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Decrease in Oxygen Concentration for the Fast Start-Up of Partial Nitritation/Anammox without Inoculum Addition

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Abstract: Initiating the partial nitritation and anammox (PN/A) process without inoculation poses a significant challenge. Thus, there is a notable amount of interest in devising a straightforward strategy for the start-up of PN/A. This study demonstrates the feasibility of achieving the rapid start-up of a one-stage PN/A process within a moving-bed sequencing batch biofilm reactor (MBSBBR) by reducing the oxygen concentrations: 3.0 mg O_2/L (Stage I), 2.0 mg O_2/L (Stage II), and 1.0 mg O_2/L (Stage III). The anammox activity was observed 15 days after a gradual decrease in the oxygen concentration and confirmed using a specific anammox activity test (5.9 mg N/gVSS·h). During Stage III, the average total inorganic nitrogen (TIN) removal efficiency was 60.6%. The relative abundance of planctomycetes, a typical phylum representing anammox microorganisms, increased almost three times from Stage I (2.8%) to Stage III (7.1%). These results demonstrate the potential of a decrease in oxygen concentration for the fast start-up of the one-stage partial nitritation and anammox process without inoculation. The implementation of the studied DO strategy has practical implications for wastewater treatment plant operators, particularly in the start-up of the PN/A processes. Additionally, batch assays allow for the rapid assessment of treatment plant performance, providing real-time insights into its functionality and, thereby, optimizing wastewater treatment practices.

Keywords: PN/A; batch assay; fast start-up; microbial community; MBSBBR

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1. Introduction

The partial nitritation and anammox (PN/A) process is a promising and energy-efficient method, where ammonia (N-NH_4) is first oxidized to nitrite nitrogen (N-NO_2) and, subsequently, the remaining N-NH_4 together with N-NO_2 are converted into nitrogen gas [1]. The first step is conducted using ammonia oxidizing bacteria (AOB) under aerobic conditions and the second using anaerobic ammonia oxidation bacteria (anammox) under anoxic conditions. The PN/A process has attracted particular attention due to the widespread pursuit of energy self-sufficiency in wastewater treatment plants. The PN/A process reduces sludge production by approximately 90%, organic carbon consumption and dissolved oxygen (DO) demand by around 60%, permitting energy savings due to aeration [2]. The process is most commonly used for treating ammonia-rich sidestream effluent. Due to biofilm's relative resistance to anammox biomass washout and easier control over the population of nitrite oxidizing bacteria [3], the most commonly used reactor for PN/A is the moving-bed biofilm reactor (MBBR) [4].

The PN/A process is highly demanding due to the impact of several factors on the process, such as the optimal dissolved oxygen concentration, pH, temperature, aeration modes, and organic compounds. The greatest challenge in initiating the PN/A process is to keep the balance among the functional microbes in the context of anammox and nitrite

oxidizing bacteria (NOB) competition for N-NO_2, in conjunction with AOB and anammox variant living conditions. Therefore, NOB suppression is critical and the most challenging aspect for the PN/A process start-up and operation stability [5]. Several factors, namely the nitrogen load rate (NLR), low dissolved oxygen (DO), temperature, and free ammonia (FA), contribute to successful NOB activity inhibition. Although the literature offers many publications regarding PN/A process start-up, operation, and control [6,7], they provide various start-up performances and durations. DO is the easiest factor to control in a typical municipal wastewater treatment plant [8]. If the DO concentration is limited, NOB cannot successfully compete with AOB due to the lower oxygen affinity [9]. Lv et al. [10] initiated a PN/A system by reducing the DO (from 0.9 to 0.4 mg O_2/L) and changing the hydraulic retention time. Besides a low DO concentration, several strategies regarding aeration control were applied to start-up the PN/A process. Huang et al. [11] achieved start-up of the PN/A process in 30 days by implementing pre- and post-non-aeration. Another start-up was reached by gradually reducing the DO and regulating the nitrite [12]. The combination of applying an intermittent aeration mode and a low DO concentration of 0.5 mg O_2/L was also reported as an effective strategy [13]. Another method combining intensifying agitation and promoting the aeration rate successfully enhanced the PN/A process. NOB suppression was achieved by applying a special type of aeration control consisting of anaerobic–oxic–anoxic modes [11]. Pereira et al. [14] reported aeration strategies of 30 and 45 min with anoxic periods in relation to 15 min aerobic periods, coupled with low air flow rates, which were better for PN/A establishment.

Anammox development in biofilm is a slow process due to anammox's low specific growth rate resulting in a prolonged PN/A reactor start-up [3]. Therefore, the vast majority of laboratory and full-scale plants tend to use inoculum in order to mitigate the risk of excessively long start-up periods. However, even after seeding the reactor with inoculum, the start-up periods can reach several months. It has been reported that with 10% seed media from an established sidestream deammonification MBBR process, start-up was completed after 120 days [15]. Kowalski et al. [3] achieved start-up of a PN/A process in 56 days by developing a partial nitritation biofilm first and then seeding it with anammox biomass from a lab-scale MBBR operated for approximately 1 year, together with using bio-primer coated media. Chen et al. [16] initiated a PN/A process within 40 days in a reactor inoculated with mixed denitrifying-anammox granular sludge at a 3:1 volume ratio. The effectiveness of inoculation relies on the anammox culture's adaptability to a new environment, enabling it to thrive in the presence of the new influent. Furthermore, the amount of anammox bacteria is crucial if external seeding is used, as a higher concentration and volume result in a shorter start-up time. This is additionally associated with a large volume of external seeding transportation [7]. The availability of an adequate amount of seed material might not always be guaranteed, especially in many countries, where there are few or no full-scale anammox installations [3]. Therefore, reducing the start-up time of the anammox process without external inoculum is of great interest. A minority of anammox projects were set up without the addition of external inoculum. Kanders et al. [7] demonstrated that start-up took 120 days at full scale and 72 days at laboratory scale by using rejected water from thermophilic anaerobic digestion. Dimitrova et al. [17] successfully started a full-scale PN/A MBBR process without seeding within 111 days through intermittent aeration (DO from 0.6 to 0.8 mg O_2/L) and operation at inhibitory FA concentrations (5–20 mg/L).

Although the sidestream PN/A process has been widely settled at full scale, however, according to many scientific papers, the long and uncertain start-up time is still considered a bottleneck for PN/A process implementation [18]. It is therefore of great interest to develop a simple strategy for fast PN/A start-up. The current study aimed to determine a strategy for PN/A fast start-up in a moving-bed sequencing batch biofilm reactor (MBSBBR) without any anammox-enriched inoculum by reducing the DO concentration (from 3.0 to 1.0 mg O_2/L). This approach may provide new insight into shortening the PN/A start-up time with the application of only a DO control. This research also contributes directly

to the reduction of energy consumption in wastewater treatment plants, thus promoting sustainable operational practices. The comprehensive methodological scope distinguishes this study from the previous one. As a tool for tracking the changes in microbial activity, three types of batch assays were conducted: (a) the ammonia utilization rate (AUR) test, for AOB activity determination; (b) the nitrite utilization rate (NUR) test, for NOB activity determination; and (c) the specific anammox activity rate (SAA) test, for anammox activity determination. In this article, the nitrogen removal efficiency was studied. Additionally, it encompasses the results from the monitoring tests, including the analysis of nitrogen conversion in a complete cycle. Furthermore, to assess the impact of microbial communities on process performance, the microbial diversity and abundance of key functional groups were investigated using next-generation sequencing (NGS) analysis and compared to the technological results in the last part of the Results and Discussion section. Through the detailed analysis of the microbial activities and community dynamics, we aim to provide insights that can be instrumental in developing sustainable strategies for nitrogen removal, a critical aspect of wastewater treatment.

Due to the low specific PN/A microorganism's growth rate, most previous publications have focused on the long-term impact of DO changes on reactor performance [19,20], which prompted the additional question of whether short-term changes can be ruled out as having an impact on the microbial community. Therefore, in this study, the effect of short-term DO changes on the course of the processes occurring in the phases of the operational cycle was verified by means of monitoring tests, including the analysis of nitrogen conversion in a complete cycle. Additionally, 24 h after the introduced change, biomass samples were collected for NGS to check whether a short-term change affects the microbial diversity. Hence, the last segment of this article involves a comparative discussion regarding microbial diversity within the context of the monitoring test results.

2. Materials and Methods

2.1. Reactor Operation and Control

A laboratory-scale MBSBBR (working volume: 28 L) was used in this study (Figure 1). EvU-Perl biofilm carriers were applied as a moving bed, with an active surface of 600 m^2/m^3. The moving bed occupied approximately 25% of the working volume of the reactor. There were three 8 h operational cycles on a daily basis. Each cycle included 10 phases (Figure S1):

- Four aerobic phases (the first 3 lasted 95 min, the last 115 min);
- Four anoxic phases with dosing (before each aerobic phase, 2.5 L of sewage was dosed into the reactor);
- Sedimentation (50 min);
- Decantation (10 min).

Synthetic wastewater was dosed using a Masterflex® L/S® peristaltic pump (Chicago, IL, USA). The reactor's content was mixed with a low-speed mechanical paddle stirrer, R-50 (CAT, Ballrechten-Dottingen, Germany). Dispersing oxygen aquarium filters located at the bottom of the reactor used as an air distribution system were connected to an air pump providing the appropriate oxygen concentration in the system. A Memosens COS81D optical probe (Endress+Hauser, Weil am Rhein, Germany) connected to a Liquiline CM442 converter (Endress+Hauser, Weil am Rhein, Germany) was responsible for the measurement of the oxygen concentration and temperature in the reactor. The air pump was switched on or off automatically, depending on the actual indication of the oxygen concentration. A digital electrode, CPS471D (Endress+Hauser, Weil am Rhein, Germany), was responsible for pH measurement. The temperature at 30 °C was maintained through a jacket on the reactor, by means of a DD-200F heating circulator (JULABO GmbH, Seelbach, Germany). The automatic control system, SCADA Wonderware InTouch (version 2017-Update 2), was responsible for the maintenance of the set oxygen concentration and conducting technological operations by activating the raw wastewater dosing pump, a stirrer, and an electromagnetic decanting valve.

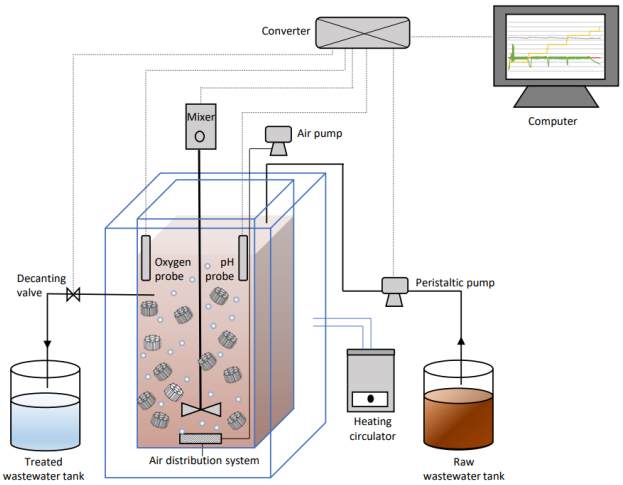

Figure 1. Schematic diagram of the MBSBBR.

2.2. Synthetic Wastewater

The wastewater contained a mixture of tap water and 15 mg/L of EDTA, 133.7 mg/L of NH_4Cl, 660 mg/L of NH_4HCO_3, 1.4 mg/L of $CaCl_2$, 60 mg/L of $MgSO_4$, 10 mg/L of $FeSO_4 \cdot 7H_2O$, 2.7 mg/L of KH_2PO_4, and 1000 mg/L of $NaHCO_3$. The influent characteristics were as follows: ammonia 154 mg N-NH_4/L, alkalinity 1000 mg $CaCO_3$/L, phosphate 2.6 mg P-PO_4^{3-}/L, temperature 30 °C, and pH 7.6–7.9. During the experiment, raw synthetic wastewater was prepared on a daily basis (except weekends).

2.3. Operation Conditions

The experiment was divided into three stages (I–III) based on different DO concentrations (Table 1). The research was initiated in a reactor, where a nitrifying biofilm had developed on carriers. The reactor facilitated complete nitrification. The process efficiency achieved prior to the commencement of this study was described in Series III.4 in another study [21]. The experimental stages were primarily selected to enable a shift from the nitrification to the PN/A reactor without inoculation and to evaluate the impact of a gradual decrease in the DO concentration on the reactor performance. In order to track how the technological changes implemented at subsequent stages affected the activity and structure of the microbial communities, batch assays were conducted, and samples of biomass were collected for next-generation sequencing.

Table 1. Reactor operation stages.

Stage	Duration (Days)	DO Concentration (mg O_2/L)
	Long-Term Change	
I	16	3.0
II	15	2.0
III	14	1.0
	Short-Term Change [1]	
A	1	2.0
B	1	1.0
C	1	3.0

[1] The effect of short-term change to the DO concentration was investigated at the end of Stage I.

Before starting Stage II, 3 additional research stages (A–C) lasting 1 day were designated in order to assess the impact of a short-term change in the DO concentration on the effectiveness of the nitrification process. Each stage involved analysis of the nitrogen conversion in one cycle.

After the completion of Stage I, the DO was reduced to 2.0 mg O_2/L (Stage A), and 16 h after the introduced change, the nitrogen conversion in one cycle was analyzed. Then, the DO was reduced to 1.0 mg O_2/L (Stage B), and the nitrogen conversion in one cycle was analyzed 16 h after the change was introduced. Then, the DO was increased to 3.0 mg O_2/L (Stage C), and the nitrogen conversion in one cycle was analyzed 16 h after the introduced change. In addition, 24 h after the change in the DO concentration, biomass samples were collected for next-generation sequencing to determine the effect of short-term changes to the DO concentration on the microbial community.

2.4. Monitoring Tests, Including the Analysis of Nitrogen Conversion in a Complete Cycle

Prior to the start of the monitoring tests, including the analysis of the nitrogen conversion in a complete cycle, raw wastewater was prepared in a manner analogous to the everyday wastewater preparation process. The test involved collecting samples from the reactor at regular intervals throughout the complete wastewater treatment cycle and analyzing the measured indicators of pollution. The first sample was collected just before the start of a new cycle to determine the baseline values of the quality indicators in the treated wastewater, before the addition of raw sewage. Samples were also collected at the end of each dosing phase, prior to the activation of the blowers that initiated the aerobic phase. The first three aerobic phases were divided into equal periods of approximately 32 min, and samples were collected at each designated time point. The final aerobic phase, due to its extended duration, was divided into four periods, with samples collected approximately every 29 min. After the sedimentation and decantation phase, the treated effluent was collected. The samples were analyzed for concentrations of ammonium, nitrite, nitrate, and alkalinity. Prior to analysis, the samples were passed through disposable syringe filters.

2.5. Batch Assays

The determination of the maximum activity of the anammox, AOB, and NOB functional groups involved performing three batch assays:

- The specific anammox activity rate (SAA) test, permitting the determination of the anammox activity;
- The ammonia utilization rate (AUR) test, permitting the determination of the AOB activity;
- The nitrite utilization rate (NUR) test, permitting the determination of the NOB activity.

Batch assays were carried out as a tool to evaluate the impact of technological changes on the activity of microorganisms. The batch test conditions are described in Table 2. The DO concentration was set at a level that was considered non-limiting for the rates in each assay. Pal et al. [22] state that achieving highly efficient nitrification in a moving-bed technology necessitates maintaining a DO concentration of at least 5 mg O_2/L.

Table 2. Batch assay operational parameters.

Parameters	SAA	AUR	NUR
Temperature (°C)	30	30	30
Total test reactor volume (L)	2	2	2
Carrier volume (L)	0.5	0.5	0.5
Initial N-NH_4 concentration (mg N/L)	15	30	-
Initial N-NO_2 concentration (mg N/L)	19.8	-	30
Dissolve oxygen concentration (mg O_2/L)	0	6	6

The biomass for the batch tests was collected from the laboratory MBSBBR and washed in dechlorinated tap water, before starting the test to remove residual substrate. A two-liter reactor filled with carriers (25% of the reactor volume) was employed in the batch assays. The test reactor was placed in a magnetic stirrer with a heating plate and kept at 30 °C. During the AUR and NUR tests, an air pump and an air stone placed in the middle of the

vessel introduced oxygen into the reactor. During the SAA test, pure nitrogen gas was introduced in the headspace phase to remove oxygen. Suitable mixing of the medium during the test was provided by a magnetic stirrer. In the AUR test, 4% NH_4Cl solution was added to the reactor in a certain quantity, providing an assumed N-NH_4 concentration. In the NUR test, a 5% KNO_2 solution was added to ensure the assumed initial N-NO_2 concentration. In the SAA test, N-NH_4 and N-NO_2 with a 1:1.32 mole ratio were used as nitrogen sources. Every 30 min, 25 mL of the liquid samples were collected and instantly filtered for the determination of the N-NH_4, N-NO_2, and N-NO_3 concentrations in the filtrate. Tests were conducted until the ammonia concentration decreased to 0 mg N-NH_4/L (AUR and SAA), and until the nitrite nitrogen concentration decreased to 0 mg N-NO_2/L (NUR). The biofilm quantity on the carriers was determined as volatile solids.

2.6. Microbial Community Analysis

Biofilm samples were collected at the end of each stage, stored at −25 °C, and used for DNA extraction and next-generation sequencing (NGS).

2.6.1. DNA Extraction

Genomic DNA was isolated using a method based on the Genomic Mini AX Bacteria + kit. The DNA was purified with an Anty-Inhibitor Kit after isolation. The DNA concentration was measured using the fluorometric method on a Qubit 4 fluorometer. The bacterial DNA was confirmed by means of real-time PCR, which was conducted in a thermocycler, CFX Connect (Biorad). The reaction employed universal starters, amplifying a fragment of the bacterial gene 16S rRNA.

2.6.2. V3–V4 Amplicon Library Preparation

Before the V3–V4 amplicon library preparation, DNA eluates were verified in terms of the quantity and quality. The libraries were prepared in accordance with the guidelines in 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B, two-stage PCR by means of Herculase II Fusion DNA Polymerase and the Nextera XT Index Kit v2. In accordance with the Illumina qPCR Quantification Protocol Guide, the library quality was verified.

2.6.3. NGS of the 16S rRNA Gene and Bioinformatics

Sequencing was performed on the MiSeq 2 × 300 bp platform (Illumina, San Diego, CA, USA) using paired-end technology by Macrogen. The bioinformatics analysis was conducted using the CLC Genomics Workbench v12 (Qiagen) and the Microbial Genomics Module Plugin v4.1 (Qiagen) programme.

2.7. Analytical Methods

Raw and treated wastewater samples were collected from the laboratory reactor to analyze the total nitrogen (TN), N-NO_2, N-NH_4, and N-NO_3. Chemical analyses were performed by means of Hach Lange cuvette tests, according to the method described by the producer. The total volatile solids in the biofilm were measured according to the Polish standard PN-EN872:2007 [23].

3. Results and Discussion

3.1. Nitrogen Removal Performance

At the beginning of the experiment, the MBSBBR was operated in the 'nitrification' mode. The average ratio of N-NO_3 production and N-NH_4 consumption (ΔN-NO_3/ΔN-NH_4) was high at 0.9. The AOB activity was maintained at a high level, with more than 90% ammonia removal efficiency (ARE) throughout Stage I (Figure 2). The average N-NO_2 accumulation was 5.89 mg/L, with a nitrite accumulation rate (NAR) of 4.4%, which was much lower when compared to 75% in a previous study [24] and 90% in another study [10], during the PN/A start-up period. This observation indicated a feeble level of inhibition of

NOB. Approximately 84.6% of the influent N-NH_4 was oxidized to N-NO_3 (average N-NO_3 was 134.3 mg/L). A high ARE, verified during Stage I, and the concomitant production of N-NO_3 confirmed the occurrence of the nitrification process.

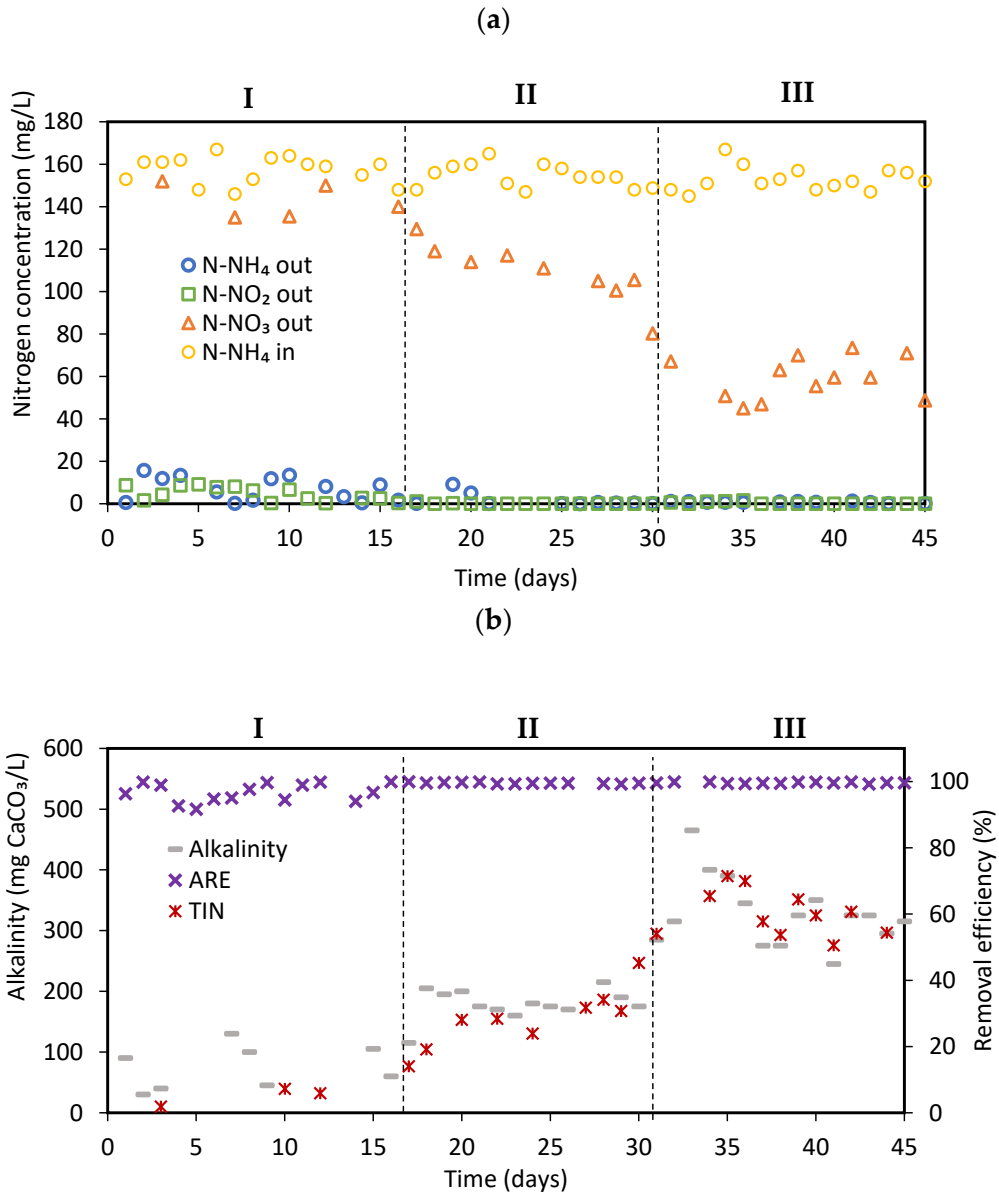

Figure 2. Overall performance of the system: (**a**) nitrogen concentrations in the reactor influent and effluent; (**b**) effluent alkalinity, total inorganic nitrogen (TIN), and ammonia removal efficiency (ARE).

Throughout Stage II, with the DO reduction to 2.0 mg O_2/L, the nitrite concentration was stable below 1.1 mg N-NO_2/L, with no accumulation. The ARE increased to 99.6%. The average ratio ΔN-NO_3/ΔN-NH_4 decreased to 0.72. During Stage II, the predominant change observed in the reactor operation was a gradual decrease in N-NO_3 from 129.5 mg/L to 80.3 mg/L, coinciding with an increase in the total inorganic nitrogen (TIN) removal from 15.2% to 45.1%. These results indicate that anammox may occur in the biofilm.

On the first day of Stage III, a steep decrease in N-NO_3 concentration to 67 mg NO_3/L was recorded, indicating the occurrence of anammox 15 days after a decrease in the DO concentration. The lack of nitrite accumulation indicates anammox presence due to N-NO_2 consumption by these bacteria. Zhang et al. [24] obtained stable partial nitritation and anammox 7 and 27 days, respectively, after the beginning of the SBBR operation by using

low activity dewatered excess activated sludge to control the aeration rate. According to Zuo et al. [12], anammox start-up can be obtained through a gradual decrease in the DO after the commencement of partial nitritation. In this study, despite ineffective NOB activity suppression, the anammox start-up was obtained through a gradual reduction in the DO. Throughout this stage, approximately 99.6% of the ammonia was removed, and the N-NO_3 concentration was in the range of 45–73 mg/L. In Stage III the greatest TIN removal performance was verified (60.6%). The TIN removal results obtained in the present study were comparable to the 70.87 ± 1.36% reported by Chen et al. [25], using aeration control as an operation strategy, and the 56.6% reported by Sun et al. [26], with intermittent aeration after 28 days operation. Additionally, Songkai et al. [6] achieved a total nitrogen removal efficiency (TNRE) of 45% by reducing the aeration rate. The average ratio of ΔN-NO_3/ΔN-NH_4 decreased to 0.38, indicating that nitrification was replaced by PN/A in the reactor. The operational conditions of the reactor and an increase in the TIN removal performance point to anammox as the main process responsible for ammonia removal from the system.

The change in biofilm color is an important parameter reflecting microbial performance and activity [27]. Annamox is rich in heme c proteins, which give biofilm its characteristic red color [28]. During the experiment, the biofilm color changed from brown to red, macroscopically indicating the anammox occurrence that was confirmed during NGS. The average alkalinity in the effluent increased with a decrease in the DO, confirming the occurrence of anammox. Alkalinity consumption is higher when all of the N-NH_4 is oxidized via nitrification, contrary to the alkalinity consumption during the anammox process [29]. All the above suggests fundamental shifts in the MBSBBR nitrogen removal processes and confirms the successful establishment of the PN/A process.

3.2. Batch Assays

As the distribution of AOB, NOB, and anammox significantly impacts the performance and efficiency of the PN/A [30], batch tests assessing the activity of these three main functional groups were employed (Figure 3).

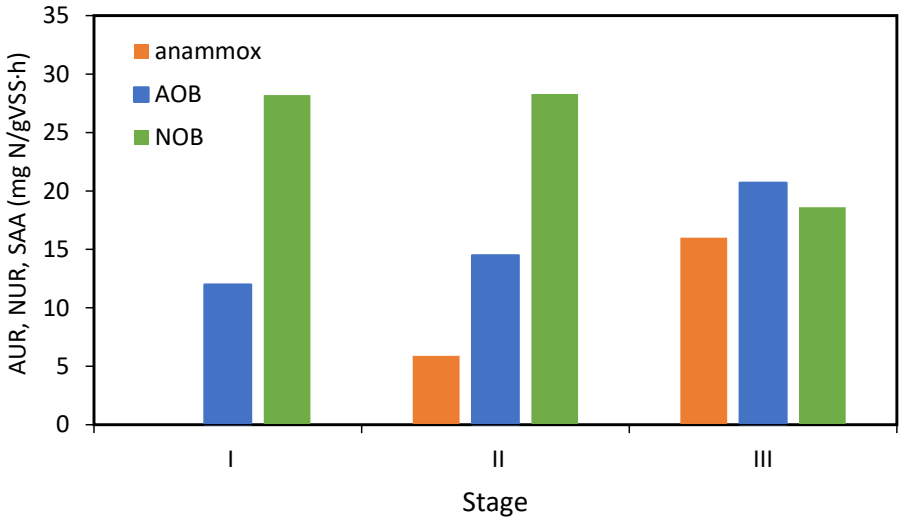

Figure 3. Batch assays results.

On the last day of Stage I, the batch test resulted in an AUR of 12.0 mg N/gVSS·h and a nitrate production rate (NPR) of 11.2 mg N/gVSS·h (Figure S2). The NPR/AUR ratio was 0.95. The NUR reached 28.2 mg N/gVSS·h.

Throughout Stage II, the AUR increased to 14.6 mg N/gVSS·h and the NUR remained at a similar level (28.3 mg N/gVSS·h), causing a decrease in the NUR to AUR rate from 2.4 to 2.0. The NPR/AUR ratio decreased to 0.68. This suggested the enrichment of AOB.

At the end of Stage II, an additional SAA test was carried out, providing important information on the actual anammox activity. The test was used as a tool to validate the anammox occurrence assumption, based on a steep decrease in the N-NO_3. The SAA test was carried out under a non-limiting substrate concentration and optimal conditions. Coinstantaneous removal of the N-NO_2 and N-NH_4 was noticed in the anammox activity test (Figure S3). The SAA value reached 5.9 mg N/gVSS·h, explaining the nitrogen removal performance and proving the existence of anammox bacteria in the reactor. Songkai et al. [6] also confirmed anammox bacteria presence after a steep loss of TN in the maximum anammox activity test. The ratio of N-NH_4:N-NO_2 consumed and N-NO_3 produced, useful measures concerning the indication of the microbial activity of anammox, was found to be −1:−1.29:−0.15, a value similar to the stoichiometry of the anammox reaction −1:−1.32:+0.26 [31] except the N-NO_3 reduction, suggesting the development of autotrophic microorganisms in the biofilm, consuming N-NO_3 under anoxic conditions.

Throughout Stage III, the AUR increased to 20.7 mg N/gVSS·h and the NUR decreased to 18.6 mg N/gVSS·h (Figure S4), which suggested that implementing a DO control is an effective strategy to prevent NOB growth [32]. Moreover, continuous aeration is typically employed to sustain a reduced DO level, aiming to enhance the activity of anammox bacteria, while inhibiting the growth of NOB [33,34]. In a study by Jiang et al. [35], the AUR was enhanced from 4.7 mg N/L·h to 5.7 mg N/L·h, while increasing the DO concentration from 1.51 ± 0.02 mg/L to 1.98 ± 0.03 mg/L. The highest N-NO_2 accumulation was recorded (3.0 mg N-NO_2/L), together with a decrease in the NUR, indicating that the assumed conditions contributed to NOB suppression. The suppression of NOB is a major challenge in obtaining effective PN/A. With a gradual decrease in the DO from 3.0 to 1.0 mg O_2/L, AOB outcompeted NOB, causing the NPR/AUR and NUR/AUR ratios to decrease to 0.36 and 0.90, respectively. The results show a relationship with other studies. When Al-Hazmi et al. [8] increased the DO concentration at each stage from 0.7 mg O_2/L to 1.5 mg O_2/L, the NPR/AUR ratio increased. The SAA reached 16 mg N/gVSS·h, constituting a value almost three times higher than that determined at the end of Stage II, suggesting that the activity of anammox was successfully enhanced due to a lower DO, giving anammox a chance to grow. The N-NH_4:N-NO_2 consumed and N-NO_3 produced ratio was found to be −1:−1.23:+0.19, a value approximate to the stoichiometry of the anammox reaction −1:−1.32:+0.26 [31]. However, a decrease in the DO concentration did not completely suppress NOB, which was also found in another piece of research [36]. Additionally, the SAA/AUR ratio was lower in Stage II (0.40) and Stage III (0.77) compared to the theoretical value (1.76) [25]. This may suggest that anammox was potentially the rate-limiting step, contrary to many studies indicating that partial nitritation is the main rate-limiting step in a one-stage PN/A [37,38]. However, the SAA/AUR ratio was comparable to the value of 0.82 reported in a previous study [25], which suggested that this phenomenon could be attributed to a low NLR, and its gradual increase may have subsequently elevated the SAA and, hence, enhanced the TN removal efficiency.

3.3. Nitrogen Conversion during a Single Cycle

3.3.1. Effect of Long-Term DO Change

The determination of the impact of a long-term change to the DO concentration on the course of the processes occurring in the phases of the operational cycle involved the analysis of the nitrogen conversion in one cycle (Figure 4).

During Stage I, the N-NH_4 decreased from 32.0 to 8.9 mg N/L. There was a linear decrease in the concentration of N-NH_4 in each phase. The ammonia oxidation rates (AOR) decreased from 12.6 to 9.1 mg/L·h in subsequent phases. In the first and third phase, the N-NO_2 concentration increased. In the second and fourth phase, the N-NO_2 concentration increased to 6.4 mg N/L and 6.9 mg N/L, respectively, and then decreased. The N-NO_3 concentration slightly increased at a rate of 12.7 ± 0.4 mg/L·h in the first and the second phase, and at a rate of 8.7 mg/L·h in the third and fourth phase.

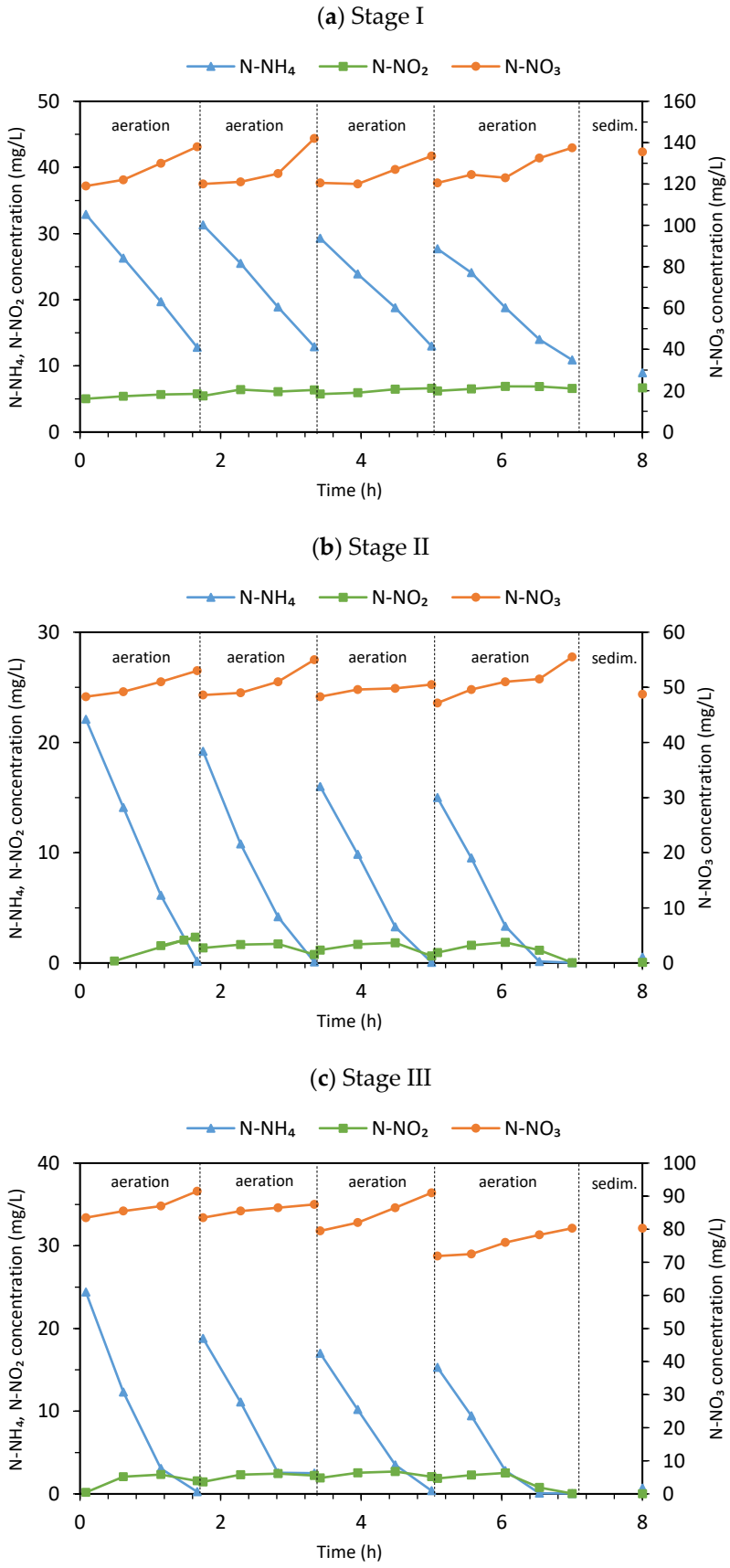

Figure 4. Performance of the MBSBBR in one operational cycle during: (**a**) Stage I, (**b**) Stage II, (**c**) Stage III.

During Stage II, the ammonia decreased from 24.4 to 0.7 mg N/L. There was an almost linear decrease in the N-NH_4 concentration in each phase. The ammonia oxidation rates were approximate to those at Stage I, except for the first phase, where the N-NH_4 oxidation rate was higher (14.3 mg/L·h), indicating higher AOB activity during this phase. The N-NO_2 concentration increased in every phase after 65 min to 2.5 ± 0.3 mg N/L, and then decreased. The N-NO_3 concentration increased in every phase at a rate of 4.8 mg/L·h (first phase), 2.5 mg/L·h (second phase), and 7.4 mg/L·h (third phase). In the fourth phase, a steep decrease in the N-NO_3 concentration was recorded. During the fourth phase, the N-NO_3 increased at a rate of 4.7 mg/L·h.

After the occurrence of anammox, the cyclic performance of the nitrogen conversion was different. A decrease in the N-NH_4 concentration was also linear. The N-NO_2 increased in every phase after 65 min and then decreased due to consumption by the anammox bacteria. The N-NO_3 concentration was at a level of 48–55 mg N/L, lower than in previous stages. In every phase, the N-NO_3 concentrations increased at a much lower rate of 2.6 ± 1.3 mg/L·h.

3.3.2. Effect of Short-Term DO Changes

At the end of Stage I, to determine the impact of a short-term change to the DO concentration on the course of the processes occurring in the phases of the operational cycle, the analysis of the nitrogen conversion in one cycle was carried out (Figure 5).

During Stage A, when the DO concentration decreased from 3.0 to 2.0 mg O_2/L, the N-NO_2 increased from 1.7 to 3.9 mg/L, the N-NH_4 decreased from 44.3 mg/L to 19.3 mg/L, and the N-NO_3 increased from 100.5 to 112 mg/L, during the complete cycle. In the first and the second aerobic phase, the N-NH_4 decreased at an approximate rate of 11.1 and 11.7 mg/L·h. During the last two aerobic phases, the N-NH_4 oxidation rates declined at a rate of 9.7 mg/L·h and 8.4 mg/L·h, respectively. The TNRE was 19%.

During Stage B, the N-NO_2 increased from 1.6 to 2.3 mg/L, and the N-NO_3 increased from 69.0 mg/L to 73.5 mg/L. In the first aerobic phase, the N-NH_4 oxidation rate was approximate to the rates from Stage A (11.7 mg/L·h), but in subsequent phases, it decreased two times to 5 mg/L·h. The N-NH_4 was much higher (52.2 mg/L), suggesting that a decrease in the DO concentration adversely affected ammonia oxidation. The TNRE was 12.5%.

During Stage C, the N-NO_2 decreased from 2.2 to 1.0 mg/L, and the N-NO_3 increased from 94.5 to 115 mg/L. In the first aerobic phase, the N-NH_4 oxidation rate was the highest (14.3 mg/L·h), although it decreased to 11.8, 11.4, and 9.8 mg/L·h in subsequent phases. The N-NH_4 decreased to 0.5 mg N/L, suggesting that an increase in the DO concentration enriched the AOB activity. According to a previous study, increasing the DO causes the enrichment of AOB activity [39]. The TNRE reached 33%.

In conclusion, the highest N-NH_4 concentration was noticed when the reactor was operated at the lowest oxygen concentration (1.0 mg O_2/L). This indicated that a short-term decrease in the DO concentration reduced the activity of the AOB and, therefore, reduced the ammonia removal efficiency. These results varied from those for a long-term decrease in the DO concentration, where the ammonia oxidation rates were high in every phase. The analysis of the course of the N-NO_2 concentration during a short-term DO change showed that only in Stage C did the nitrite concentration decrease in the cycle. This suggests that NOB were the most active at a DO concentration of 3.0 mg O_2/L. These results coincided with the highest N-NO_3 production rate in every phase during Stage C. During long-term changes in the DO concentration, nitrate production was the most effective in Stage I, where the DO concentration was 3.0 mg O_2/L. This highlights that higher DO values indirectly promote complete nitrification by enriching the AOB and NOB activity.

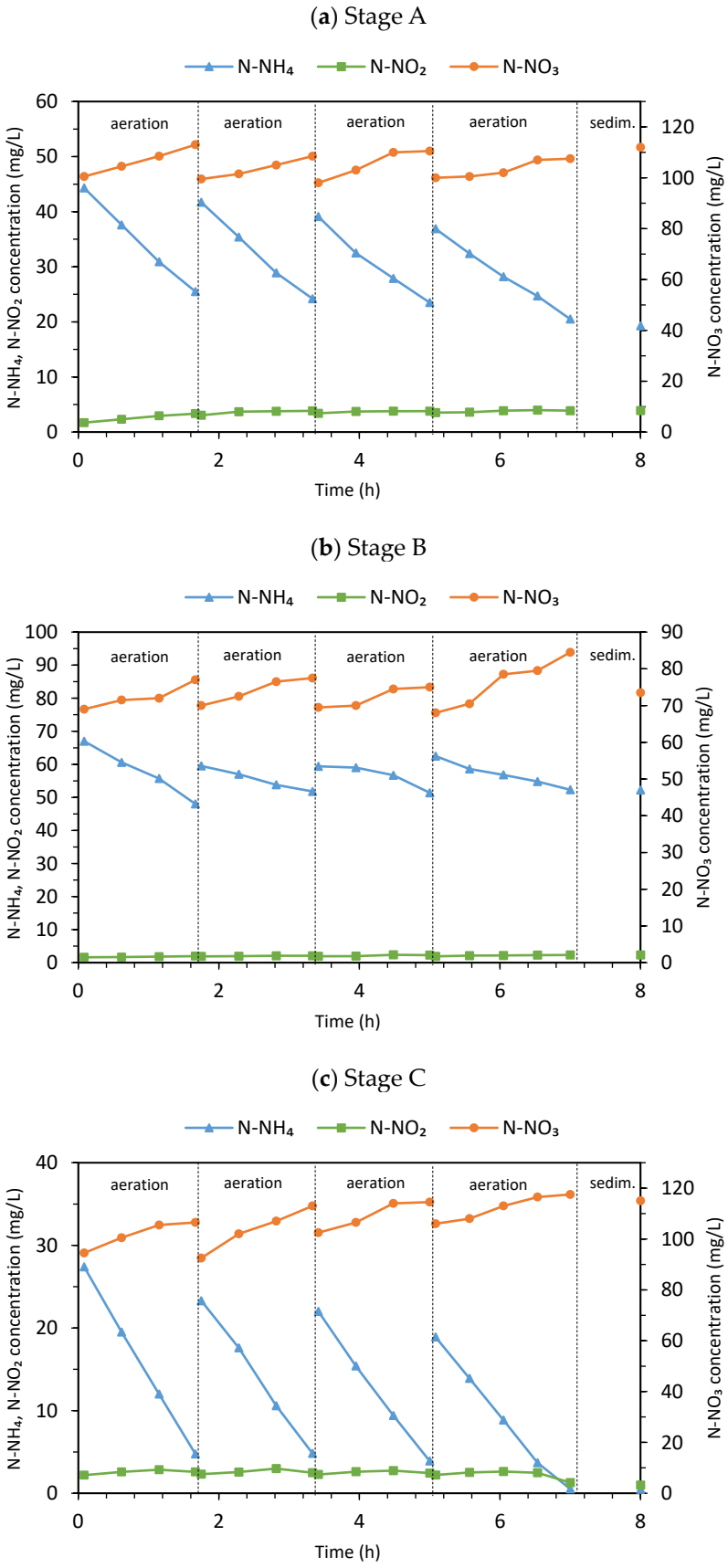

Figure 5. Performance of the MBSBBR in one operational cycle during: (**a**) Stage A, (**b**) Stage B, (**c**) Stage C.

3.4. The Microbial Community in the Context of the Technological Results

For an insight into the microbial community, NGS was carried out. The microbial community structure analysis generated from 206.378 to 351.762 16S rRNA gene (V4–V5) amplicon raw reads. Out of these, an average of 99.96% of reads were merged. The diversity of the collected samples was also estimated (Table 3). The number of operational taxonomic units (OTU) varied between 52,062 and 88,417 in the biofilm samples. The Chao1 index assesses the richness of a bacterial community, while the Shannon index assesses its diversity. The results showed differences in the richness index and diversity index between the microbial communities in different stages. During a long-term DO concentration change, the richness in the reactor decreased in Stage II and increased in Stage III. The greatest richness was observed in Stage I, with a DO concentration of 3.0 mg O_2/L (with a Chao1 index of 143.89). During a short-term DO concentration change, the richness in the reactor shifted significantly, revealed by the decreasing trends from 155.84 to 79.05 of the estimated Chao1. The microbial diversity in the PN/A reactor may have a significant effect on the anammox activity [3]. During both short-term and long-term DO concentration changes, the highest diversity was noticed when the DO was 1.0 mg O_2/L.

Table 3. Richness and diversity of the microbial community.

Stage	Total Reads	OTUs	Chao1 Index	Shannon Index
I	283.346	81,313	143.89	3.93
II	318.762	88,417	72.30	1.93
III	351.762	74,614	123.15	4.00
A	206.378	52,062	155.84	4.45
B	215.258	52,301	139.18	4.59
C	305.248	84,636	79.05	2.07

The microbial community composition directly determines the reactor efficiency. Microorganisms in the system responded to the introduced changes to the DO concentration. The microorganism diversity identification by phylum on the biofilm samples collected in each operational stage is shown in Figure 6. The most dominant phyla in the biofilm in Stage I were *Firmicutes* (46%) and *Proteobacteria (36%),* followed by *Actinobacteria* (4.2%), *Cyanobacteria* (3.8%), *Planctomycetes* (2.8%), *Chlamydiae* (2.3%), *Bacteroidetes* (1.3%), and *Chloroflexi* (1.1%). In Stage II, the most dominant phylum was *Proteobacteria* (96%), while the other phyla were *Chloroflexi* (1.6%), *Actinobacteria* (0.77%), and *Planctomycetes* (0.34%). *Proteobacteria* are considered to be affiliated with ammonia oxidizing bacteria, nitrite oxidizing bacteria, and denitrifying bacteria, and are responsible for ammonia removal and the accumulation of nitrite [40]. It is important to emphasize that despite the reduction in *Planctomycetes* in Stage II compared to Stage I, a batch test confirmed the anammox activity (5.9 mg N/gVSS·h). Moreover, a gradual decrease in the TIN concentration in the reactor effluent was observed during Stage II. This may suggest that a batch test is a useful tool for anammox process monitoring. The obtained results indicate that the abundance of bacteria capable of performing anaerobic ammonia oxidation was sufficient at that time to achieve a TIN removal efficiency of 45.1%.

In Stage III, the most dominant phyla were *Proteobacteria* (64%), *Chloroflexi* (12%), and *Planctomycetes* (7.1%), followed by *Chlamydiae* (2.6%), *Actinobacteria* (1.7%), *Bacteroidetes* (1.4%), *Chlorobi* (1.4%), *Acidobacteria* (1.1%), and *Firmicutes* (1.1%). Other studies using anammox-based reactors usually describe comparable microbial groups in relation to the dominant phyla [41]. *Chloroflexi* often occurs in anammox reactors without organic carbon compounds [42]. Because *Planctomycetes* are typical, representing anammox microorganism phylum [43], the sequencing results confirmed the observation from the nitrogen removal performance and the batch tests showing that anammox occurred in Stage III. *Bacteroides*, *Chloroflexi* and *Chlorobi* phyla members are omnipresent and play a significant role in anammox bioreactors [44]. *Chloroflexi* and *Bacteroidetes* participate in the maintenance of a stable and solid backbone for bacterial aggregates [11]. In Stage III, among anammox affiliated

genera, unknown genera from the family *Pirellulaceae* (3.9%) and *Candidatus Brocadia* (1.0%) were predominant.

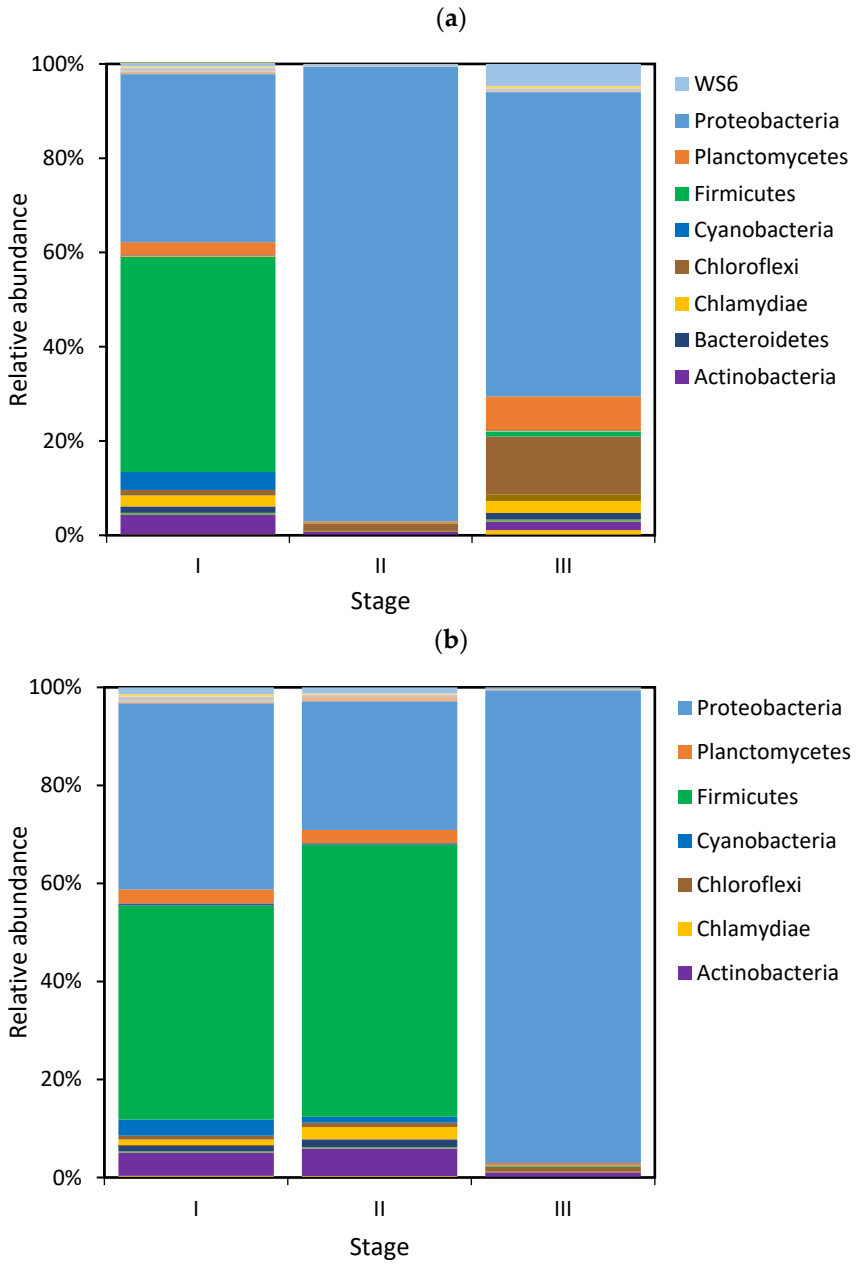

Figure 6. Relative abundance of the microbial community structure at the phylum level during: (**a**) Stages I–III, (**b**) Stages A–C.

The results from the microbial community analysis confirmed the observation from the batch tests indicating anammox occurrence and are proof of the fast PN/A process start-up. The relative abundance of *Planctomycetes* containing anammox bacteria increased almost three times from Stage I to Stage III. The essential synergy between anammox and AOB for PN/A process was supplied. The abundance of *Proteobacteria*, a common dominant phylum in PN/A systems [45], increased during Stage II, and then decreased in Stage III when anammox occurred. The same relationship was found in another piece of research [6]. An increase in *Proteobacteria* abundance during Phase II confirmed the results from the batch test, where AUR increased, suggesting the enrichment of AOB. The results show that inoculation is not necessary to start-up the PN/A process, as also presented in another study [7].

The microbial community during a short-term DO concentration change was also analyzed. Moreover, 24 h after DO reduction to 2.0 mg O_2/L (Stage A), the microbial community did not change significantly compared to the microbial community in Stage I (with DO of 3 mg O_2/L). The most dominant phyla in the biofilm were *Firmicutes* (44%) and *Proteobacteria* (38%), followed by *Actinobacteria* (4.7%), *Cyanobacteria* (3.3%), *Planctomycetes* (2.8%), *Chlamydiae* (1.1%), and *Bacteroidetes* (1.1%). In addition, 24 h after DO reduction to 1.0 mg O_2/L (Stage B), *Firmicutes* abundance increased to 56%, and *Proteobacteria* abundance decreased to 26%. *Planctomycetes* abundance remained at a similar level of 2.7%. Then, 24 h after the DO increased to 3.0 mg O_2/L (Stage C), the microbial community changed significantly. The abundance of *Proteobacteria* increased vastly to 96%, while *Actinobacteria* and *Chloroflexi* decreased to 1%. The anammox correlated phylum *Planctomycetes* decreased to 0.43%. The obtained results indicate that within 24 h after a change to the DO concentration, the microbial community varied. *Proteobacteria* abundance was the highest in Stage C (DO of 3.0 mg O_2/L), and the lowest in Stage B (DO of 1.0 mg O_2/L). The change in the abundance affiliated with ammonia oxidizing, nitrite oxidizing, and denitrifying bacteria phylum, confirmed the results from the monitoring tests suggesting that a short-term increase in the DO concentration enriched AOB and NOB activity. Feng et al. [18] reported that a high DO concentration can promote NOB growth. An increase in the DO concentration to 3.0 mg O_2/L (Stage C) caused a vast decrease in *Planctomycetes* abundance. This indicates that anammox is sensitive to high DO concentration, and an accidental failure to increase the DO concentration can inhibit anammox [18]. High DO concentrations may irreversibly inhibit anammox metabolism [46]. This assumption agrees with the results obtained from the nitrogen conversion in a typical cycle in Stage C, where during each phase the highest N-NO_3 was recorded.

The obtained results indicate that variations in the operational factors, such as the DO concentration, may disrupt the balance between the ammonia oxidizing bacteria, nitrite oxidizing bacteria, and anammox in a one-stage PN/A. Therefore, the analysis of the PN/A performance using batch tests during disturbances in the DO concentrations can be useful for future applications in practical engineering. Further studies should focus on PN/A start-up and performance in full-scale installations.

4. Conclusions

One-stage PN/A was successfully started up in an MBSBBR only, due to a gradual decrease in the DO concentration, with an average TIN removal efficiency of 60.6%. It shows that the PN/A process can be purposefully initiated in a relatively short time with no biomass containing anaerobic ammonia oxidizing bacteria inoculation if appropriate conditions are applied. Anammox activity was confirmed in the SAA test (5.9 mgN/gVSS·h). Planctomycetes affiliated with anammox phylum were enriched to an abundance of 7.1%.

In practical terms, the introduction of the DO reduction and control regime explored in this study, holds promising practical applications for wastewater treatment plant operators, offering a sustainable and efficient alternative for initiating PN/A processes, which is pivotal for achieving long-term environmental and economic sustainability. Such a strategy can be applied to various types and configurations of systems, continuous and sequential, providing a universally applicable solution that can be tailored to meet the unique demands of different facilities, thereby promoting sustainability on a broader scale.

The results also indicate that batch tests can be a straightforward and practical tool for wastewater treatment plant operators, for indirectly tracking changes in microbial communities, aligning with sustainable monitoring practices. The real-time and rapid information provided by these tests empowers operators to make swift and informed decisions, contributing to the sustainable and efficient operation of wastewater treatment plants. Moreover, the ability to promptly detect variations in test values renders batch tests a valuable instrument for investigating microbial activity, while traditional microbiological studies may take considerable time to yield results. Researchers and practitioners can explore and compare different microbiological methods, refining tests to extract specific

information. Continuous efforts to enhance and tailor these tests will unlock their potential to uncover nuanced insights into wastewater treatment processes. This ongoing exploration and optimization can further contribute to the advancement of wastewater treatment practices, ensuring their effectiveness and sustainability in the long run.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16020622/s1, Figure S1: The MBSBBR complete cycle duration; Figure S2: Batch test results during Stage I, (a) AUR, (b) NUR; Figure S3: Batch test results during Stage II, (a) AUR, (b) NUR, (c) SAA; Figure S4: Batch test results during Stage III, (a) AUR, (b) NUR, (c) SAA.

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