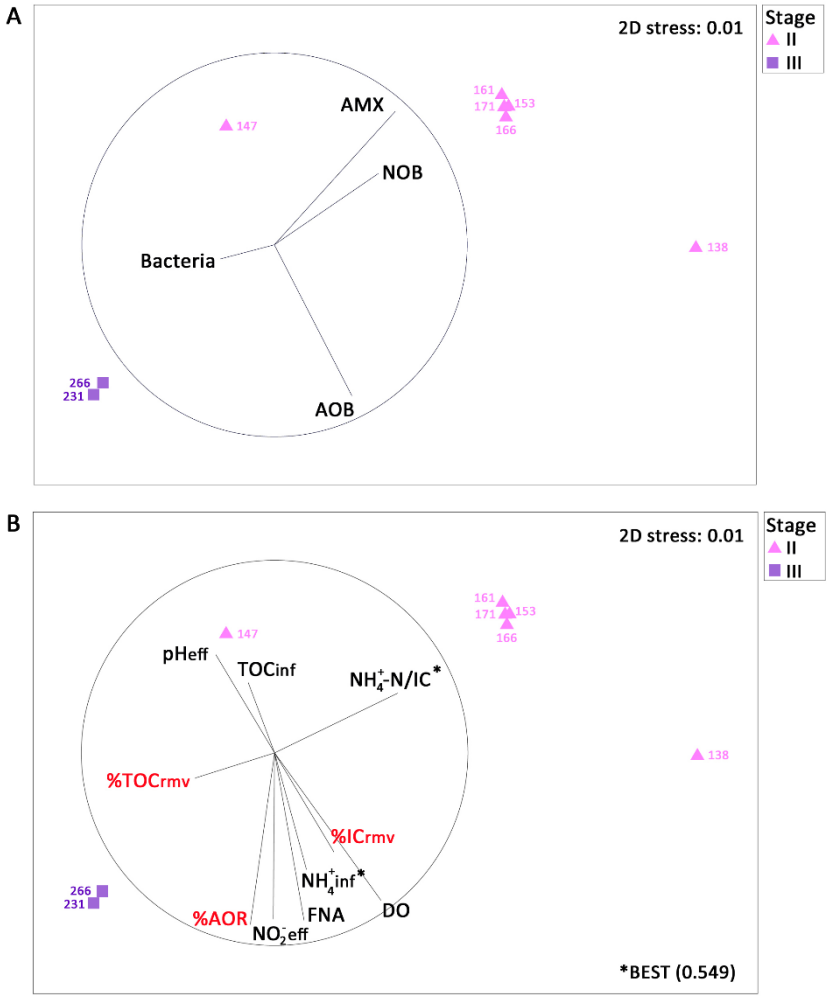


Figure 5. Non-metric multidimensional scaling (MDS) ordination of the SBR samples from Stages II and III, according to the abundance of copies of gene markers of total Bacteria, AOB, NOB and AMX. Vectors on plots represent the trends of the abundance of each gene marker (**A**) and the strength and directional influence of the operational parameters (**B**) throughout the samples' ordination. %AOR, %TOC and %IC removal rates were also included in plot **B** to illustrate the correlations among SBR performance and the abundances of the targeted bacterial groups. The operation parameters which best explained the distributions of the biological data according to BIO-ENV analysis are marked with an asterisk (*).

399 The abundances of NOB and AMX displayed similar trends through the samples' ordination,
400 and opposite to the behaviour observed for Bacteria (Figure 5A). According to BEST analysis,
401 the concentration of NH_4^+ in the influent and the NH_4^+-N/IC ratio contributed a major
402 explanation (54.9 %) to the ordination of samples (Figure 5B, Table S2). Consistently with their
403 metabolic features, both groups were strongly favored by higher NH_4^+-N/IC ratios ($r > 0.900$),
404 and displayed moderate negative correlations with the FNA concentrations ($r = -0.415$ and $r= -$
405 0.616 for NOB and AMX, respectively) in agreement with the reported inhibitory activity of this
406 compound to both groups of chemolithotrophs within the range of concentrations achieved in
407 the SBR [7]. Contrary to the patterns observed for AOB, the correlation of the abundance of
408 total Bacteria with the % TOC removal was robust and positive ($r = 0.999$) (Figure 5, Table S2).

409 **3.6. Diversity of Bacteria in the SBR identified by Illumina sequencing and links to**
410 **operating parameters in the SBR**

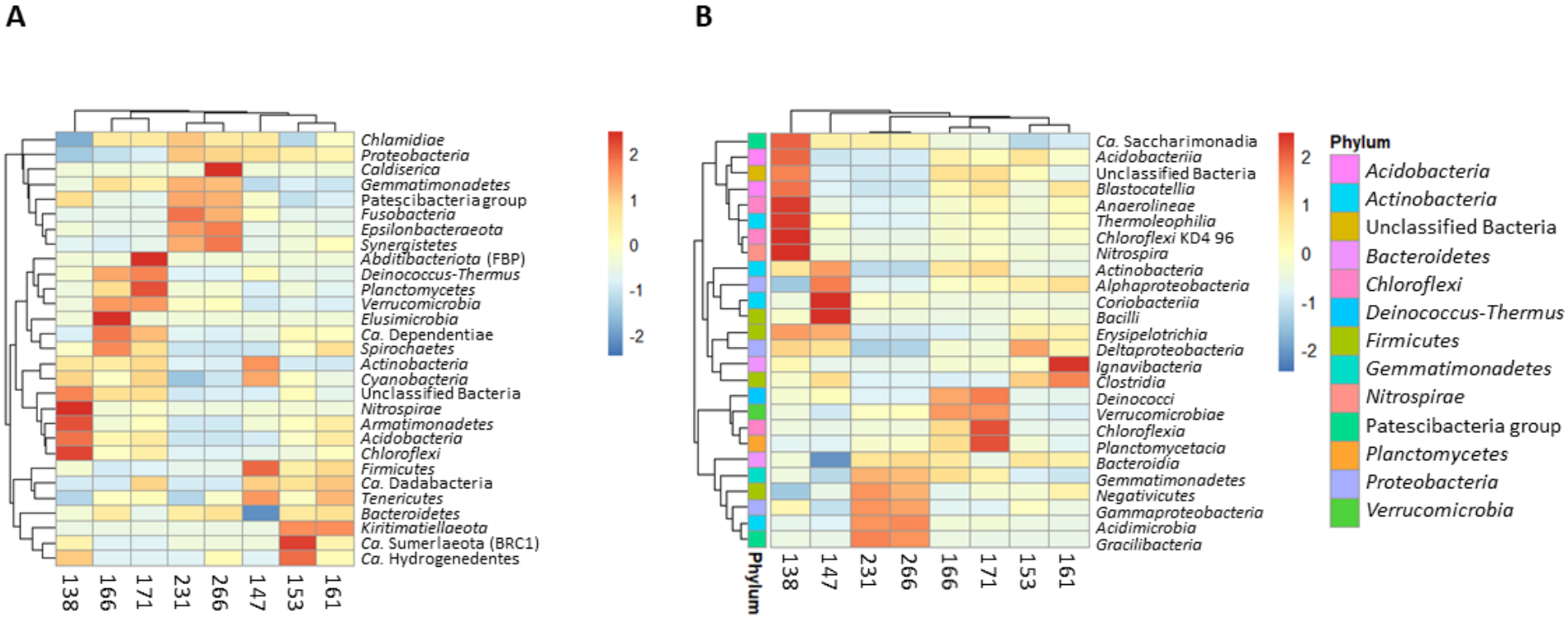
411 To complete the characterization of the bacterial community in the SBR, Illumina sequencing
412 of partial 16S-rRNA genes was conducted on the same set of samples that qPCR. The average
413 numbers of reads, Good's coverage, and diversity indices (richness, S; Simpson, D; and
414 Shannon-Wiener, *H'*) are summarized in Table S3. Overall, the indices described bacterial
415 communities displaying high species diversity and low functional organization, although the
416 samples of Stage III had significantly lower values of S and *H'* and higher values of D, compared
417 to those of Stage II, showing that the community was less diverse and became more
418 specialized after the anoxic starvation, comprising a narrower spectrum of numerically
419 prevalent populations.

420 The relative abundances of Bacteria identified in the SBR samples at the Phylum and Class
421 levels are displayed in Fig. S3 (complete data set available in Table S4). A total of 4288 OTUs
422 were classified in 29 different Phyla, while 528 OTUs (4.6 % of sequence reads) could not be

423 assigned to any known Phyla (Figure S3A). Overall, the bacterial community was dominated by
424 members of the *Proteobacteria* (relative abundance 38.4 ± 0.73 - 59.5 ± 0.2 %), followed by
425 *Bacteroidetes* (5.8 ± 0.1 - 23.8 ± 0.1 %), *Actinobacteria* (7.7 ± 0.2 - 19.8 ± 0.1 %), *Firmicutes* (2.1
426 ± 0.1 - 11.5 ± 0.3 %), and *Chloroflexi* (0.35 ± 0.0 - 7.2 ± 0.0 %), in accordance with previous
427 descriptions of the diversity at the Phylum level in aerobic WWTPs, either based on
428 conventional or advanced technologies [38, 39]. Members of 74 Classes were identified, of
429 which 25 displayed a relative abundance > 0.1 % (Figure S3B). The prevalent Classes were
430 *Gammaproteobacteria* (16.5 ± 0.1 - 45.4 ± 0.1 %), *Alphaprotebacteria* (6.4 ± 0.2 - 39.3 ± 0.2 %),
431 *Actinobacteria* (2.8 ± 0.0 - 18.5 + 0.2 %), *Bacteroidia* (5.7 ± 0.1 - 23.2 ± 0.4 %) and *Clostridia* (1.4
432 ± 0.0 - 6.0 ± 0.0 %). Among the *Gammaproteobacteria*, most OTUs (429) fell within the Order
433 *Betaproteobacteriales* (6.2 ± 0.2 - 38.4 ± 0.2 %).

434 Figure 6 shows the hierarchical clustering of the samples according to the relative abundances
435 of taxa at both Phylum and Class levels. The sample taken on day 138, when municipal
436 wastewater started to be fed to the SBR, clustered apart in both heatmaps, and was
437 characterized for the over-representation of Phyla *Acidobacteria*, *Armatimonadetes*,
438 *Chloroflexi*, and *Nitrospirae*, and Classes *Acidobacteriia*, *Anaerolineae*, *Blastocatellia*, *Ca.*
439 Saccharimonadia, *Chloroflexi* KD4 96, *Erysipelotrichia*, *Nitrospira,* and *Thermoleophilia*. From
440 day 138 onwards, population succession lead to significant changes of community structure,
441 and by day 147, Classes *Actinobacteria*, *Alphaproteobacteria*, *Bacilli* and *Coriobacteriia*
442 increased their representation, while *Chloroflexia*, *Deinococci*, *Planctomycetacia* and
443 *Verrucomicrobiae* became more abundant by the end of Stage II (days 166 and 171).

444



445

446 **Figure 6.** Heat maps showing the relative abundances of Bacteria at phyla (**A**) and class (**B**) levels (when higher than 0.1 % in at least one sample)

447 derived from Illumina sequencing data of the SBR samples retrieved during Stages II and III. Hierarchical clustering is based on average linkage

448 (unweighted pair group method using arithmetic averages).

449 The samples taken during Stage III (days 231 and 266) were characterized by higher-than-
450 average relative abundances of OTUs classified within the Phyla *Caldiserica*, *Chlamydiae*,
451 *Epsilonproteobacteraeota*, *Fusobacteria*, *Gemmatimonadetes*, Patescibacteria group,
452 *Proteobacteria*, and *Synergistetes*, and Classes *Acidimicrobia, Bacteroidia,*
453 *Gammaproteobacteria, Gemmatimonadetes, Gracilibacteria* and *Negativicutes,* indicating that
454 the starvation stage favored the prevalence of these taxa once the SBR operation was
455 restarted.

456 The shifts in the bacterial community structure taking place throughout Stages II and III were
457 also reflected by the MDS ordination of the samples based on their community diversity
458 (Figure 7). The BEST analysis indicated that the NH_4^+-N/IC ratio and the concentration of NO_2^- in
459 the effluent provided the best explanation (51.1 %) to the observed changes of community
460 patterns (Figure 7B). These variables significantly drove the shifts of *Nitrospirae* abundance,
461 which were strongly favored by higher NH_4^+-N/IC ratios ($r > 0.990$) and displayed moderate
462 negative correlations with the concentration of NO_2^- in the effluent and %AOR (Table S5), in
463 agreement with the results observed for qPCR data. Shu, et al. [40] previously described a
464 positive relationship between the relative abundances of both AOB and NOB and higher NH_4^+-
465 N/IC ratios, as well as negative correlations between the relative abundance of *Nitrospirae* and
466 the NO_2^- concentration, in the effluent of a laboratory-scale simultaneous anammox and
467 denitrification system.

468 Those Phyla which were over-represented in the initial sample of Stage II (*Acidobacteria*,
469 *Armatimonadetes*, *Chloroflexi*, *Nitrospirae*) were favoured by the low input of organic matter
470 to the SBR (Table S5). Conversely, 7 of the 18 Phyla displaying a relative abundance >0.1% in at
471 least one sample were strongly favored by higher TOC concentrations in the influent ($r > 0.800$,
472 Table S5). Among these, the numerically dominant groups *Proteobacteria* and *Bacteroidetes*
473 comprise a majority of organoheterotrophic bacteria and are generally regarded as key players

for organic matter removal in biological wastewater processes based on different technologies [41, 42]. However, a strong correlation with TOC removal efficiency was only observed in the case of *Bacteroidetes* ($r > 0.919$, Table S5), indicating that members of these Phyla may play a particularly significant role under the conditions tested here. In this sense, *Bacteroidetes* are well known to specifically contribute to the hydrolysis and degradation of polymeric molecules in WWTPs [42].

Strong negative correlations were found among the concentrations of either NO_2^- or FNA and the relative abundances of *Firmicutes* ($r < -0.90$) and *Actinobacteria* ($r < -0.6$) (Fig. 7, Table S3). Nitrite is known for long as a potent inhibitor of species of *Firmicutes* [43], particularly those of the class *Clostridia* [44], which were the most abundant *Firmicutes* in the PN bioreactor (Figure S2). The biocidal effect of NO_2^- is thought to be related to the generation of FNA under acidic pH [44]. The effects of NO_2^- and its derived FNA on the community diversity of activated sludge were previously investigated in a PN/AMX system devoted to mainstream wastewater treatment [45] and a bioreactor for the acidic fermentation of waste sludge [46]. In both studies, the relative abundance of *Actinobacteria* decreased at higher FNA concentrations, while in the waste sludge fermentation system, FNA increased the overall relative abundance of *Firmicutes*, although it either favored or hampered that of particular *Clostridia* species. Accordingly, in a study conducted with pure cultures of ruminal bacteria it was found that *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens* were sensitive to nitrite concentrations as low as 2 mg NO_2^--N/L, while *R. albus* growth was unaffected [43]. The susceptibility to nitrite or FNA of microorganisms involved in wastewater treatment is acknowledged to vary widely, in agreement with the diversity of mechanisms through which this compound exerts its growth inhibitory and biocidal effects [44, 47].

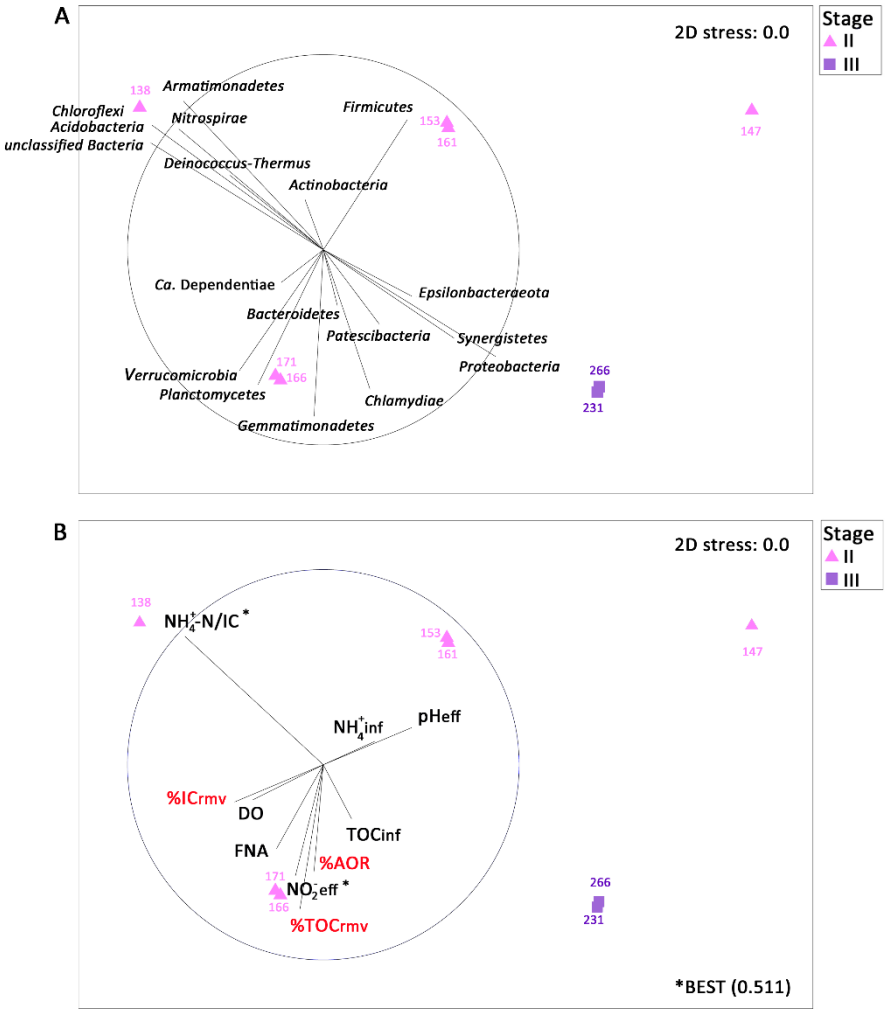
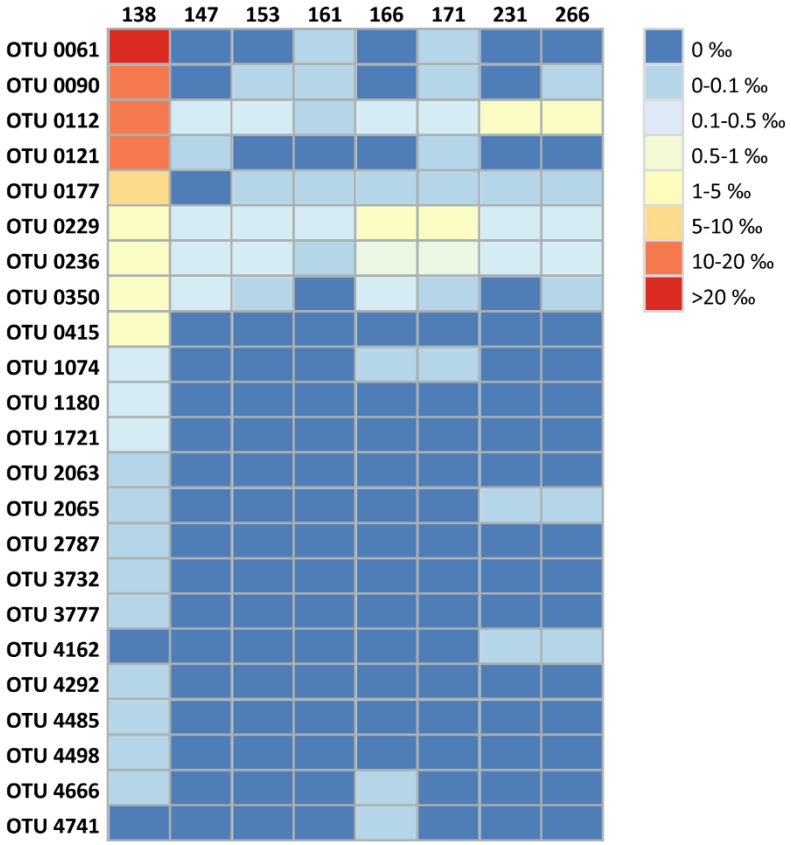


Figure 7. Non-metric multidimensional scaling (MDS) ordination of the SBR samples in Stages II and III, according to the relative abundance of bacterial Phyla identified by Illumina sequencing. Vectors on plots represent the trends of the relative abundance of each Phylum (A) and the strength and directional influence of the operational parameters (B) throughout the samples' ordination (only Phyla with relative abundance >0.1% in at least one sample are shown). %AOR, %TOC and %IC removal rates were also included in plot B to illustrate the correlations among SBR performance and the relative abundances of the different Phyla. The operation parameters which best explained the distributions of the biological data according to BIO-ENV analysis are marked with an asterisk (*).

507 The subset of Illumina sequencing reads identified as phylogenetically close to known AOB and
508 NOB genera were further analyzed to investigate the changes of the diversity of these
509 functional groups occurring throughout Stages II and III (Figure 8). The relative abundance of
510 AOB-related OTUs was 9.1 % on day 138, and ranged 0.04 - 0.26% in the rest of samples,
511 following trends consistent with those observed for the absolute quantification of *amoA* gene
512 copies. Twenty-three OTUs were classified as *Nitrosomonas*, while only one OTU (OTU 4839)
513 occurring in low abundance and in a single sample (day 161) was closely related to
514 *Nitrosospira*. The AOB community was more diverse on day 138 (21 OTUs), compared to the
515 rest of the samples (5-9 OTUs) (Figure 8).

516 The consensus sequences of 14 *Nitrosomonas*-related OTUs, which occurred in at least one
517 sample with a frequency ≥ 5 sequence reads, were manually introduced in the BLASTn search
518 tool of the NCBI nucleotide database and aligned to entries of 16S rRNA gene sequences of
519 cultivated *Nitrosomonas* spp. strains, and a Neighbour-Joining tree was constructed to display
520 their phylogeny (Figure S4). Eight OTUs (OTUs 0112, 0177, 0236, 0350, 1180, 1721, 2063 and
521 2065) grouped with Cluster 6a strains (*N. oligotropha-N. ureae*), while 5 OTUs (OTUs 0061,
522 0090, 0121, 0415 and 1074) fell within Cluster 7 (*N. europaea-N. eutropha*), and OTU 0229 lied
523 between Clusters 6a and 6b, closest to 16S rRNA sequences of *N. marina* and *N. oligotropha*
524 type strains (95.3 and 94.9% similarity, respectively). The OTUs dominating the community on
525 day 138 (OTU 0061, OTU 0090 and OTU0121; 29, 18 and 14 ‰ relative abundances,
526 respectively) belonged to Cluster 7 and were soon displaced after the change of feeding and
527 cycling regime of the SBR by Cluster 6a-related OTUs, which kept dominating the AOB
528 community after the anoxic starvation step (Figure 8, Figure S4). In Stage III, the AOB
529 community became particularly specialized, being dominated by a single OTU (OTU 0112, 75.1
530 - 81.7 % of sequence reads related to *Nitrosomonas* in days 231 and 266).

Nitrosomonas spp. of Clusters 7 and 6a frequently co-occur in laboratory- and full-scale nitrifying bioreactors (ie., [48, 49]), but their specific distribution patterns are highly influenced by the operational conditions [50]. Both cluster-level conserved features and strain-specific traits are determinant for niche differentiation and population succession [51]. Cluster 7 members are regarded as r-strategists, having a low affinity for ammonia and prevailing in WWTPs with higher N loads, while Cluster 6a strains are considered K-strategists, displaying a higher affinity for their electron donor and being favored when ammonium concentrations become limiting for r-strategists [52]. In addition, genomes of Cluster 7 strains encode several proton pumping terminal oxidases with different affinities for DO, which provide a competitive advantage over Cluster 6a AOB under fluctuating DO concentrations [51], such as those provided when aeration was fully simultaneous to organic matter feeding in the SBR and AOB were forced to compete for DO with organoheterotrophs (days 138-143). The AOB community in the SBR was structured under autotrophic conditions during Stage I with an average concentration of NH_4-N of *ca.* 50 mg/L (Figure 2B), and at the beginning of Stage II displayed a high species diversity but was dominated by Cluster 7-related *Nitrosomonas* (Figure 8). After organic matter started being fed and cycle configuration was changed (days 147-171), the abundance of AOB and their population diversity were drastically reduced, with Cluster 6a members becoming steadily prevalent throughout the rest of Stages II and III. In this sense, Ma, et al. [32] reported that Cluster 6a phylotypes dominated the nitrifying biomass of an SBR treating semi-synthetic wastewater bearing similar ranges of concentration of organic matter and ammonia; in addition, these authors found that Cluster 6a-related populations survived well to anoxic starvation and were able to fast recover the ammonia oxidation activity after resuming operation, in line with the observations presented here.



554
555 **Figure 8**. Heatmap displaying the relative abundances (‰) of OTUs classified within the genera
556 *Nitrosomonas* and *Nitrosospira*, according to the Illumina sequencing data of the total Bacteria
557 communities in the SBR samples retrieved during Stages II and III. Data represent mean (n=2)
558 of two independent amplicon samples, SD for the classification levels were less than 1%.

559

560 The relative abundance of *Nitrospira*-related OTUs within the total bacterial community was
561 >100-fold reduced in samples of days 231 and 266, in agreement with the trends observed
562 when *nxrB* copies were quantified by qPCR. The NOB community displayed low diversity since
563 only three OTUs related to members of this functional group were identified. Alignment of
564 their consensus 16S rRNA sequences against the EMBL database revealed that OTU0157 and
565 OTU0324 were close to *Candidatus* N. defluvii (97 % and 99.3 % similarity with accession No.
566 DQ059545.1, respectively) while OTU1760 was 99.1 % similar to the sequence of *Candidatus* N.

567 moscoviensis (accession No. AF155153.1). OTU0157 and OTU0324 coexisted in all samples of
568 Stage II while OTU1760 occurred in very low abundance in the middle phase of Stage II.
569 Relative abundances of the OTU0157 and OTU0324 abruptly decreased from day 147 onwards,
570 after the cycle configuration was modified. OTU0324 was the only NOB-related phylotype
571 detectable in Stage III. According to these results, the diversity of the NOB community was
572 only slightly modified and not drastically influenced by operational changes, unlike reported by
573 other authors, who observed NOB population succession at the genus level (from *Nitrospira* to
574 *Nitrotoga*) in response to FNA [10].

575 **3.7. Robustness and feasibility of the *in-situ* FNA inhibitory concentration strategy**

576 In the present study, the nitritation process was started up using a sludge with negligible NOB
577 activity. Then, stable long-term nitritation process (354 days, 191 treating municipal
578 wastewater) was achieved by maintaining *in-situ* FNA inhibitory concentrations, from 0.02 to
579 0.20 mg HNO_2-N/L, while treating primary settled municipal wastewater at 15 ± 1 °C (Figure 1).
580 Moreover, no adverse effects associated with the presence of organic matter were observed.
581 Indeed, an 80 % average organic matter conversion was achieved, limiting the possible growth
582 of heterotrophic denitrifying bacteria that would compete for nitrite with AMX in the following
583 anoxic unit.

584 Despite the wastewater composition variability (Table 1), the *in-situ* FNA production-based
585 strategy was proved to be very robust even when during Stage II the FNA concentration was
586 below the 0.02 mg HNO_2-N/L (no NOB activity was observed) or higher than 0.2 mg HNO_2-N/L
587 (AOB were not suppressed). Although NOB were present during the entire operational period
588 (Figure 4) its activity inside the reactor was not observed, confirming the robustness of the *in-*
589 *situ* FNA strategy. On the contrary, Ma, et al. [10] observed that when the nitrogen
590 concentration decreased by 28 %, the excess of aeration caused the decrease of NAR from 75

591 to 10 %. In their study, after the increase of influent ammonium concentration, NAR was not
592 restablished and the increases of FNA concentration (from 0.25 to 1.00 mg N/L) and sludge
593 treatment fraction (10 to 30 %) caused an AOB activity reduction while nitrate remained as the
594 main oxidation product. Jin, et al. [5] achieved long-term stable nitritation operation (260
595 days) based on the ammonium valley control treating municipal wastewater. Nevertheless,
596 when a failure in the control system occurred, the DO concentration increased over 2 mg O_2/L
597 and nitrate production was observed. In the present strategy, the DO concentration was not
598 controlled and it only affected the oxidation ratio, while similar nitrate production was not
599 observed when feeding concentration varied or a failure in the feeding pump or aeration
600 occurred.

601 Moreover, with the *in-situ* FNA production strategy, no long-term adaptation of NOB to the
602 suppression factor has been observed (Figure 1), as it has been widely reported when FNA
603 treatment is applied in an external unit [9]. For example, Ma, et al. [10] noticed a dominant
604 NOB population shift from *Nitrospira* to *Nitrotoga* genus after the establishment of the
605 nitritation process by treating the sludge in a FNA treatment unit using 0.75 HNO_2-N/L and
606 nitrate build-up was observed. Therefore, it seems that the strategy of NOB activity
607 suppression by *in-situ* FNA inhibitory concentration is more robust than *ex-situ* treatment.

608 The proposed *in-situ* FNA production-based strategy depends on the N/IC ratio. Pedrouso, et
609 al. [11] explained that for N/IC ratios between 0.6 to 1.0 g N/g C in incoming wastewater, the
610 limited alkalinity decreases the pH leading to high FNA concentrations. The feasibility of this
611 strategy was demonstrated in this study with a N/IC ratio between 0.61 - 0.80 g N/g IC. The
612 strategy succeeded even when organic matter, traditionally considered as a challenge, was
613 present. Indeed, *Nitrospira* spp. (the only NOB genus detected) abundance was found
614 positively correlated to the N/IC ratio of influent wastewater (Figure 7, Table S5). Thus, the

615 application of the *in-situ* FNA inhibitory concentration strategy in those regions characterized
616 by hard waters (N/IC< 0.6 g N/g IC) would not be adequate or would require control of pH with
617 the associated high costs. Nevertheless, more than 50 % of the water in Europe is classified as
618 soft o slightly hard. Apart from the wastewater hardness (associated with that of freshwater),
619 the incoming nitrogen and IC concentrations are also influenced by the previous treatment
620 stages in the WWTP. The most common approaches to remove the organic matter content
621 from the wastewater are: high rate activated sludge (alkalinity is not significantly affected),
622 wastewater anaerobic digestion (alkalinity is produced), and/or chemical enhanced pre-
623 treatment (consumes alkalinity) [53]. Thus, the treatment train must be considered globally to
624 define the correct operational strategy of the subsequent nitritation unit.

625 To sum up, the proposed strategy enables to maintain stable nitritation process at low
626 temperature and low nitrogen concentration making possible its application on mainstream
627 WWTPs and potentially to reach the energy autarky. The advantages of the in-situ FNA
628 production strategy rely on the proven robustness against wastewater composition, and a
629 complex control system is not required either chemical addition for pH adjustment, among
630 others.

631 **4. Conclusions**

632 A nitritation process was successfully established and maintained in an SBR by the *in-situ* FNA
633 accumulation-based strategy (up to 0.2 mg HNO_2/L) to treat municipal wastewater containing
634 organic matter (up to 1.2 g TOC/N) at low temperature (15 ± 1 °C). The optimization of the
635 operational cycle in the SBR allowed coping with the changes in nitrogen and organic matter
636 concentrations in the municipal wastewater. The results showed that a NAR close to 100 % and
637 an organic matter removal of 80 % can be achieved in the same unit. Moreover, the long-term
638 stability (354 days) and robustness of the process were proven despite the influent

wastewater composition fluctuations, with N/IC ratios varying from 0.61 to 0.89 g N/g C. Despite analysis based on microbial molecular markers revealed the occurrence of NOB, their activity was suppressed due to the high FNA levels achieved by the self-regulation of pH during the nitritation process. Quantification of bacterial groups by qPCR and analysis of bacterial diversity by massive parallel sequencing evidenced that both AOB abundance and community structure were significantly influenced by the concentration of organic matter and the adjustment of the cycling conditions, while anoxic starvation inflicted a loss of the overall diversity and promoted specialization of the Bacteria community; however, these changes were not negatively reflected in the SBR performance, due to functional redundancy within both the chemoheterotrophic and ammonia oxidizing communities.

Acknowledgements

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