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Nitrification upon Nitrogen Starvation and Recovery: Effect of Stress Period, Substrate Concentration and pH on Ammonia Oxidizers' Performance

Leila Abbaszadeh [1], Eleni Koutra [1], Konstantina Tsigkou [1], Maria Gaspari [2], Panagiotis G. Kougias [2] and Michael Kornaros [1,*]

[1] Laboratory of Biochemical Engineering & Environmental Technology (LBEET), Department of Chemical Engineering, University of Patras, 26504 Patras, Greece
[2] Soil and Water Resources Institute, Hellenic Agricultural Organisation Demeter, Thermi, 57001 Thessaloniki, Greece
* Correspondence: kornaros@chemeng.upatras.gr

Abstract: Nitrification has been widely applied in wastewater treatment, however gaining more insight into the nitrifiers' physiology and stress response is necessary for the optimization of nutrient removal and design of advanced processes. Since nitrification initiates with ammonia oxidation performed by ammonia-oxidizing bacteria (AOB), the purpose of this study was to investigate the effects of short-term ammonia starvation on nitrogen uptake and transformation efficiency, as well as the performance of starved nitrifiers under various initial substrate concentrations and pH values. Ammonium deprivation for 3 days resulted in fast ammonium/ammonia accumulation upon nitrogen availability, with a maximum uptake rate of 3.87 mmol $g_{protein}^{-1}$ min^{-1}. Furthermore, a delay in the production of nitrate was observed with increasing starvation periods, resulting in slower recovery and lower nitrification rate compared to non-starved cells. The maximum accumulation capacity observed was 8.51% (*w*/*w*) independently of the external nitrogen concentration, at a range of 250–750 mg N L^{-1}, while pH significantly affected ammonia oxidizers' response, with alkaline values enhancing nitrogen uptake. In total, ammonia accumulation after short-term starvation might serve as an important strategy that helps AOB restore their activity, while concurrently it could be applied in wastewater treatment for effective nitrogen removal and subsequent biomass utilization.

Keywords: nitrification; ammonia-oxidizing bacteria (AOB); starvation; recovery; ammonia accumulation

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1. Introduction

Nitrification represents an established two-step biological process performed by two types of chemolithoautotrophic microorganisms, namely, ammonia-oxidizing bacteria (AOB) that convert ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) that convert nitrite to nitrate [1]. However, there are several deviations from traditional nitrification [2], including archaea capable of ammonia oxidation, anammox bacteria, which stand for ammonia oxidizers under anaerobic conditions, as well as comammox bacteria that have been recently discovered proving that direct oxidation of ammonia to nitrate can be also accomplished by a single microorganism, as in case of naturally isolated *Nitrospira* species [3,4]. Ammonia oxidizers constitute an indispensable part of ecosystems significantly contributing to the nitrogen cycle, atmospheric equilibrium, and wastewater treatment [5]. However, current processes often fail to effectively remediate all types of wastewaters in a sustainable way; therefore, a combination of different bioprocesses, such as partial nitrification, anammox, comammox, and denitrification can lead to advanced wastewater treatment [6,7]. Within nitrification, ammonia oxidation by AOB, also known as nitritation, mostly determines process velocity and is catalyzed by two types of enzymes, ammonia monooxygenase

(AMO), resulting in hydroxylamine (NH_2OH) as an intermediate, and hydroxylamine reductase (HAO), which oxidizes NH_2OH to nitrite [8], while both enzymatic levels can be affected under stress conditions [9].

In nature, as well as in engineered environments, such as wastewater treatment plants (WWTP), AOB are occasionally exposed to substrate surplus, restriction, or actual starvation, attributed to high competition with heterotrophic bacteria, other AOB and plants, variation in pH value, low mineralization rates, and changes in sewage concentration over periods of inactivity and complete shutdowns [9]. Furthermore, AOB in WWTP are usually encountered in clusters or flocs, with individual cells facing different substrate levels depending on the layer of the aggregate, which calls for various adaptations within ammonia oxidizers [9–11]. In this respect, there are several physiological and molecular traits, such as low energy demand and decay rate, cell-to-cell signals, cell mobility, stable enzymes, and RNAs that allow AOB to survive and maintain ammonia-oxidizing activity under unfavorable conditions [9]. Interestingly, different strategies toward N-source levels can explain the occurrence of AOB in oligotrophic or N-rich habitats [12], as well as the prevalence of specific species, e.g., heterotrophic AOB depending on the prevailing environmental conditions [13].

Investigation of the impacts of starvation on nitrifiers' behavior and cellular mechanisms of adaptation and recovery has been in the limelight of scientific research for several decades; however, there has been a renewed interest, with a view not only to investigate the adaptability of ammonia oxidizers, but also to select microbial consortia with desired characteristics and optimize wastewater treatment technologies [2,9]. Under this scope, the nitrogen removal efficiency of a partial nitrification-anammox reactor treating digested blackwater reached 95%, when subjected to successive periods of starvation and recovery [14]. Furthermore, the ammonia-oxidizing activity of non-starving *Nitrosospira briensis* cultures could reach up to 250 μM N h^{-1}, while 10 days of starvation negatively affected AMO mRNA levels, which were rapidly restored upon the addition of fresh substrate [15]. Similarly, in contrast to 16S rRNA and HAO that were retained during starvation [16], AMO mRNA was also affected in cases of an enriched culture of freshwater ammonia oxidizers (AOB-G5-7); however, ammonia-oxidizing activity was restored after a starvation period of 50 days [17]. A very fast recovery has been also described in the case of *Nitrosomonas europaea*, even after 342 days of starvation, indicating that stable cellular components ensure immediate utilization of the energy source as soon as it becomes available [18,19]. Lastly, a rapid uptake of ammonium in the cells prior to recovery of ammonia oxidation activity has been observed, which probably served as a maintenance strategy in the case of low ammonium concentration [20].

However, the stress responses observed within AOB are mostly species-specific and depend highly on the previous condition of a culture [18] and the starvation period [15], as well as the existence of cells in biofilms or suspensions [21], or the co-existence with other nitrifiers in mixed populations that are encountered in nature [5]. Furthermore, emphasis has been previously given to single species or different stress conditions, as well as to partial nitrification, which is subsequently combined with other processes. Therefore, there is limited knowledge on the effect of short-term starvation on enriched nitrifying cultures. Under this scope, the purpose of the present study was to investigate the effect of nitrogen starvation and recovery on enriched nitrifying cultures, derived from a WWTP treating municipal wastewater. To this end, the uptake and transformation efficiency of all nitrogen forms (total nitrogen, ammonium, nitrite, and nitrate) were studied in correlation with short-term starvation, different starvation periods, as well as with various initial substrate concentrations and pH values. The ultimate goal was to shed light on nitrifiers' physiology and behavior, as well as their responsiveness to starvation and exposure to different ammonium concentration levels, thus unraveling nitrification under various conditions, usually encountered both in natural habitats and in WWTPs, concurrently offering opportunities for enhanced treatment processes.

2. Materials and Methods

2.1. Isolation and Enrichment of Nitrifying Microorganisms

Seed sludge was sampled from the WWTP located on the University campus in Patras (Rio, Patras, Greece). The synthetic growth medium used for the enrichment of nitrifying microorganisms included ammonium chloride (NH_4Cl) as a nitrogen source. In addition, potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were used both to buffer pH in the range of 7.3–7.4 and to provide a phosphorus source for the sludge. Sodium hydrogen carbonate ($NaHCO_3$) was added in excess, to ensure that the nitrification process was not limited by alkalinity. The detailed composition of the synthetic wastewater was: 0.956 g L^{-1} NH_4Cl, 10.52 g L^{-1} K_2HPO_4, 4.72 g L^{-1} KH_2PO_4, 3.52 g L^{-1} $NaHCO_3$, and 1 mL L^{-1} trace elements, including 1 g L^{-1} $FeSO_4 \cdot 7H_2O$, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.25 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.25 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.1 g L^{-1} H_3BO_3, and 5 mL L^{-1} H_2SO_4. During the isolation procedure followed, 50 mL of the initial amount of active sludge was mixed with 450 mL of synthetic growth medium in a Duran flask (Schott). The flask was incubated in a stirred water bath, at 25 °C and 100 rpm. To ensure aerobic conditions, air was provided by means of a ventilation pump under a constant flow rate of 2 L min^{-1}. Upon complete oxidation of ammonia to nitrate, biomass was collected through centrifugation and was inoculated in a fresh growth medium. This process was repeated over 8 months, and lastly, the enriched nitrifying granules were dispersed through sonication prior to experimentation.

2.2. Experimental Setup

The effect of short-term starvation, starvation period, and the initial concentration of ammonium and pH on the performance of AOB and nitrification process was investigated in the present study. Duplicate experiments were performed in a total volume of 250 mL, TSS concentration of 500 mg L^{-1}, temperature of 25 °C, at pH of 7.2–7.8, and subjected to continuous aeration conditions by ventilation pump. Concerning starvation, the enriched nitrifying bacterial culture was cultivated in a nitrogen-depleted medium for 1, 2, 3, 4, and 5 days, respectively, and was subsequently exposed to 250 mg NH_4^+-N L^{-1}. Concurrently, the performance of non-starved cells was used as a control test. Furthermore, the effect of various ammonium concentrations, 100, 250, 500, and 750 mg NH_4^+-N L^{-1}, on substrate uptake was tested on 3-day-starved cells. The range of ammonium concentration chosen was higher than usually measured in conventional municipal WWTPs. However, wastewater may contain a much wider range of ammonia concentrations, depending on its origin and type [22,23], as, for example, in the case of mature landfill leachate in which the ammonia concentration usually exceeds 1500 mg NH_4^+-N L^{-1} [7]. Lastly, the effect of pH on nitrification was assessed by inoculating 3-day-starved cells in a growth medium containing an ammonium concentration of 250 mg NH_4^+-N L^{-1}, at pH values of 6.5, 7, 7.5, 8, and 8.5. In all experiments, nitrogen removal and nitrogen transformation were evaluated for 4 h upon inoculation.

2.3. Analytical Methods and Statistical Analysis

In order to assess the process of nitrification, determination of nitrite (NO_2^-) and nitrate (NO_3^-) was performed by Ionic Chromatography (DIONEX ICS-3000) while ammonium nitrogen was measured according to the Phenate method [24]. Total Nitrogen was measured by a TOC-TN analyzer (TOC-V_{CSH}-TNM-a, SHIMADZU), after which, nitrogen types were transformed to NO_3^--N through digestion. Protein determination was performed through Bradford assay [25] after biomass pre-treatment, which included the addition of 0.5 M NaOH, sonication for 30 min, and incubation at 100 °C for 120 min, as previously described [26]. Biomass concentration was measured as dry cell weight (DW), according to a modified method of *Standard Methods* for estimation of Total Suspended Solids (TSS) [24], using GF/F grade filters. The specific accumulation rate of ammonium (mmol $g_{protein}^{-1}$ min^{-1}) was estimated by dividing the amount of ammonium removed from the medium, excluding the amount converted to nitrite and nitrate, with grams of

protein per time. In addition, specific nitrogen accumulation capacity (%) was estimated by dividing the difference in nitrogen concentration in the medium with TSS (mg L^{-1}). Lastly, nitrification rate was expressed as the amount of nitrate-nitrogen produced per mg TSS and min (mg NO_3^--N/mg TSS min). All the experiments were performed in duplicate and average values ± standard deviation (SD) are presented in the results. One- and two-way ANOVA at a confidence level of 95% (p-value > 0.05) as well as Tukey's post-hoc test were used for the statistical analysis of the experimental results (Minitab 19).

2.4. DNA Extraction and Sequencing Data Analysis

A representative sample of the culture (35 mL) was collected, while 25 mL were used for genomic DNA extraction (DNeasy PowerSoil Pro kit, QIAGEN, Germantown, MD, USA). More details about the procedure, the protocol, the quantification, and the qualification assessment can be found in the work of Tsigkou et al. [27]. Raw reads obtained from 16S rRNA gene sequencing were deposited in Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov, accessed on 2 August 2022) under the Bioproject PRJNA865123, with the SRA accession number of SAMN30098149, 206,392 raw reads, 82,792 assigned reads, and 354 OTUs.

3. Results and Discussion

3.1. Microbial Analysis of the Culture

Besides natural occurrence, nitrification represents an established technology widely applied in WWTP worldwide. However, microbial performance during nitrification at large-scale applications can be further enhanced, while an emphasis should be given to the changes observed in population dynamics and performance, in order to ensure stable nitrification [22]. Therefore, gaining more insight into the physiology of the consortia involved and their adaptations to critical environmental factors is of high importance for the optimization of nutrient removal and design of advanced processes.

Regarding the identification of the microbial community, only bacteria were present in the enriched inoculum, and more specifically, 50 species were found in relative abundance in the total microbiome, while no archaea were detected. As presented also in Figure 1, Bacteroidetes dominated (42% of the total bacterial community), while Proteobacteria, Actinobacteria, Firmicutes, and Deinococcus-Thermus phyla followed, which were identified at lower percentages. Concerning the most abundant species, *Nitrosomonas eutropha 44*, belonging to the Proteobacteria phylum, accounted for 6.65% of the total microbiome. *Nitrosomonas eutropha* is a non-pathogenic, salt-tolerant AOB, which prefers high concentrations of NH_4^+-N [28,29]. Furthermore, two new species of the Firmicutes phylum were dominant, at 5.92% (*Firmicutes sp. 37*) and 14.09% (*Firmicutes sp. 58*) of the total microbiome, respectively. According to literature, microorganisms belonging to Firmicutes that participate in microbial nitrification can resist in extreme environments, while, specifically, some heterotrophic nitrifying Firmicutes are able to produce extracellular enzymes, facilitating the hydrolyzation and utilization of solid biopolymers [30,31], as well as two new species, categorized in the phylum of Bacteroidetes. More specifically, these species belonged to Bacteroidetes, but in different families. The most abundant, at 22.64%, was the *Weeksellaceae sp. 5*. Bacteria of such a family are Gram-negative, non-spore-forming, chemoorganotrophic pathogenic, with probable fermentative metabolism and variable oxidase and catalase activities [32,33]. The second dominant species of the Bacteroidetes phylum belonged to the Chitinophagaceae family, with a total amount of 5.80% (*Chitinophagaceae sp. 20*). Chitinophagaceae bacteria are heterotrophic AOB and have been also reported in the literature as dominant nitrifiers during the total nitrogen removal from anaerobic digestate [34].

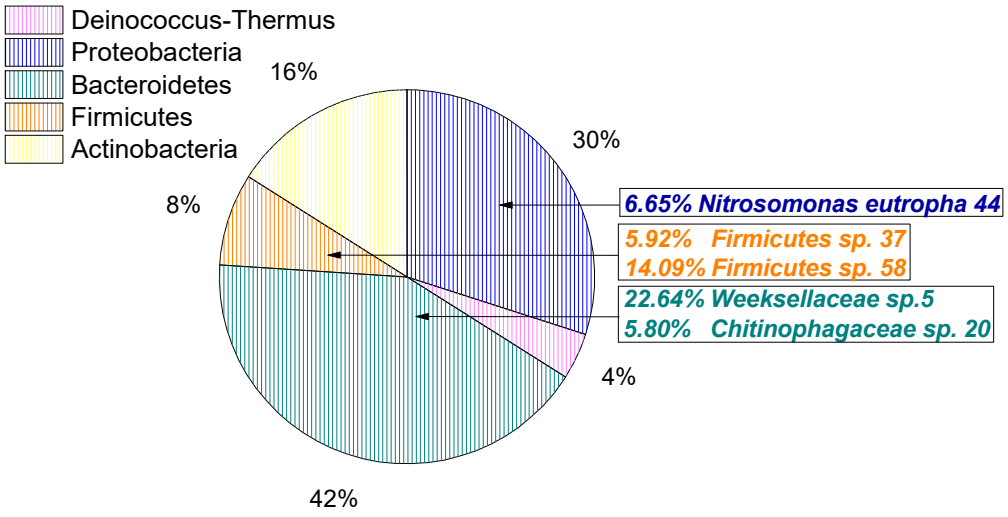

Figure 1. Taxonomic composition of the enriched inoculum bacterial community at phylum levels (the percentages refer to the distribution of the detected bacteria to the relevant phyla) and an indication of the most abundant microorganisms (in terms of the total microbiome).

3.2. The Effect of Short-Term Starvation and Starvation Period on Substrate Uptake

The enriched nitrifying culture was initially deprived of ammonium nitrogen for 3 days, after which cells were exposed to 250 mg N L^{-1} through the addition of NH_4Cl, in order to evaluate the effect of starvation on ammonia oxidation and nitrification, compared to non-starved cultures, which served as control (exposed though at a slightly higher initial concentration of 280 mg NH_4^+-N L^{-1}). As shown in Figure 2, the decrease in the concentration of ammonium (278 mg N L^{-1}) in the case of non-starved cells was accompanied by the simultaneous production of nitrate (257.5 mg N L^{-1}), while the concentration of total nitrogen (calculated as the sum of NO_2^-, NO_3^-, and NH_4^+ nitrogen) was almost constantly decreasing due to nitrogen assimilation in the nitrifying biomass (Figure 2a). In addition, 7.4% of the starting ammonium nitrogen was calculated to be assimilated by both AOB and NOB at the end of this batch experiment. In contrast, when nitrifying bacteria were exposed to short-term starvation, a fast decrease in the concentration of ammonia was observed within the first few minutes upon ammonia addition, from 250 mg N L^{-1} to 238.5 mg N L^{-1} after just 5 min, while neither nitrate nor nitrite was concomitantly produced (Figure 2b). Through Total Nitrogen determination, it was confirmed that 27.5 mg N L^{-1} in the form of ammonia was accumulated by the culture within the first 240 min, while nitrification by starved cells was completely restored by the end of the experiment, similar to the non-starved culture, accompanied by a gradual increase in total nitrogen concentration. Furthermore, 5.6% of the starting ammonium nitrogen was calculated to be assimilated by the nitrifying culture at the end of this run. These findings are in agreement with previous results showing a fast recovery of AOB activity in response to short-term starvation (up to 10 days) [15]. Furthermore, a rapid increase in AMO activity of washed and stored overnight *Nitrosomonas europaea* cells has been described upon inoculation in 50 mM ammonium, followed by a slow decrease to the initial levels [35]. Such a response was attributed to the ability of the ammonia oxidizer tested to maintain a standard level of enzyme activity not only for cell maintenance but also for fast recovery when nitrogen becomes available again, while the increase was affected by ammonia levels [35]. Furthermore, a rapid increase in the ammonia removal efficiency of a nitrifying infiltration system up to 90.6% was also observed upon recovery after 7 days of starvation, which was subsequently reduced to 63.5% [36].

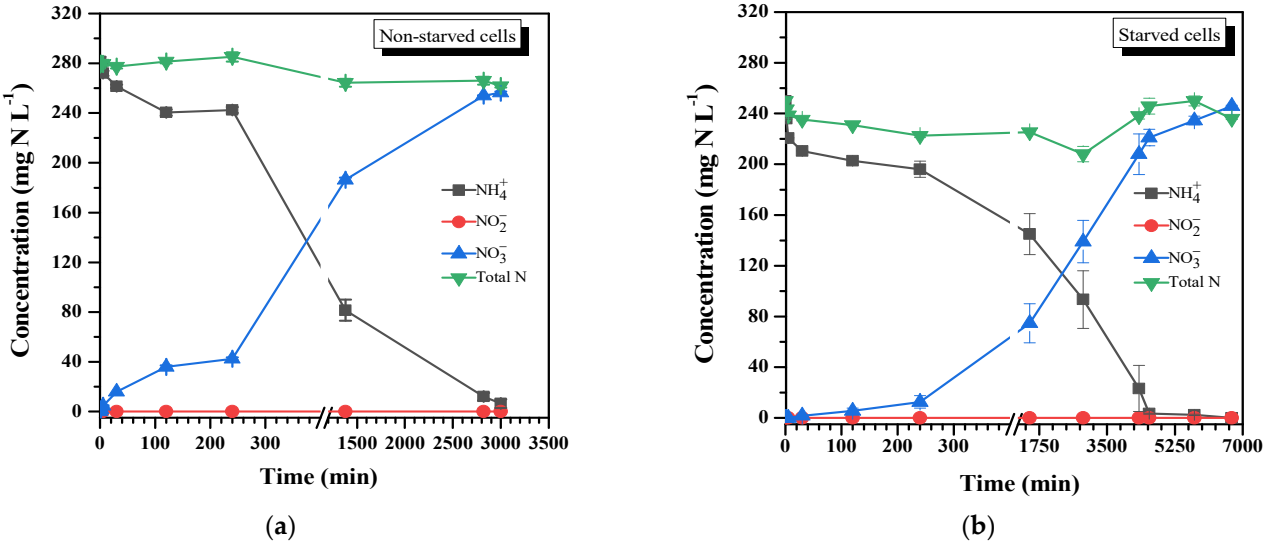

Figure 2. The process of nitrification performed by non-starved (**a**) and 3-day-starved (**b**) cells of enriched nitrifying cultures. Incubation conditions included temperature of 25 °C, initial NH_4^+-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

Subsequently, in order to investigate the impact of the starvation period on the ammonia/ammonium accumulation, the enriched nitrifying culture was incubated for 0, 1, 2, 3, 4, and 5 days in the absence of ammonium prior to the addition of 250 mg N L^{-1} in the medium. Figure 3a depicts the decrease in total nitrogen with time within the first 240 min of these experiments. Such a decrease corresponds to the amount of nitrogen removed from the liquid phase and transferred inside the cells, either for assimilation or accumulation. Regarding the statistical analysis of the results, the *p*-value was equal to zero for both variables (time and starvation days), indicating statistical significance, after two-way ANOVA. However, pairing (no statistically significant differences) of the results was observed for time between 0 and 2 min, 2 and 5 min, as well as 5 and 240 min. As expected, in non-starved cells, i.e., 0 days of starvation, no decrease was observed since the amount of nitrogen assimilated by the culture is negligible within such a short timeframe. However, in the case of 1-day-starved cells, the concentration of the ammonium in the medium decreased from 250 mg L^{-1} to 246.3 mg L^{-1} within 2 min, and to 243.5 and 236 mg L^{-1} for 2 and 3-day-starved cells, respectively. In addition, by further increasing the duration of starvation from 3 to 5 days, the accumulated amount of ammonia/ammonium increased slightly and remained constant, indicating no statistically significant differences for longer starvation periods (Figure 3a). Interestingly, nitrogen accumulation was observed within the first 5 min after nitrogen addition without simultaneous nitrite or nitrate formation, in contrast to non-starved cultures in which nitrate production started almost immediately upon inoculation (Figure 3b). A delay in the production of nitrate was observed in the case of all short-term starved cultures, with increasing starvation periods resulting in slower recovery and lower values of nitrification rate compared to non-starved cells. Both variables exhibited statistically significant differences (*p*-value = 0), after two-way ANOVA, indicating pairing for the results of 0–2–5–30 min, and the starvation periods of 0–1 days and 2 to 5 days. In fact, a 92.3% decrease in nitrate production (1.2 10^{-4} mg NO_3^--N mg TSS^{-1} min^{-1}) was realized in the case of 5-day-starved cells compared to the non-starved culture. Similarly, a previous study on the effects of 1, 2, and 3 weeks of starvation and recovery showed that longer periods of nitrogen deprivation resulted in more pronounced effects on nitrification activity and slower recovery upon fresh substrate addition [36]. In contrast, nitrite production from *N. europaea* started within up to 2 h after ammonium addition, independently of a starvation period between 1 and 10 weeks [12]. Furthermore, the maximum specific ammonium accumulation rate demonstrated in the present study was 3.87 mmol $g_{protein}^{-1}$ min^{-1} in

the case of the 3-day-starved cells, while no further increase was observed in the case of 4- and 5-day-starved cultures (Figure 4). In contrast, the uptake rate by non-starved cells was negligible. Similar to these results, Schmidt et al. [20] also reported an ammonium accumulation rate of 3.1 mmol $g_{protein}^{-1}$ min^{-1} in the case of starved cells of *N. europaea* without concomitant nitrite production, possibly preparing AOB for coping with famine.

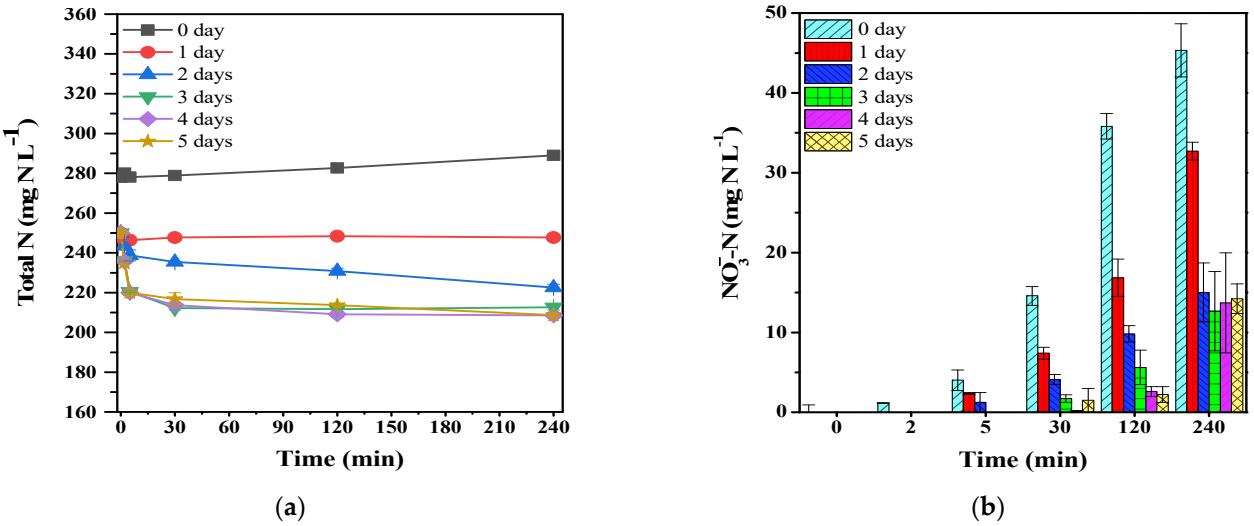

Figure 3. Variation in Total Nitrogen concentration (mg L^{-1}) (**a**) and nitrate production (**b**) in non-starved nitrifying cultures and after 1 to 5 days of starvation. Incubation conditions included temperature of 25 °C, initial NH_4^+-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

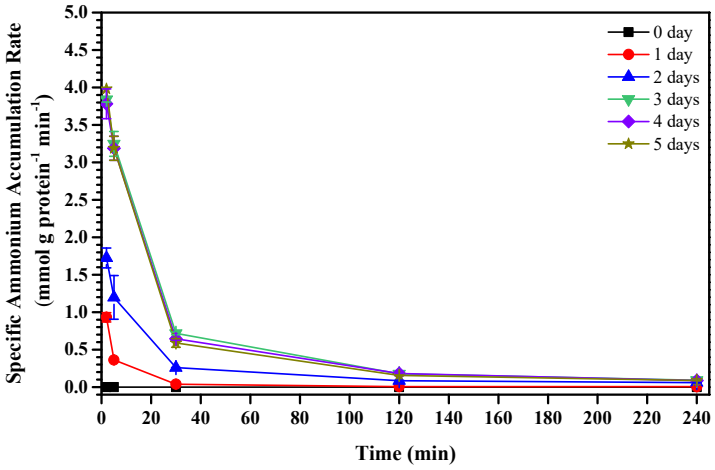

Figure 4. Effect of starvation period on the Specific Ammonium Accumulation Rate (mmol $g_{protein}^{-1}$ min^{-1}) upon inoculation in 250 mg N L^{-1}. Incubation conditions included temperature of 25 °C, initial NH_4^+-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

3.3. The Effect of Substrate Concentration on Nitrogen Removal by Starved Cells

In order to further evaluate the accumulation capacity of ammonium by AOB, 3-day-starved cells were incubated in batch reactors with different initial ammonium concentrations, namely, 100, 250, 500, and 750 mg N L^{-1}, respectively. As presented in Figure 5, at external concentrations between 250 and 750 mg N L^{-1} the maximum accumulation capacity observed was 8.51% (mg N mg TSS^{-1}), independently of the ammonium concentration in the cultivation medium (p-value = 0.889). Therefore, no higher internal nitrogen content could be observed by increasing ammonium levels in the culture medium,

in accordance with previous findings, indicating that AOB fail to establish an internal ammonium pool higher than 1 M even in the presence of external nitrogen concentration up to 1000 mg N L^{-1} [20]. In contrast, when the external ammonium concentration was relatively low (~100 mg N L^{-1} or lower), the maximum specific ammonium accumulation rate decreased from 3.9 to 2.19 mmol $g_{protein}{}^{-1}$ min^{-1} and the maximum content reached 3.32% (mg N mg TSS^{-1}). In addition, the actual nitrification substrate for AOB has been investigated in chemostats with an effluent concentration between 1 and 1000 mg N L^{-1} [37], which in the case of values lower than 100 mg N L^{-1}, an ammonium transport system was shown to be activated for ammonium uptake, in contrast to high ammonium/ammonia nitrogen levels in the environment, when ammonia is preferred. In addition, at low substrate concentrations, the production of specific bacterial transport proteins has been previously described in order to meet nitrogen requirements [38,39]. Therefore, the lower ammonium accumulation rate and internal nitrogen content observed in this study at low (~100 mg N L^{-1} or lower) ammonium cultivating conditions compared to the respective rates and nitrogen content at higher ammonium concentrations could be probably attributed to differences in the form of nitrogen taken up by the AOB at different external ammonium concentrations.

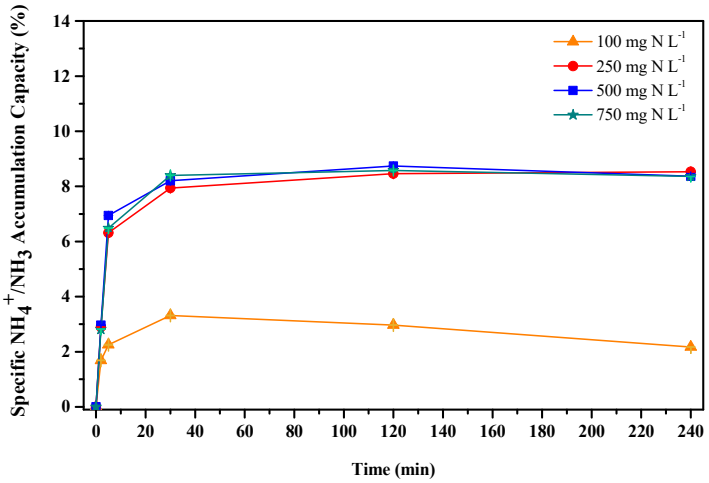

Figure 5. Specific ammonium/ammonia accumulation capacity (%) of 3-day-starved cells upon inoculation in various substrate concentrations Incubation conditions included temperature of 25 °C, initial $NH_4{}^+$-N concentration of 250 mg N L^{-1}, and cell density of 600 mg TSS L^{-1}, equivalent to 1.29×10^9 cells mL^{-1}.

Furthermore, in the present study, 'true' nitrification (conversion of ammonium ultimately to nitrate nitrogen) started approximately 30 min after the onset of the experiments. However, following the previous observations on ammonium accumulation, the specific nitrification rate was constant at 0.0065 mg $NH_4{}^+$-N mg TSS^{-1} h^{-1}, irrespective of the initial ammonium concentration in the range from higher than 100 up to 750 mg $NH_4{}^+$-N L^{-1}; being almost twofold lower (0.003 mg $NH_4{}^+$-N mg TSS^{-1} h^{-1}) at concentrations lower than 100 mg $NH_4{}^+$-N L^{-1}. Similar to these findings, a constant specific ammonia oxidation rate has also been reported at initial ammonia concentrations between 5.6 and 90 mg L^{-1} [40]. Moreover, an increase in AMO activity has been previously observed in *N. europaea* in response to increasing $NH_4{}^+$ concentrations from 5 to 50 mM [35]. However, the molecular and physiological responses of AOB towards the environmental nitrogen levels seem to vary significantly, as in the case of AMO mRNA levels in *N. europaea*, which increased in the presence of $(NH_4)_2SO_4$ between 0.013 and 2 mM, while no further increase was observed at higher substrate concentrations [41]. Interestingly, the maximum nitrification rate of an enriched population of nitrifiers, 218 mg N L^{-1} d^{-1}, only slightly varied at initial ammonium concentrations between 500 and 3000 mg N L^{-1}, indicating the minor effect of the initial substrate concentration at a specific range, on nitrification kinetics [42].

3.4. The Effect of pH on Substrate Uptake by Starved Cells

Free ammonia availability, which is strongly determined by pH values, can either have a stimulatory or inhibitory effect on the activity of AOB [8]. Subsequently, in the present study 3-day-starved cultures were exposed at the same initial ammonium nitrogen concentration (approx. 250 mg N L^{-1}) but at different pH values, ranging from 6.5 to 8.5 therefore to different ratios of NH_3 to NH_4^+, since at nearly neutral pH the equilibrium of NH_3-NH_4^+ is shifted towards NH_4^+ [35]. As presented in Figure 6a (detailed experimental data can be found in the Supplementary Materials (Figures S1 and S2)), Total Nitrogen remained almost constant in the case of non-starved cells, independent of the pH value of the medium. Similarly, at almost neutral pH (6.5 and 7) Total Nitrogen appeared also unaffected in the case of 3-day-starved cells, in contrast to higher pH values, which resulted in nitrogen accumulation by AOB (Figure 6b). In total, the specific accumulation rate of the ammonium/ammonia nitrogen calculated during the first 5 min of cultivation varied with different pH values, starting from a negligible rate at pH 6.5 and reaching a maximum of 3.2 mmol $g_{protein}{}^{-1}$ min^{-1} at pH 8 (Figure 7). The maximum specific accumulation rate did not exhibit statistically significant differences (p-value = 0.2) and is in agreement with previous findings [20]. However, by taking into consideration that this work was carried out using an enriched nitrifying culture, the maximum value of the accumulation rate presented by the AOB used in this study is higher than the value of 3.1 mmol $g_{protein}{}^{-1}$ min^{-1}, which was previously reported for pure *Nitrosomonas europaea* cultures [20]. In total, high pH values, characterized by higher levels of free ammonia in the cultivation medium, resulted in higher accumulation rates probably due to the tendency of AOB to uptake ammonia rather than ammonium for their cellular metabolism [20,37]. In contrast, at pH values lower than neutral-alkaline levels, the concentration of free ammonia tends to decrease, resulting in reduced growth and substrate uptake, as well as reduced enzyme activity [1]. The significant effect of pH on nitrification has been also highlighted in the case of the overnight-starved *N. europaea* incubated at various ammonia levels owing to different pH values (6.3, 7.5, and 8.5), which was characterized by decreased AMO activity at a less alkaline pH [35].

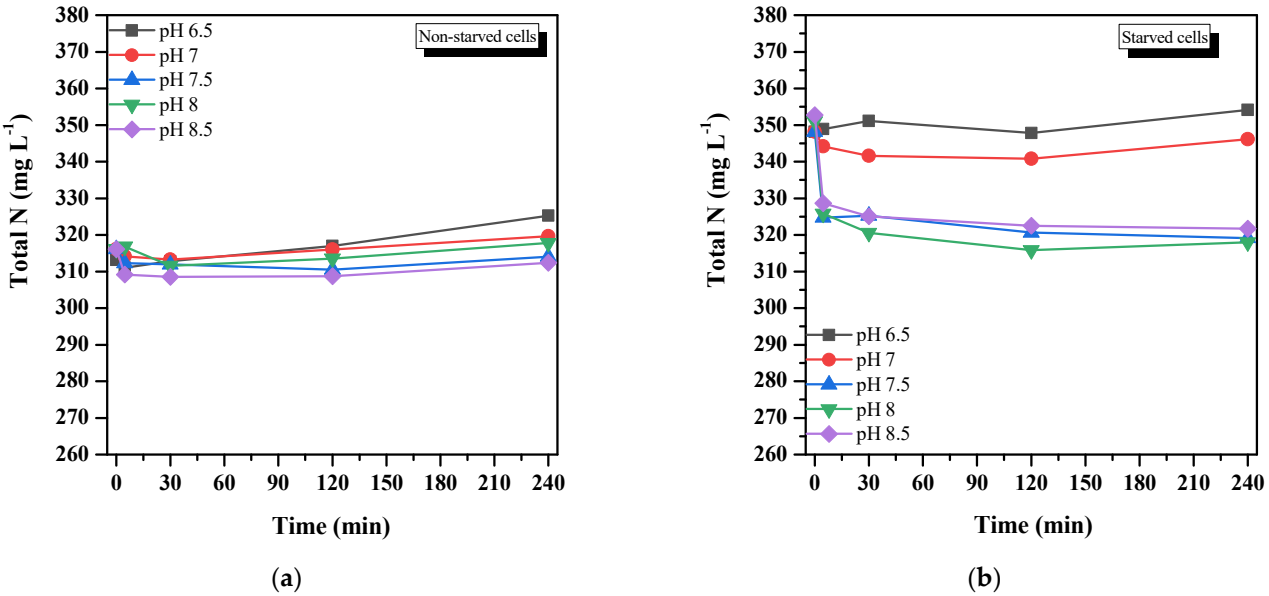

Figure 6. Impact of medium pH on variation in Total Nitrogen concentration (mg L^{-1}) in non-starved (**a**) and 3-day-starved cells (**b**) of nitrifying cultures. Incubation conditions included temperature of 25 °C, fresh NH_4^+-N addition of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

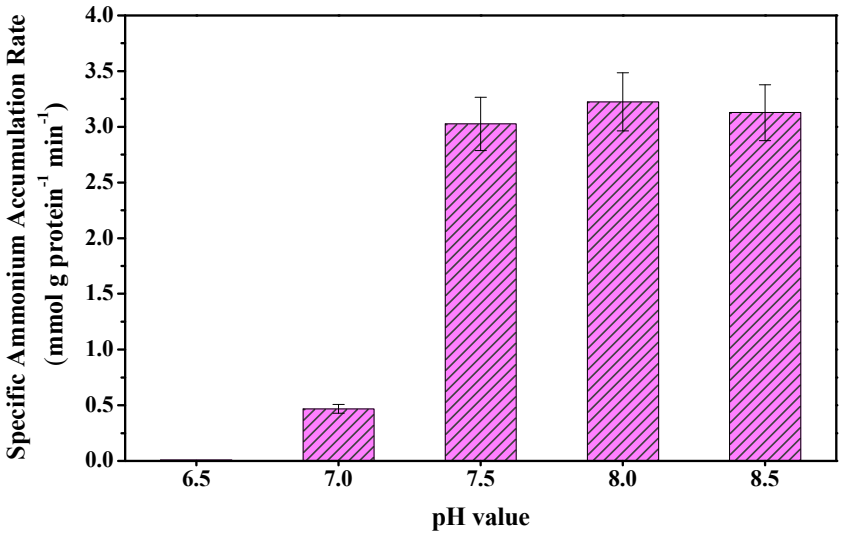

Figure 7. Effect of pH on the maximum Specific Ammonium Accumulation Rate (mmol $g_{protein}{}^{-1}$ min^{-1}) upon addition of 250 mg $NH_4{}^+$-N L^{-1}. Incubation conditions included temperature of 25 °C, initial $NH_4{}^+$-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

4. Conclusions

In the present study, nitrification was investigated upon nitrogen starvation and recovery, highlighting the effects of stress period, substrate concentration, and pH on an enriched ammonia-oxidizing culture. In contrast to typical AOB behavior, nitrogen starvation resulted in fast ammonium/ammonia accumulation, while a delay in the production of nitrate was observed with an increasing starvation period, resulting in slower recovery and a lower nitrification rate compared to non-starved cells. In this sense, such conditions (exposure of nitrifiers to nitrogen starvation) may have adverse implications on the overall nitrogen removal rate in WWTPs. In addition, based on the findings of this study, the maximum accumulation capacity remained constant at external ammonium concentrations between 250 and 750 mg N L^{-1} (p-value = 0.889), while nitrogen accumulation by starved AOB varied significantly with different pH values (p-value = 0). Based on the above, prompt nitrogen uptake by starved nitrifiers upon ammonium availability might serve as a survival strategy that helps AOB restore their activity and increase their metabolism after a period of nitrogen limitation. In this respect, ammonia accumulation might represent an important advantage in terms of the competition for substrates between similar bacterial groups, while it could be also used as a strategy applied in wastewater treatment for effective nitrogen removal and concomitant production of valuable nitrogen-loaded biomass, which could be further valorized, for instance, as a biofertilizer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080387/s1, Figure S1: The process of nitrification performed by non-starved cells of enriched nitrifying cultures at different pH values. Incubation conditions included temperature of 25 °C, initial addition of $NH_4{}^+$-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}; Figure S2: The process of nitrification performed by 3-day-starved cells of enriched nitrifying cultures at different pH values. Incubation conditions included temperature of 25 °C, initial addition of $NH_4{}^+$-N concentration of 250 mg N L^{-1}, and cell density of 500 mg TSS L^{-1}, equivalent to 1.29 × 10^9 cells mL^{-1}.

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