Journal of Environmental Chemical Engineering

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--Manuscript Draft--

Manuscript Number:	JECE-D-21-05305R2
Article Type:	Research Paper
Keywords:	partial nitritation; AOB-enrichment; NOB repression; Nitrifying bacterial dynamics, Nitrotoga
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Abstract

One of the main challenges for the full-scale implementation of the autotrophic biological nitrogen removal process is to achieve a stable long-term partial nitritation under the mainstream conditions of urban wastewater treatment plants. Here, the performance of partial nitritation process under mainstream conditions was investigated based on the change of the nitrifying community developed. A lab-scale airlift reactor was operated for 430 days with a granular sludge highly enriched in ammonia oxidizing bacteria (AOB) performing partial nitritation treating a mainstream-mimicked synthetic influent. The changes in solid retention time (SRT), dissolved oxygen (DO) concentration and temperature strongly affected to the microbial community developed, especially to the dynamics of the nitrite oxidizing bacteria (NOB) population, which triggered to instabilities in the partial nitritation process. The extremely high values of SRT applied (SRT=∞) and long-term exposure to low temperature (10°C) promoted the growth of NOB *Nitrotoga* genus and development of nitratation activity*;* and the subsequent variability in

temperature (between 10–30 °C) and in DO concentration (between 0.4 ± 0.1 and 1.4 ± 0.3 mg O_2 L^{-1}) influenced the competitiveness between nitrifiers and the growth of species previously undetected in the culture, as *Nitrospira* genus. Anaerobic ammonia oxidizing bacteria were also identified during cultivation and assisted to the repression of the NOB activity at 25-15°C. The results of the present study uphold that understanding the niche differentiation and competitiveness of NOB will be key to develop strategies for NOB repression in order to make possible full-scale implementation of partial nitritation process at mainstream conditions.

Keywords

Partial nitritation; AOB-enrichment; NOB repression; Nitrifying bacterial dynamics, *Nitrotoga*

1. Introduction

The autotrophic biological nitrogen removal process consists of the partial nitritation of ammonia, performed by ammonia oxidizing bacteria (AOB), followed by the autotrophic denitrification performed by anaerobic ammonia oxidizing bacteria (AnAOB). The implementation of this process in the main water line of urban wastewater treatment plants (WWTP) can make these facilities as energy-neutral or even energy-positive [1,2]. However, the main bottleneck so far for the full-scale implementation has been to achieve high nitrogen removal rates with stable long-term operation of the partial nitritation process, avoiding the growth of nitrite oxidizing bacteria (NOB) [3–11]. The main challenge is enhancing the growth of AOB in detriment of the growth of NOB at mainstream conditions (i.e. low temperature and low nitrogen loading), due to NOB maximum growth rates are higher than AOB growth rates at temperature lower than 25 °C [12]. This is particularly relevant for the implementation of such system in moderate climates, where wastewater temperatures varies between 5-25 °C [4]. Operational parameters such as pH, temperature, free ammonia and free nitrous acid concentrations, sludge retention time (SRT), and dissolved oxygen (DO) concentration affect to the kinetics of nitrifying bacteria [13], and different strategies based on these parameters or combinations thereof have been applied in bioreactors in order to favour AOB over NOB growth [14–18]. In parallel to the research about the implementation and optimization of wastewater engineering systems, bioreactors were used to investigate the competitions and the microbial distribution of nitrifying bacteria in dynamic ecosystems as per mixed cultures with many different cultivation conditions [19–21].

Functional redundancy in nitrifying cultures was commonly found (e.g. several species of AOB and NOB cohabitating and performing similar tasks) and it was reported to be beneficial to maintain the stability and performance of nitrifying bioreactors [22,23]. The competition and

microbial dynamics between AOB and NOB in bioreactors performing partial nitritation was mainly studied with *Nitrobacter* and *Nitrospira* genera as the main NOB players [24], because they were the most frequently identified NOB in lab-scale nitrifying bioreactors and WWTPs, respectively. Nonetheless, seven different genera of NOB were defined so far: *Nitrobacter*, *Nitrospina*, *Nitrospira*, *Nitrotoga*, *Nitrolancea*, *Nitrococcus*, and *Candidatus Nitromaritima* [25]; and their niche differentiation is still an unsolved matter influenced by the environmental parameters [26]. Recently, *Nitrotoga* genus was reported as the main NOB in WWTPs, alone or cohabitating with *Nitrospira* genus [27], and since then it was more and more frequently identified in partial nitrification systems [9,28]. Likewise, Li and co-workers [8] observed the change of NOB community towards a *Nitrotoga*-dominance when imposing free ammonia inhibiting conditions, evidencing the resilience of *Nitrotoga* genus under extreme environments of nitrogen removal. In this sense, the high metabolic and physiologic versatility of NOB makes them as very robust candidates to confront variable environmental conditions, and in fact, novel NOB species with functions within the nitrogen cycle are still being identified [29].

NOB ecological niches were studied in complete nitrification processes with mixed cultures [24,30], but studies on NOB dynamics and/or competitiveness between NOB performing partial nitritation at mainstream conditions are limited. These studies are of paramount importance since such potential microbial competitions will affect to the development of operation strategies pursuing NOB suppression and stable partial nitritation. For instance, some strategies applied so far were based on the application of low concentrations of DO in the bulk liquid by considering that NOB generally present lower oxygen affinity than AOB [4,15,31]; however, it was later demonstrated that *Nitrospira* genus presented higher oxygen affinity than AOB [16]. Then, low DO concentrations may not be used as effective strategy to provide competitive advantage for

AOB over NOB neither in *Nitrospira*-dominated systems nor in cultures where NOB population might change to *Nitrospira* genus. In the present study, a nitrifying culture which proved success on achieving long-term stable partial nitritation at mainstream conditions [32] was used as a reference culture to investigate the nitrifying community dynamics in partial nitritation process under unfavourable conditions. On one hand, the availability of a highly AOB-enriched culture was key to mimic in the long-term a mixed culture developed in a partial nitritation bioreactor, and on the other hand, the changing operational conditions were used to naturally induce changes in the microbial population.

The main aim of this study was to investigate the impact of the nitrifying population shifts, triggered by the change in key operational parameters, on a partial nitritation system under mainstream conditions. The nitratation activity was assessed in terms of the bioreactor performance and the microbial dynamics after changing SRT, temperature and DO concentration.

2. Materials and Methods

2.1. Bioreactor set-up and starting culture

A lab-scale airlift reactor with a total working volume of 5.2 L was used for cultivation. The detailed description of the reactor set-up and the monitoring system were as indicated in a previous work of the authors [32]. In the present study, the air flow was continuous and the DO concentration in the bulk liquid and the temperature were manually changed according to the experimental period. pH was controlled at 8.0 ± 0.1 by dosing a Na_2CO_3 0.5 M solution. The synthetic medium used as influent contained: 70 mg $N\text{-}NH_4^+$ L^{-1}, 45 mg KH_2PO_4 L^{--1}, 784 mg $NaHCO_3$ L^{-1}, 80 mg NaCl L^{-1}, 40 mg $CaCl_2$ L^{-1}, 90 mg $MgCl_2$ L^{-1} and 1 mL of trace elements

solution per litre of influent [33]. The ammonium concentration was chosen in the high range of an urban WWTP influent since this is the most usual situation in dry weather zones and in urban WWTPs where the reject water coming from the dewatering of digested sludge is recirculated to the mainstream line. Both situations are usual in Spain.

The nitrifying culture consisted of granular sludge highly enriched in AOB (ca. 90% of relative abundance according to fluorescence *in situ* hybridization analysis) adapted to low temperature in previous studies of the authors [17,32]. The initial sludge concentration in the reactor was 2.5 g VSS L^{-1}. Before starting the present study, the nitrifying culture performed stable partial nitritation at 10 ºC for 250 days, with an average nitrogen loading rate of 0.63 ± 0.06 g N L^{-1} d^{-1} and a nitrate concentration in the effluent of 0.6 ± 0.3 mg N-NO_3 L^{-1} [32]. A picture of the nitrifying culture is shown in Figure S-1 in Supplementary Material.

2.2. Cultivation strategy and operational conditions in the bioreactor

The airlift bioreactor was operated in continuous mode and partial nitritation process was pursued as cultivation strategy. Stable partial nitritation favouring the growth of AOB in detriment to the growth of NOB can be achieved in granular systems at low DO/ammonium concentrations ratio in the bulk liquid [34]. In the present study, ammonium and nitrate concentrations in the bulk liquid were measured on-line (AN-ISE sc probe with a Cartrical cartridge plus, Hach Lange, Germany) and inflow rate was automatically controlled to maintain a stable concentration of ammonium of 32 ± 4 mg N-NH_4^+ L^{-1} during the entire operation. Hence, DO/ammonium concentrations ratio was always maintained at low values (0.03 ± 0.01 mg O_2 mg^{-1} N). From now, this cultivation strategy will be referred as DO/ammonium strategy.

The shifts of the microbial population in the nitrifying culture triggered by the changes in SRT, temperature and DO concentration were investigated. According to the cultivation conditions

applied, five different periods of operation were defined in the bioreactor (Table 1). Conditions were maintained at least for 75 days before changing period, although the length of each period was selected depending on the response of the system. Between days 314 and 334, several operational problems took place coinciding with holiday's season, leading to scarce data, so this period was not considered in the study.

Table 1 Periods of operation of the lab-scale airlift reactor performing partial nitrification. (SRT: solids retention time; T: Temperature; DO: dissolved oxygen). Between days 314–334 the reactor presented problems of operation and data were not considered.

Period	**Days**	**SRT (days)**	**T (ºC)**	**DO (mg O_2 L^{-1})**
I	0 – 72	80 ± 20	10	1.4 ± 0.1
II	73 – 171	∞*	10	1.2 ± 0.2 from day 73 to day 131 0.8 ± 0.1 from day 132 to day 171
III	172 – 249		10 from day 172 to day 202 20 from day 203 to day 249	0.4 ± 0.1
IV	250 – 346	10 ± 10	20 from day 250 to day 289 30 from day 290 to day 346	0.4 ± 0.1 from day 250 to day 275 0.8 ± 0.3 from day 276 to day 346
V	347 – 430		30 from day 347 to day 380 Progressive decrease to 10 from day 381 to day 430	1.4 ± 0.3

*The calculated average value of SRT was as high as 600 ± 400 days, so SRT was considered ∞

2.3.Bioreactor batch test under anoxic conditions

A batch test to evaluate the occurrence of AnAOB activity was performed in the lab-scale airlift reactor on day 353 (Figure S-2 in Supplementary Material). First, reactor feeding was stopped and aeration was maintained until ammonium concentration was ca. 10 mg $N\text{-}NH_4^+$ L^{-1}. Then, air supply was changed by nitrogen gas supply through the air diffuser placed at the bottom of the reactor. When DO concentration was lower than 0.03 mg O_2 L^{-1} a pulse of nitrite and ammonium was added in a concentrations ratio adequate for AnAOB growth (ca. 1.3 g $N\text{-}NO_2^-$ g^{-1} $N\text{-}NH_4^+$, [35]), being the initial concentrations 37 mg $N\text{-}NO_2$ L^{-1} and 26 mg $N\text{-}NH_4^+$ L^{-1}). pH was maintained at 8.0 ± 0.3 by dosing H_2SO_4 10% (v/v). Bulk liquid samples were periodically withdrawn from the bioreactor to measure ammonium, nitrite and nitrate off-line. The batch test was finished when ammonium concentration was ca. 10 mg $N\text{-}NH_4^+$ L^{-1}. Then the air supply was restarted. The values of the nitrite to ammonium consumption ratio and the nitrate produced to ammonium consumed ratio were used as indicators of AnAOB activity and were calculated as indicated in the Supplementary Material.

2.4.Microbial community analyses

Relative abundances of AOB, NOB and AnAOB were determined by using fluorescence *in situ* hybridization (FISH) coupled to confocal laser scanning microscopy (CLSM). The nitrifying bacteria targeted were: Betaproteobacterial AOB, *Nitrobacter* genus (NOB), *Nitrospira* genus (NOB) and *Nitrotoga* genus (NOB). Biomass samples were directly grabbed from the reactor and granules were homogenized by grinding before fixation and hybridization. Sample fixation, hybridization protocol and details of the 16S rRNA-targeted oligonucleotide probes utilised are described in Supplementary Material.

Microbial community development was examined by metagenomic sequencing of the nitrifying culture. 16S-rRNA gene amplicon sequencing was performed by using Illumina platform. Total

DNA was extracted from approximately 0.2 g of sludge by using a MoBio PowerBiofilm™ DNA extraction kit (MoBio Laboratories, USA) following the manufacturer's instructions. Quality and quantity of the DNA were measured using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, USA). DNA samples from day 160 were sent to *Research and Testing Laboratory* (Lubbock, TX, US) and DNA samples from day 346 were sent to *AllGenetics* (Galiza, Spain). The steps of DNA dilution, PCR, library preparation and sequencing were performed by these companies. For library preparation, a fragment of the bacterial 16S V3-V4 ribosomal RNA gene of ca. 400 bp was amplified using the primers 357F (5' CCT ACG GGN GGC WGC AG') and 785R (5' GAC TAC HVG GGT ATC TAA TCC 3'). Libraries were prepared using barcoded primers and purified to subsequently be sequenced in a fraction of an Illumina MiSeq PE300 run. The companies provided the Illumina paired-end raw data generated. Then, the demultiplexed FASTQ files (forward and reverse reads) were processed using Galaxy (https://usegalaxy.org/). As of here, reads were pre-processing to clean-up and to reduce potential sequencing errors before assembling. Processing details and quality reports are shown in Supplementary Material. Paired-end assembly, alignment and taxonomic assignment were done by using the Mothur Tool Suite [36] implemented in Galaxy. Before taxonomic assignment, sequences were clustered into Operational Taxonomic Units (OTU) following the *de novo* approach, with OTU identity threshold of 97%. Ribosomal Database Project database [37] was used to perform the taxonomic classification. More details of the bioinformatics analyses can be found in Supplementary Material.

2.5. Analytical methods and calculations

Samples of the bulk liquid of the reactor were periodically withdrawn and the concentrations of the different nitrogenous compounds were directly measured after sampling. Total ammonia nitrogen concentration was analysed with an ammonium analyser (AMTAX sc, Hach Lange,

Germany) and nitrite and nitrate concentrations were analysed with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Only nitrogen as ammonium, nitrite and nitrate were considered to calculate the nitrogen balance as indicated in Equation 1. Nitrogen loses associated to the incorporation into new cells and to the potential production of nitrogen oxides (e.g. N_2O) were assumed negligible in comparison to the rest of terms of the N-balance. Therefore, the N-balance fulfilment was calculated as indicated in Equation 2.

$$N - NH_4^+(inf) = N - NH_4^+(eff) + N - NO_2^-(eff) + N - NO_3^-(eff) \quad \text{(Eq. 1)}$$

$$N - balance\ fulfilment\ (\%) = \left(\frac{N-NH_4^+(eff)+N-NO_2^-(eff)+N-NO_3^-(eff)}{N-NH_4^+(inf)} \times 100\right) \quad \text{(Eq. 2)}$$

Total suspended solids (TSS) and volatile suspended solids (VSS) were analysed according to Standard Methods [38]. The SRT was determined by dividing the amount of VSS inside the bioreactor by the amount of VSS washed out with the effluent and with the purge, as indicated in Equation 3.

$$SRT = \frac{[VSS]_{reactor} * V_{reactor}}{([VSS]_{effluent} * Q_{effluent}) + ([VSS]_{reactor} * Q_{purge})} \quad \text{(Eq. 3)}$$

Where, $[VSS]_{reactor}$ and $[VSS]_{effluent}$ are the VSS concentrations in the reactor and the effluent, respectively, $V_{reactor}$ is the reactor volume and $Q_{effluent}$ and Q_{purge} are the effluent and purge flow rates, respectively.

The average diameter of the granules was periodically measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). Samples were directly collected from the bulk liquid of the reactor, diluted with water and immediately transferred to the particle analyser's cuvette. The analyser was utilised according to manufacturer indications.

3. Results

3.1. Performance of the granular sludge reactor in terms of the nitrifying capacity

A lab-scale airlift reactor was operated for 430 days treating a nitrogen-low-strength synthetic influent with an average loading rate of 0.4 ± 0.1 g N L^{-1} d^{-1}. Throughout the reactor operation a low DO/ammonium concentrations ratio (0.03 ± 0.01 mg O_2 mg^{-1} N) was maintained as strategy to achieve a stable partial nitritation [17,34]. The size of the granules was maintained stable during the entire operation, with an average diameter of 700 ± 100 μm. Several changes on operational conditions (SRT, temperature and DO concentration) were carried out to evaluate the response of the nitrifying culture performing partial nitritation. These changes defined five different operational periods (Table 1, Figure 1).

During the nitrification process, ammonium is oxidized to nitrite and nitrite is oxidized to nitrate in a way that nitrogen balance is fulfilled for the whole process. Overall, as shown in Figure 1, the nitrogen balance was fulfilled during the entire operation of the airlift bioreactor with an average value of 95 ± 7%, with the exception of the end of Period IV and beginning of Period V, when the nitrogen balance was clearly unfulfilled with an average value of 68 ± 3%. At the end of Period V the nitrogen balance was recovered to original values.

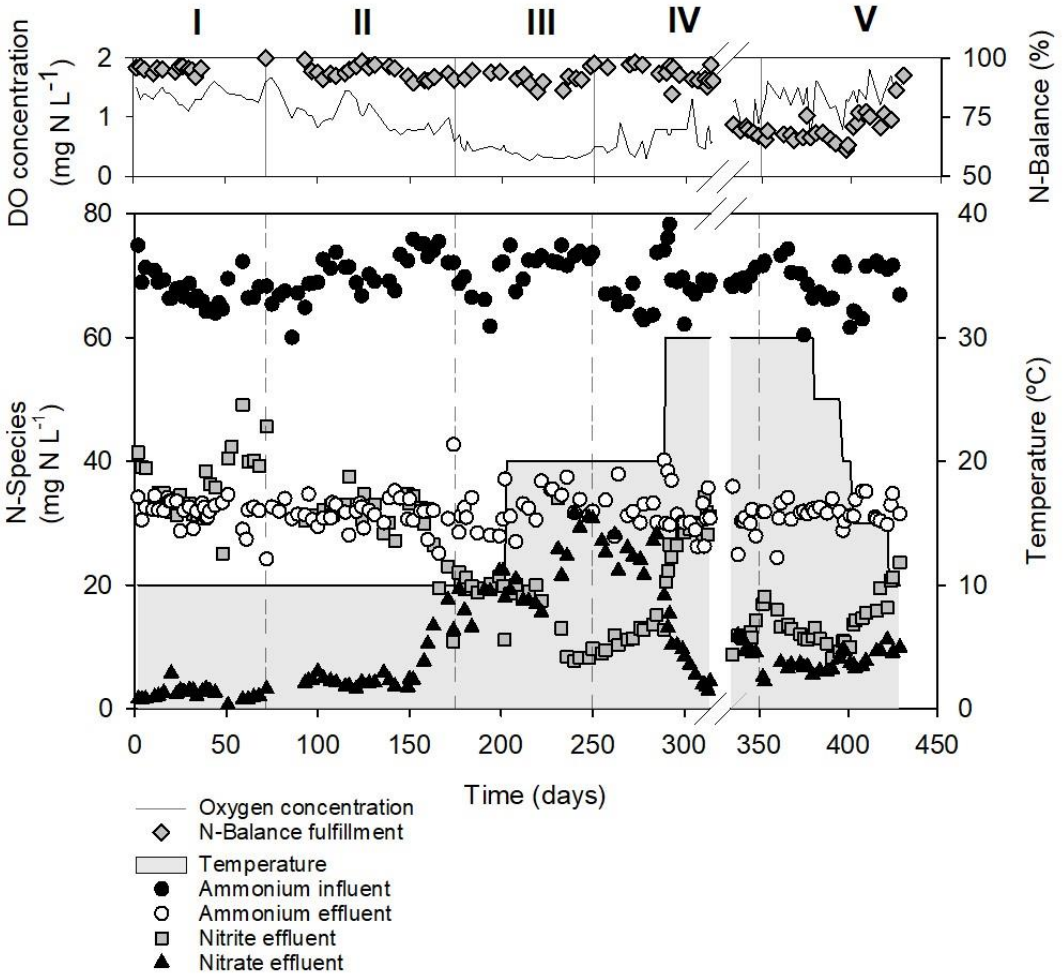

Figure 1 Follow-up of the nitrogenous compounds and the nitrogen balance fulfilment in the bulk liquid of the lab-scale airlift bioreactor during the long-term cultivation of the AOB-enriched nitrifying sludge performing partial nitrification

Stable partial nitritation at 10 ºC, with effective repression of nitratation activity, was maintained during Period I and the average effluent nitrate concentration was 2 ± 1 mg $N\text{-}NO_3^-$ L^{-1}. In Period II, SRT was vastly increased (SRT=∞) to allow for the growth of potentially slow-growing nitrifiers and nitratation activity slightly increased (4 ± 1 mg $N\text{-}NO_3^-$ L^{-1}, on average, were produced between days 73-150). In addition, DO concentration was gradually decreased from 1.2 to 0.8 mg O_2 L^{-1}. At the end of Period II a sharp increase in nitrate concentration occurred, with

an equivalent decrease of nitrite concentration in the bulk liquid of the bioreactor. In Period III, DO concentration in the bulk liquid was lowered until 0.4 ± 0.1 mg O_2 L^{-1} to impose stronger oxygen limitations to the culture and the operation stabilized with very similar accumulation of nitrite and nitrate concentrations. After one month of stable operation, temperature was increased from 10 to 20 ºC with the aim of enhancing nitritation activity in detriment of nitratation activity (canonical NOB present in wastewater show a more sensitive temperature dependence than AOB, [39]). Operation continued stable for three weeks but after that the nitrate concentration suddenly increased until achieving the highest value of the entire reactor operation (30.7 mg $N\text{-}NO_3^-$ L^{-1}), whereas nitrite concentration resulted as low as 9.7 mg $N\text{-}NO_2^-$ L^{-1}. In spite of maintaining a low DO/ammonium concentrations ratio at 20°C, NOB activity repression was not achieved at the exceptionally high SRT applied and such low DO concentration. During these three operational periods (I, II and III), biomass concentration was stable with an average value of 2.8 ± 0.5 g VSS L^{-1} and the ratio VSS/TSS was maintained in 0.95 ± 0.03.

In Period IV, SRT value was decreased until 10 ± 10 days. After this change, the abrupt increase of nitrate concentration in the bulk liquid stopped and a trend leading to recover the partial nitritation process was observed for a month, with a slow increase of nitrite concentration together with a slow decrease of nitrate concentration. However, the nitrifying culture still showed high nitratation activity. Thus, temperature was increased from 20 to 30 °C to enhance nitritation activity over nitratation, and DO concentration was increased from 0.4 ± 0.1 to 0.8 ± 0.3 mg O_2 L^{-1} to maintain stable the ammonium concentration in the bulk liquid. The system immediately responded and the partial nitritation process was recovered. Unfortunately, from day 314 onwards the bioreactor was intermittently stopped and started-up throughout a 20-days period, due to several operational failures associated to influent pumping and due to proper attention during

holidays. During this troubled period, a proper DO/ammonium concentrations ratio could not be maintained in the airlift reactor due to the successive stops of the influent pumping and the consequent decrease of the ammonium concentration in the reactor until values proximate to zero. When the operational problems were solved, normal operation continued maintaining the values of SRT, temperature and DO concentration as before the troubled period. However, such problems caused the increase of the average nitrate concentration from 4 ± 1 mg $N\text{-}NO_3^-$ L^{-1}, before the troubled period, to 11 ± 1 mg $N\text{-}NO_3^-$ L^{-1}, afterwards. Furthermore, nitrite concentration decreased during this period from an average value of 31 ± 3 mg $N\text{-}NO_2^-$ L^{-1} to 11 ± 1 mg $N\text{-}NO_2^-$ L^{-1}. In Period V, DO concentration was increased up to 1.4 mg O_2 L^{-1} to maintain the same values that those of Period I, and temperature was first maintained at 30 °C to favour nitritation activity over nitratation and then progressively decreased from 30 to 10ºC. On one hand, the nitratation activity was stable throughout the Period V, even during the temperature decrease occurred from the day 381. On the other hand, nitrite concentration increased up to an average of 20 ± 3 mg $N\text{-}NO_2^-$ L^{-1} with the decreasing of temperature. However, partial nitritation with effective repression of nitratation activity was not completely recovered at such low temperature despite of maintaining low SRT and a proper DO/ammonium concentrations ratio. In Periods IV and V, the biomass concentration decreased to 0.6 ± 0.2 g VSS L^{-1} due to the biomass purge performed from Period IV onwards, needed to maintain a low SRT, and the ratio VSS/TSS was maintained in 0.95 ± 0.03.

3.2. Microbial characterization of the AOB-enriched culture

A microbial characterization of the AOB-enriched granular sludge was performed to further investigate the instabilities of the nitrifying processes occurred during the long-term cultivation in the lab-scale airlift reactor. The variations of the nitratation activity were aimed to be explained

according to the dynamics of the nitrifying community, mainly according to the NOB abundance and NOB-species distribution.

3.2.1. Follow-up of the nitrifying population by using FISH-CSLM

FISH-CLSM analyses were performed with samples of the granular sludge from inoculum (beginning of Period I), day-171 (end of Period II), day-248 (end of Period III), day-346 (end of Period IV) and day-430 (end of Period V), to determine the relative abundance of AOB, NOB and AnAOB during cultivation (Figure 2). Nitrifying bacteria, i.e. AOB and NOB, were detected in all samples, although their relative abundance significantly changed with the time of cultivation. Figure 3 shows the percentage of variation of the relative abundance of each nitrifying population detected with respect to its own maximum abundance achieved during cultivation.

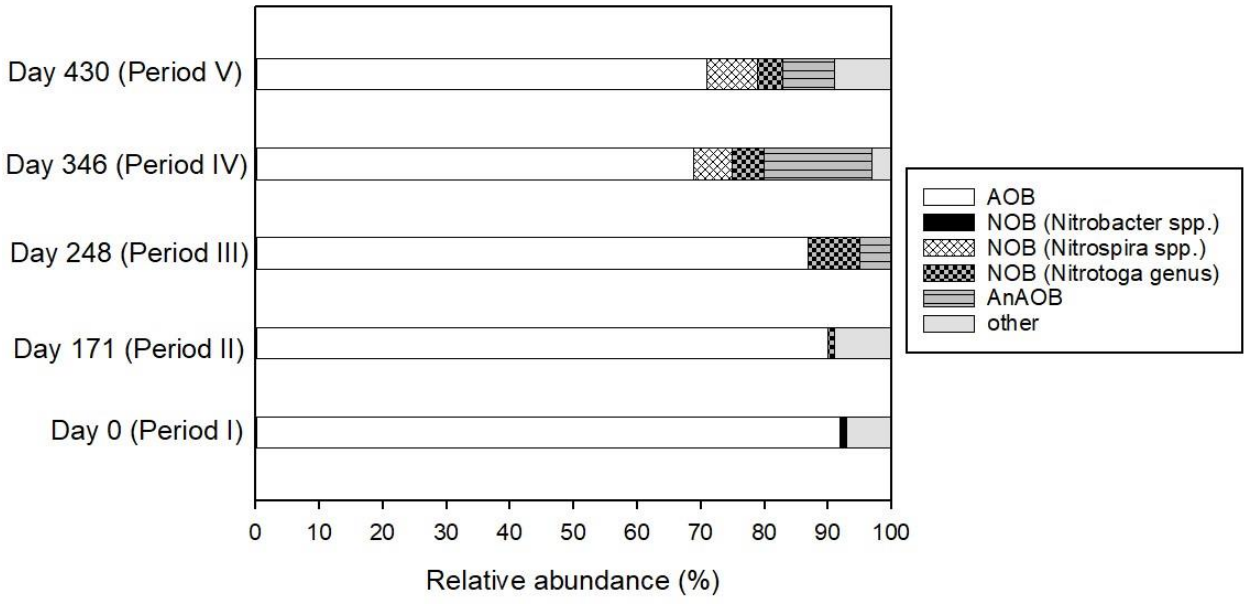

Figure 2. Relative abundance of AOB, NOB and AnAOB in the nitrifying culture at the different periods of cultivation as determined by FISH-CLSM. Error bars were not plotted but standard error was lower than

4% for AOB, Nitrospira and AnAOB relative abundance measurements and lower than 2% for Nitrotoga and Nitrobacter relative abundance measurements.

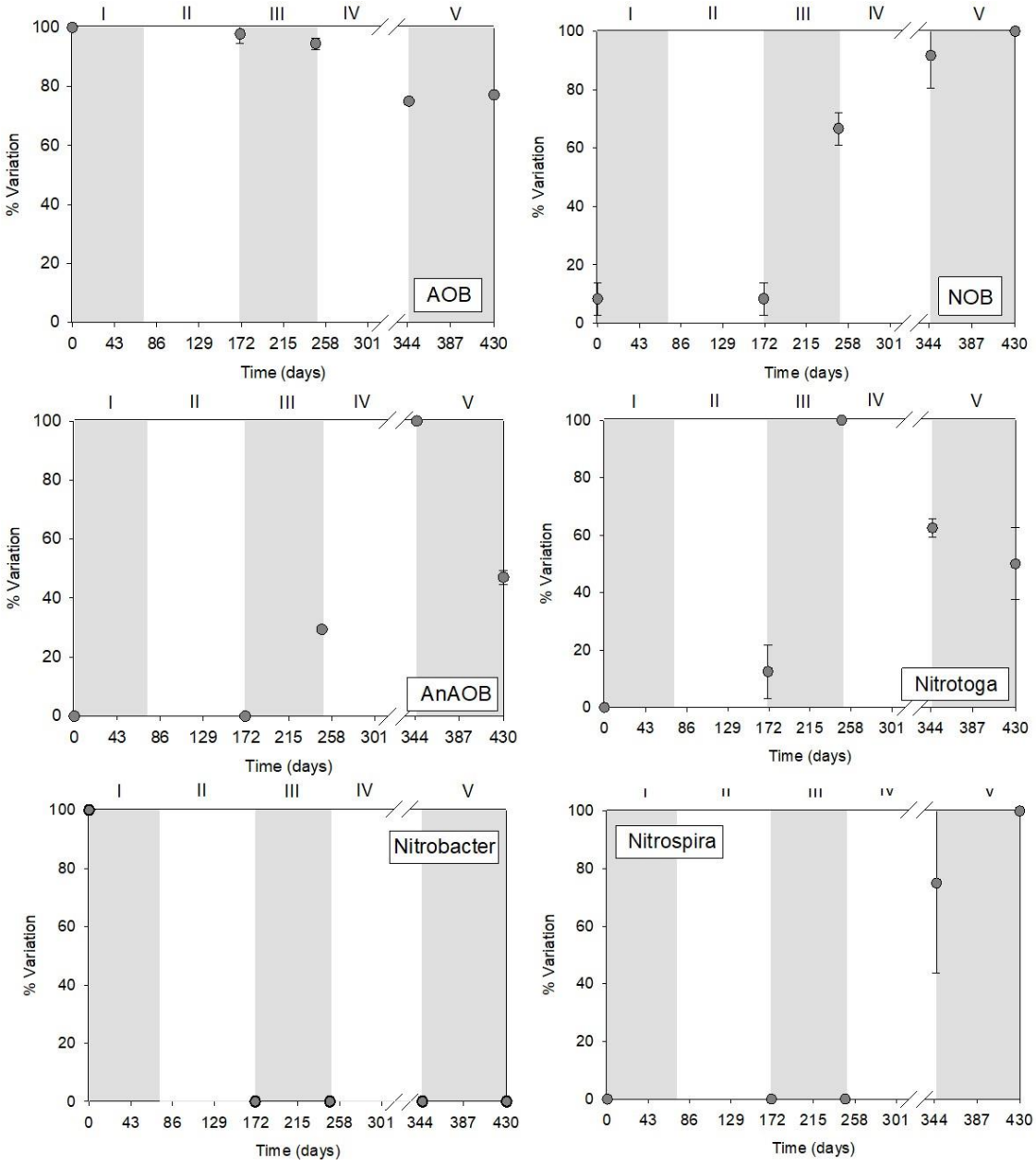

Figure 3. Variation of the AOB, NOB and AnAOB populations according to FISH results. Percentage of variation was calculated respect to their own maximum value detected. NOB includes the sum of the relative abundance of Nitrobacter, Nitrospira and Nitrotoga spp. Error bars were calculated with the error propagation simplification. Error bars are not visible if the error value is too low.

On one hand, a high enrichment in AOB was maintained during the entire operation of the granular sludge airlift reactor (Figure 2). In Periods I-III, the relative abundance of AOB was around 90% of the total population as detected by FISH-CLSM, and despite it decreased until 71 ± 3% at the end of the study, AOB were still the most predominant bacteria in the granular nitrifying culture. On the other hand, NOB were detected as the less abundant nitrifying population in the culture, although their abundance significantly increased with respect to their starting point after Period II of cultivation (Figure 3). Still, the changes in the abundance of the different NOB genera detected behaved in a very different way from one genus to other. Three different NOB genera were identified by FISH-CLSM: *Nitrotoga*, *Nitrobacter* and *Nitrospira*. Overall, NOB were effectively repressed at least until day 171, when only *Nitrotoga* genus was detected with a relative abundance of 1 ± 1% and *Nitrobacter* genus were not detected anymore, despite being in the inoculum. Nevertheless, from Period II onwards the relative abundance of NOB (first *Nitrotoga* genus and then *Nitrospira* genus) increased in the culture correlating with the increase of nitratation activity. *Nitrotoga* genus relative abundance presented a maximum value somewhere in between Periods III and IV, and then it decreased until the end of the study (Figure 3). *Nitrospira* genus was detected for the first time in the culture in Period IV, with a relative abundance of 6 ± 4%, and then it increased to 8 ± 2% at the end of the study.

AnAOB were detected for the first time in the granular sludge at the end of Period III, with a relative abundance of 5 ± 2% as detected by FISH-CSLM, and this abundance increased up to 17 ± 2% on day 346 (end of Period IV). To further investigate the presence of AnAOB activity in the nitrifying culture, a batch test with ammonium and nitrite as substrates (substrates of AnAOB) in the bulk liquid devoid of oxygen was performed on day 353 (Figure S-2 in Supplementary Material). The high consumption of both substrates under anoxic conditions, together with the

nitrite to ammonium consumption and nitrate production to ammonium consumption ratios obtained (1.03 ± 0.07 mol NO_2 mol^{-1} NH_4 and 0.22 ± 0.07 mol NO_3 mol^{-1} NH_4, respectively), which were close to the expected stoichiometry [35,40], indicated that AnAOB activity developed in the bioreactor. Nevertheless, at the end of the study (Period V) AnAOB abundance decreased until 8 ± 1%. The curve of variation of the AnAOB relative abundance correlated accordingly to the temperature and the DO concentration changes applied in the bioreactor and to the nitrogen misbalance occurred in Periods IV and V (Figure 1).

3.2.2. Microbial community assessment by using 16S rRNA gene amplicon sequencing

The metagenomic analysis of the culture was performed by using the Illumina platform. Amplicon sequencing of the 16S rRNA gene of DNA extracted from samples of days 160 (end of Period II) and 346 (end of Period IV) was used to identify the microbial population developed in the nitrifying culture. Taking into account the sequences with relative abundance higher than 0.1% of the total sequences, the main taxonomic assignments up to genus level are summarized in Figure 4 and Tables S-4 and S-5 in Supplementary Material.

A high enrichment in *Nitrosomonas* genus (AOB) was shown in both samples, although its relative abundance decreased with cultivation time, from 76% on day 160 to 31% on day 346. In addition, an increase in microbial diversity was shown if comparing data between both days. On day 160 only 9 different taxonomies of a total of 128 were identified with relative abundance higher than 1%, while on day 346 they resulted 19 of a total of 153. Furthermore, the rarefaction curves showed that both samples were expected to have a high richness and only a small fraction of them was covered by the bioinformatics analyses (Figure S-4 in Supplementary Material). Nitrifying community was dominated by AOB, specifically *Nitrosomonas* genus and any *Nitrosospira* genus was detected. Regarding NOB, on day 160 any genus of NOB was detected with relative

abundance higher than 0.1%. There was a 3.7% of relative abundance corresponding to unclassified Betaproteobacteria class, however no high alignment was found when comparing its representative sequence to 16S rRNA genes of *Nitrotoga* genus nor *Nitrosomonas* genus. Further bioinformatics analyses were done on the hits with less than 0.1% of relative abundance, and 19 sequences (0.008% of the total sequences) were found to be affiliated with *Nitrobacter* genus and 2 sequences (0.001% of the total sequences) with *Nitrospira* genus. No sequences affiliated to other NOB were identified. Still, there was up to 5179 sequences (2% of the total) belonging to the same phylotype, identified only up to Alphaproteobacteria class, and 1909 sequences (0.8% of the total sequences) belonging to the same phylotype of the bacteria kingdom, which were not classified as cultured bacteria. Conversely, on day 346 there was a 3% of sequences which belonged to *Nitrospira* genus, while *Nitrobacter* genus was still residual, with 0.009% of the total sequences. Regarding AnAOB population, there were no sequences classified as AnAOB on sample from day 160, whereas on day 346 *Candidatus Kuenenia* genus was identified with an 8% of relative abundance and also 191 sequences (0.5% of the total sequences) were assigned to the family *Candidatus* Brocadiaceae.

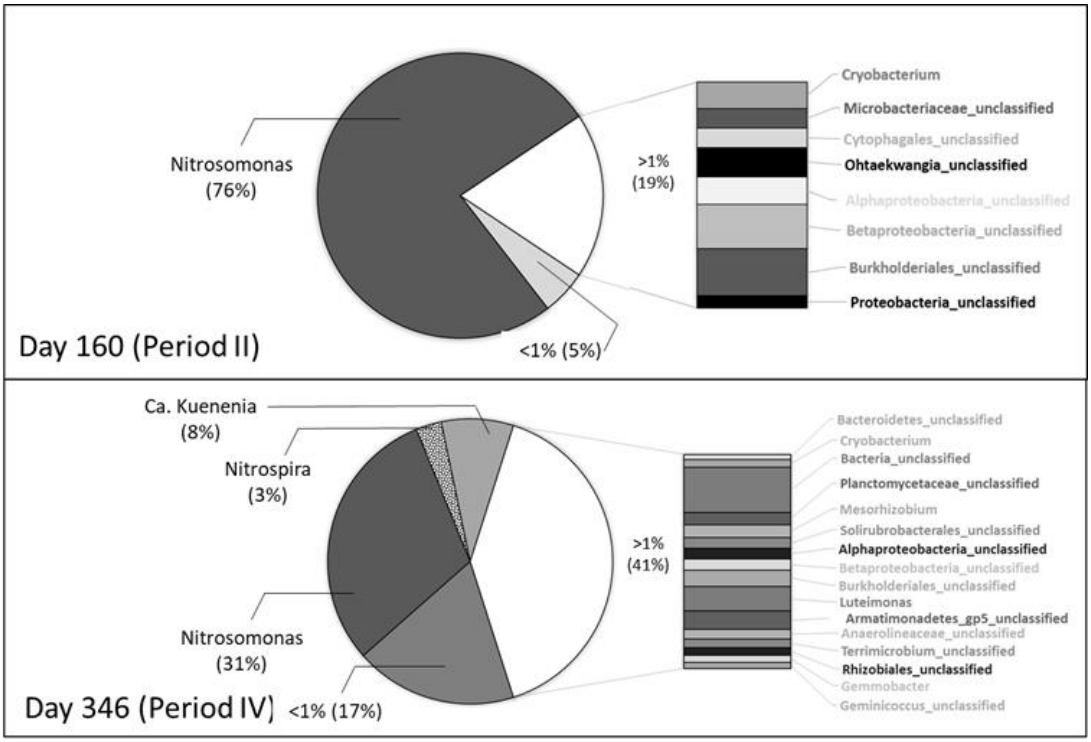

Figure 4 Relative abundance of the genera identified in samples from days 160 and 346 of operation. Depicted percentages were calculated only considering the sequences with relative abundance higher than 0.1%. Genera with relative abundance lower than 1% were grouped and not further represented in the pie chart. More detailed results of the detected population can be found in Supplementary Material.

4. Discussion

4.1. Importance of the NOB species dynamics for the stability of the partial nitritation

The shifts in the nitrifying community of the AOB-enriched culture, triggered by the changing operational conditions in the granular sludge airlift reactor, strongly affected to the stability of the partial nitrification. The changes in SRT, DO concentration and temperature especially affected to the dynamics of the NOB population, leading to instabilities in the nitrate production.

Previous studies of the authors showed that when DO concentration and temperature were changed during a long-term cultivation of granular AOB-enriched sludge performing partial nitritation, a successful suppression of nitratation activity could be achieved by maintaining the DO/ammonium strategy [17,32]. Here, this strategy was pushed to the limit by imposing an extreme increase in SRT, to allow for a potential change of the microbial population involved in the nitrification process. NOB repression was successfully achieved for 72 days when SRT was maintained in 80 ± 20 days (Period I), but nitratation activity slightly developed after SRT was vastly increased (SRT = ∞) and it sharply developed after 90 days operating at such high SRT values (Period II). The delay in observing the high nitratation activity increase after the change in SRT was likely related to the fact that when operating at such high values of SRT the response of the system is expected to be later noticeable. When working with nitrifying granular sludge, the SRT applied is expected to be higher than the minimum SRT needed for NOB growth, since bacteria grow in stratified layers in the granules and can remain in the inner part for a long time without being detached [41]. One example of this slow detachment in the present study is that *Nitrobacter* genus was never completely washed out of the system despite of being no longer active in the culture (it was detected with the metagenomic analysis on day 171 (end of Period II) and day 346 (end of Period IV) but not detected by FISH). Hence, in this study SRT was probably always higher than the minimum SRT needed for NOB growth and the success in repressing the nitratation activity during Period I was based on the strong effectiveness of the DO/ammonium strategy in limiting the NOB growth. Thus, even when the NOB abundance was very low according to FISH results, a residual amount of nitrate was always being produced in the reactor, indicating a very limited although persistent nitratation activity. In this sense, Ahn et al. [42] suggested that some NOB populations remained viable in partial nitritation bioreactors and could proliferate when the

optimal conditions arose. Therefore, the cultivation strategy applied during Period I succeed on avoid NOB proliferation although allowed a limited NOB growth (Figure 1); however, once the SRT increased up to a value close to infinite, the DO/ammonium strategy applied could not cope with it, leading to an increase of nitratation activity and NOB further proliferation. In addition, when SRT was maintained in extremely high values, neither the decrease of DO concentration nor the increase of temperature aiming to repress NOB were enough to stabilize the nitratation activity (Period III). In this respect, the extremely high value of SRT applied was demonstrated to be the key affecting the first development of nitratation activity in the culture and, consequently, affecting the stability of the partial nitritation. We hypothesize that the microbial wash out at such high values of SRT was negligible and NOB growing in suspension in the bulk liquid could attach to the granules and remain in the reactor. In addition, the low DO concentration imposed in Period III was expected to decrease the oxygen diffusion through the granule, limiting the stratified growth of AOB in the most external layers and NOB in the most inner layer of the granules, and favouring the NOB distribution in the surface.

The extremely high SRT and the subsequent changing conditions applied eased the proliferation of a novel NOB population that was not previously detected in the culture: *Nitrotoga* genus. The presence of *Nitrotoga* genus was reported in full-scale WWTPs and correlated with low temperature operations, between 7–16°C [27]. The nitrifying culture used here was subjected to 10°C for more than one year ([32] plus Periods I and II of this study), which could give *Nitrotoga* genus an adaptive advantage over other NOB. However, despite this advantage, its growth was expected to be slow and hence favoured when working at the extremely high SRT. Lücker and co-workers [27] reported that the cellular ribosome content of *Nitrotoga* genus may be below the limit of the FISH protocol when *Nitrotoga* genus are not functionally important in the culture. This fact

explains why *Nitrotoga* genus was detected by FISH when nitratation activity increased in the airlift reactor and not in the inoculum, although the reason why they were not detected with 16S rRNA gene sequencing remained unclear. One hypothesis would be that the unclassified phylotype affiliated to Betaproteobacteria (Tables S-4 and S-5 in Supplementary Material) was related to *Nitrotoga* genus, and in fact this unclassified phylotype significantly decreased from Period II to IV, as *Nitrotoga* genus did. However, the coverage of the corresponding sequence was low, and the identification could only be confident up to the class level, i.e. to Betaproteobacteria. In any case, since the relative abundance of total NOB population was the same in Periods I and II, we hypothesized that a key for breaking the previous stability of the partial nitritation process was the shift of NOB-population from *Nitrobacter* genus to *Nitrotoga* genus followed by the significant increase of *Nitrotoga* genus in the nitrifying culture (Figures 2 and 3). The high ecophysiological versatility of NOB allows for their pronounced niche differentiation [29] and it is known that the microbial composition of the nitrifying granular sludge may differ significantly even if the macroscopic output (e.g. nitratation activity) is very similar [23]. Thus, the produced nitrate in the bioreactor during Period I and II, expected to be produced by the *Nitrobacter* genus detected at the beginning of the study, could come from other NOB genus, e.g. *Nitrotoga*, which could be able to potentially outcompete AOB under the new conditions imposed to the system. At the beginning of Period III, the increase of temperature and the decrease of DO concentration temporarily slowed down the nitratation activity (nitrate concentrations in the bulk liquid were maintained stable for several weeks) but nitratation activity (presumably due to *Nitrotoga* genus) eventually increased in spite of the high temperature and low DO concentrations applied, and *Nitrotoga* genus only decreased after SRT was lowered (Period IV). The high metabolic flexibility of *Nitrotoga* genus [43] could explain its competitive advantage regardless the changes of temperature and DO

concentration when SRT was extreme. In fact, recent research on *Nitrotoga* genus evidenced their widespread ecological niches: (i) regarding temperature, *Candidatus Nitrotoga fabula* was shown to prefer temperatures higher than 20°C [43] despite *Nitrotoga* genus enriched from wastewater engineering systems were initially described as cold-adapted microorganisms; (ii) regarding nitrite affinity, *Nitrotoga* genus showed a medium to high affinity, in general lower than *Nitrospira* genus but higher than *Nitrobacter* genus [26] and it was detected in many different nitrite habitats [44,45]; (iii) regarding oxygen affinity, *Nitrotoga* genus showed genomic potential for survival under low-oxygen environments, allowing its growth on a wide oxygen habitat range [45].

Nitrotoga genus was detected in WWTPs co-aggregating with AOB and cohabitating with *Nitrospira* genus [27]. Studies on the niche competitiveness with the latter were reported [26,46–48]; however, to the authors knowledge, research on the competition between *Nitrotoga* and *Nitrobacter* genera was very limited, with only reported insights about the influence of temperature [30]. Furthermore, *Nitrotoga* genus was not commonly found coexisting with *Nitrobacter* genus and, until now, there were scarce studies reporting such co-occurrence [8,9]. In this study, the growth of *Nitrotoga* genus increased when *Nitrobacter* genus were no longer detected by FISH-CSLM in the culture (i.e. *Nitrobacter* genus activity was expected to be nil or very limited) but it remains unclear if an active population of *Nitrobacter* genus could outcompete *Nitrotoga* genus under the applied cultivation conditions. In any case, *Nitrotoga* genus was outcompeted by other nitrifiers in the culture after the SRT was lowered, as shown by the decrease of its population (Figure 3). Achieving the total suppression of nitratation activity once SRT was lowered and *Nitrotoga* genus abundance decreased seemed to be a slow process, as shown by the slow decreasing of nitrate concentration in the bulk liquid of the reactor (beginning of Period IV), and

a fast washout of *Nitrotoga* genus would be needed to completely recover the partial nitritation activity of the culture.

When temperature increased to 30ºC in Period IV, the partial nitritation process was instantly recovered because of the favouring conditions for AOB kinetically outcompete NOB [13]. Moreover, these operational conditions (SRT=10 days; T=30ºC and DO=0.8 ± 0.3 mg O_2^- L^{-1}) were expected to further decline the *Nitrotoga* population. However, the new conditions together with the troubled 20-days period occurred from day 314 led to extra population changes at the end of Period IV: *Nitrotoga* genus decreased, AnAOB increased and a new NOB population, *Nitrospira* genus, was detected with a significant relative abundance (Figure 2).

AnAOB population was first detected at the end of Period III but its activity was only noticed at the end of Period IV when the nitrogen balance was clearly unfulfilled due to the N_2 removal caused by the autotrophic denitrification (Figure 1). The low DO concentration (0.4 ± 0.1 mg O_2^- L^{-1}) imposed during Period III allowed the growth of AnAOB despite of operating under aerobic conditions in the bulk liquid, because a gradient of oxygen was expected to be generated inside the granules leading to anoxic zones where the AnAOB could develop. This is the principle applied in the widely spread CANON process, where a co-culture of AOB and AnAOB growing in aggregated form is established under microaerobic conditions to avoid inhibition of AnAOB by oxygen and to achieve appropriated conditions to obtain partial nitritation [13]. The AnAOB population had not an impact on the airlift operation until operational conditions derived from the technical problems of Period IV caused an increase of its relative abundance, probably also enhanced by the temperature increase to 30 ºC. Still, the increase of DO concentration and decrease of temperature to 10ºC at the end of the study hindered the AnAOB activity, as shown by the recovery of N-balance and decrease of their relative abundance.

At the end of Period IV, part of the ammonium and nitrite of the bulk liquid was expected to be consumed by AnAOB, and the capacity of NOB to oxidize nitrite was then limited. AnAOB activity assisted to NOB repression and the less nitrite availability could stir up the change in the NOB population, decreasing *Nitrotoga* genus and increasing *Nitrospira* genus. In fact, the competition between different NOB is strongly affected by the availability of nitrite [44]. *Nitrobacter* genus was characterized as r-strategist and was reported to grow when substrate concentrations are in excess or when there are punctual accumulations [49]; while *Nitrospira* genus was characterized as k-strategist and reported to grow under nitrite-limiting conditions, even with nitrite concentrations in the order of nanomolar [50]. Regarding *Nitrotoga* genus, Nowka et al. [44] and Wegen et al. [26] recently reported strong physiological data and analysed competition between *Nitrotoga* genus and other NOB. Accordingly, *Nitrotoga* genus was reported to have moderate nitrite affinities with values of k_{m,NO_2^-} in the range of 43-60 µM, lower than those of *Nitrobacter* genus (k_{m,NO_2^-}=49-554 µM) but higher than those of *Nitrospira* (k_{m,NO_2^-} = 9-27 µM); and were reported to present relative low maximum growth rates (μ_{max}=18-48 µmol NO_2^- mg^{-1} protein h^{-1}) which tilted the balance in characterizing it as k-strategist. In the present study, the high concentration of nitrite in the bulk liquid of the airlift reactor was expected to guarantee the availability of nitrite in the granules, despite of the internal mass transfer limitations, and thus, allow for the growth of an r-strategist NOB population, such as *Nitrobacter* genus. However, when AnAOB developed, nitrite limitations could occur inside the granules due to the competition for nitrite between NOB and AnAOB, favouring the growth of k-strategists NOB-populations, such as *Nitrospira* and *Nitrotoga* genera. The main driver for the successful niche occupation of *Nitrotoga* genus when co-habitating with *Nitrospira* genus was reported to be the low temperature [26]. Hence, in the present study, we hypothesized that *Nitrobacter* genus was outcompeted by

Nitrotoga genus due to the influence of the long-term exposure to low temperature at the extreme SRT applied, and *Nitrospira* genus could outcompete *Nitrotoga* genus when temperature was increased, SRT was lowered and nitrite availability was probably limited due to the growth of AnAOB. This competitiveness between the different NOB triggered the instabilities of the partial nitritation process in the present study, which points out the importance to further research on the competition between the different species of NOB in AOB-enriched cultures performing partial nitritation.

4.2. Practical implications in wastewater treatment

The strategy of applying a low DO/ammonium concentrations ratio by maintaining high ammonium concentration in the bulk liquid of nitrifying granular sludge bioreactors was reported to succeed in achieving stable partial nitritation under many different operational conditions [17,32,34,51,52]. Due to the high nitrite concentrations maintained in the bulk liquid, NOB of *Nitrobacter* genus (r-strategists) were usually detected in the bioreactors applying such strategy. The success of this cultivation strategy lies in (i) maintaining a residual ammonium concentration in the bulk liquid, which is required to keep the specific AOB growth rate higher than that of NOB [53]; and (ii) imposing strong oxygen limiting conditions in the stratified granules to favour AOB growth in detriment of *Nitrobacter* genus growth, since AOB present higher oxygen affinity than *Nitrobacter* spp. [54] and with enough ammonium in the bulk liquid they will consume all the available oxygen. NOB generally present lower oxygen affinity than AOB [55] but there are important exceptions to this assertion, e.g. some *Nitrospira* spp. present a competitive advantage over AOB in oxygen limited environments [16]. In addition, for aggregated sludge systems (e.g. granules) it was reported that the oxygen affinity was also determined by the microcolony size and growth yield of the nitrifiers [56]. It was known and also shown in Period I of the present study

that the DO/ammonium cultivation strategy allowed to sustain AOB over NOB growth at low temperature and low nitrogen concentrations while the main NOB belonged to *Nitrobacter* genus, however its effectiveness has not been described yet when *Nitrotoga* genus leads the NOB community. This genus was also detected in our previous study [57] where nitratation activity was observed despite of using the DO/ammonium strategy. Hence, the taxonomy of the NOB population developed in the nitrifying culture must be considered when applying an operational strategy aiming to repress NOB and, as demonstrated in this study, is key for guaranteeing the success of the partial nitritation process.

Here, the substantial operational perturbations imposed in the long-term triggered the change in the NOB community which led to instabilities in the partial nitritation process; however it is important to notice that such disturbances are not expected to occur in full-scale systems, where operational parameters are extensively monitored and controlled and short-term events are faced straightaway. Even none-controllable environmental instabilities (e.g. temperature changes) are usually mild and gradual and do not last long periods of time. In any case, as pointed out in the present study, when applying the DO/ammonium strategy in a granular sludge reactor performing partial nitritation, both SRT and DO concentration must be monitored and maintained in appropriate values to avoid the proliferation of AnAOB and slow-growing NOB. A feasible threshold for both parameters could be a SRT lower than 80-100 days and a DO concentration higher than 0.5 mg O_2 L^{-1}. Still, both (i) the viable presence of NOB in nitrifying sludge performing partial nitritation despite of their activity repression [42], and (ii) the potential migration of exogenous NOB with the influent of bioreactors [58], can be a source of an undesirable NOB proliferation, which, if occurring, must be quickly addressed.

The present study pointed out that investigating the instabilities of the partial nitritation process at mainstream conditions based on the community distribution of nitrifiers is key to succeed on the engineering of the process. Furthermore, understanding the resilience of AOB-enriched nitrifying cultures to disturbances is key to predict the response to the change of microbial populations in full-scale systems, such as WWTPs.

5. Conclusions

The substantial changes in SRT, DO concentration and temperature applied during the long-term continuous operation of an AOB-enriched granular sludge airlift reactor motivated important microbial population shifts which led to instabilities of the partial nitritation process. SRT maximum increase (SRT=∞) allowed for the growth of novel NOB in the nitrifying culture, and temperature and DO concentration influenced as well its nitritation capability and the development of different species of NOB and AnAOB previously undetected in the culture.

The long-term exposure to low temperature and the high SRT applied were suggested to promote the growth of *Nitrotoga* genus. All *Nitrobacter*, *Nitrotoga* and *Nitrospira* genera were detected in the culture, although their respective cohabitation and abundance changed depending on the cultivation period, pointing out their expected different ecological niches.

The results of the present study showed that the change of NOB population can increase the nitratation activity of a granular sludge bioreactor which previously showed a successful strategy to achieve NOB repression. Further research on NOB competition should be addressed under partial nitritation conditions to successfully design strategists for the implementation of full-scale engineering systems.

6. Acknowledgements

This study was funded by the Spanish Ministerio de Economía, Industria y Competitividad (MINECO), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER, EU), CTQ2017-82404-R. Clara Reino acknowledges the Xunta de Galicia (Spain) and the CRETUS Institute of Santiago de Compostela for her postdoctoral fellowship (ED481B-2019-008). The authors are grateful to Dr Julio Pérez for helpful discussions during the course of this research.

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