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# Changes in Nitrification Kinetics and Diversity of Canonical Nitrifiers and Comammox Bacteria in a Moving Bed Sequencing Batch Biofilm Reactor—A Long-Term Study

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**Abstract:** A lab-scale pure moving bed sequencing batch biofilm reactor (MBSBBR) was employed to investigate changes in nitrification kinetics and microbial diversity. The MBSBBR operated under different aeration strategies (defined by the ratio of the duration of the subphases with ($t_1$) and without ($t_2$) aeration (R = $t_2/t_1$)) – continuous (R = 0) and intermittent (with constant time of non-aerated subphases ($t_2$ = 10 min) and variable duration of subphases with aeration ($t_1$ = 40 min–R = 1/4, $t_1$ = 30 min–R = 1/3, $t_1$ = 20 min–R = 1/2) and dissolved oxygen (DO) concentrations (6 mg/L; 3.5 mg/L). Moreover, the reactor's organic (OLR) and nitrogen (NLR) loading rates were changed in the following ranges: OLR—537–402 gCOD/$m^3$·d, NLR—64–48 gN/$m^3$·d. The obtained results showed that, irrespective of changes introduced in particular series, a highly effective nitrification process (93.36 ± 2.13%) was achieved. The activity of bacteria capable of oxidizing ammonia nitrogen changed differently from that of bacteria capable of oxidizing nitrites (NOB). An increase in R was the primary factor changing the activity of ammonia-oxidizing microorganisms. NOB activity was affected only by the reduction of OLR and NLR. NOB were the predominant bacterial group, consistent with the kinetics studies. A DO decrease caused an increase in the abundance of AOB, NOB, and Comammox bacteria. Comammox bacteria were the most abundant at R = 1/2 and DO = 3.5 mg/L.

**Keywords:** moving bed; nitrification kinetics; ammonia oxidation rate; nitrite oxidation rate; aeration strategy; Comammox bacteria



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## 1. Introduction

As a result of intensive agricultural and industrial activities, as well as the expansion of urban areas, increasing amounts of nitrogen compounds (in particular, ammonia nitrogen) enter the natural environment, causing its degradation. In order to prevent this as much as possible, new and modernized wastewater treatment plants (WWTPs) are being designed globally, and processes aimed at biological nitrogen removal are being developed [1]. Biofilm-based wastewater treatment systems are gaining prominence because they demonstrate considerable resilience against variations in hydraulic loads, pH levels, temperature fluctuations, and pollutant concentrations [2]. Leyva-Diaz et al. (2015) assert that biofilm-based systems achieve a higher nitrification rate compared to traditional activated sludge systems [3]. Biofilm-based wastewater treatment processes facilitate the accumulation and retention of biomass without the necessity for external devices for separation and retention. These technological solutions are especially advantageous for retaining slow-growing nitrifying bacteria due to their extremely high solids retention time (SRT) [4]. This is particularly important due to the fact that the nitrification process is one of the key links in the natural nitrogen cycle and, at the same time, the limiting stage.

If there are problems with the efficiency of nitrification, it will lead to challenges in the subsequent steps of wastewater nitrogen removal. Therefore, it is imperative to properly select the operating parameters of biological reactors and to ensure that the biomass used for wastewater treatment fosters the robust growth of nitrifying organisms.

One example of a biofilm-based wastewater treatment system is pure moving bed technology. This is a simple and efficient wastewater treatment technology, with biomass immobilized on moving plastic carriers. Compared to systems based on activated sludge technology or those with fixed beds, this solution offers many advantages, such as: no sludge bulking, small size of compact units, increased treatment capacity, complete solids removal, and reduced sludge production [5]. The implementation of pure moving bed technology is also associated with easy operation and low maintenance and energy requirements [6]. As reported by Safwat (2018), this technological solution prevails over conventional biological wastewater treatment systems in terms of greater microbial activity, diversity, and stability [7]. Pure moving bed technology is used in both continuous-flow systems—moving bed biofilm reactors (MBBR) as well as moving bed sequencing batch biofilm reactors (MBSBBR). The former solution has already been widely described in the literature [8], whereas MBSBBR has only been reported in several papers to date [9].

The most frequently studied factors affecting the course and effectiveness of wastewater treatment using pure moving bed technology are the following: intermittent aeration (IA) [10], temperature [11], dissolved oxygen (DO) concentration [12], and type of media used [13]. It is worth emphasizing that these studies have mainly focused on continuous-flow systems. A comparison of MBBR with MBSBBR in the context of the impact of influent characteristics and feeding regime (continuous and sequencing-batch) on the nitrification process and nitrifying biofilm development on different types of moving bed biofilm reactors can be found in the work of Bassin et al. (2012) [14]. For instance, these authors observed that in the sequencing batch system, the time required to develop a thick biofilm was shorter than that in MBBR. Moreover, the conditions under which wastewater treatment processes are conducted can affect the composition and activity of particular bacterial communities present in the systems. A review of the literature on wastewater treatment using pure moving bed technology also indicates that a little-recognized issue is how a community of nitrifying organisms in biofilm, especially Comammox bacteria, changes in response to different operating conditions of wastewater treatment systems and how they affect the activity of particular groups of nitrifying bacteria. For example, Zhao et al. (2022) observed selective enrichment of Comammox bacteria in the MBBR, further proving that they can even dominate the canonical ammonia oxidizers [15]. Another group of researchers confirmed the presence of Comammox bacteria in various full-scale WWTPs based on continuous-flow pure moving bed technology [16]. However, these reports still do not permit a clear identification of the factors influencing the development of Comammox bacteria in reactors operating with pure moving bed technology, highlighting the need for more research in this area. To the best of our knowledge, there have been no attempts to describe the abundance of Comammox bacteria in pure moving bed sequencing batch reactors, which creates a significant gap regarding the occurrence of these microorganisms. In our previous studies, we explored the presence of Comammox bacteria and canonical nitrifiers. However, these studies were conducted in a moving bed sequencing batch biofilm reactor operating as a hybrid technology with biomass in the form of activated sludge and biofilm [17,18]. The focus was on analyzing how changes in aeration strategy and DO concentration affected the size of their populations and activities. Promising results led the authors to conduct further research in a reactor employing pure moving bed technology.

In light of this background, in this study, a MBSBBR operated in pure moving bed technology with nitrification/denitrification was employed to investigate the effect of different aeration strategies (defined by the ratio of the duration of the subphases with ($t_1$) and without ($t_2$) aeration ($R = t_2/t_1$), and dissolved oxygen concentration in aerated subphases), as well as the organic (OLR) and nitrogen (NLR) loading rates on the course of the nitrification process and microbial diversity. The specific objectives are as follows:

(1) to assess the impact of the aforementioned factors on the ammonia oxidation rate (AOR) and nitrite oxidation rate (NitOR) in biofilm; (2) to assess changes in the frequency of occurrence of particular groups of nitrifying microorganisms in biofilm as a result of the introduced changes; (3) to characterize the structures of communities of nitrifying microorganisms that develop in the form of biofilm; (4) to determine the abundance of Comammox bacteria in biofilm; and (5) to correlate changes in activity of particular groups of nitrifying microorganisms with microbial community composition in biofilm. These studies were conducted continuously for over a year (445 days), which undoubtedly distinguishes them compared to previously published research.

## 2. Materials and Methods

### *2.1. Reactor Description*

The experiment was conducted in a laboratory-scale plexiglass MBSBBR with an active volume of 28 L (Figure 1).

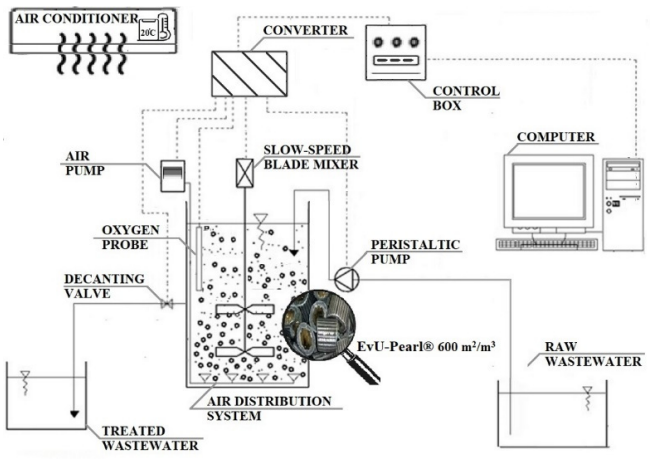


**Figure 1.** Schematic of the moving bed sequencing batch biofilm reactor with accessories.

As a carrier of biofilm, an EvU-Pearl® moving bed was applied, in the shape of a cylinder with an active surface area of 600 $m^2/m^3$ ($\Phi$ = 5 mm, h = 8 mm) (EvUInnovative Umwelttechnik GmbH, Gröditz, Germany) (Figure 2). The moving carriers constituted 25% of the active volume of the reactor, i.e., 7 L.

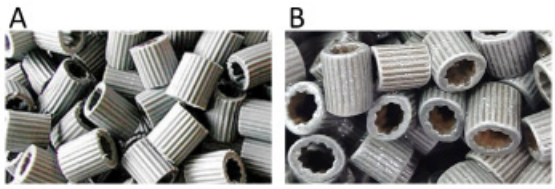


**Figure 2.** Photographs of the EvU-Pearl® moving carriers (**A**) before and (**B**) after biofilm formation.

The content of the reactor was stirred using a slow-speed blade mixer R-50D (CAT, Ballrechten-Dottingen, Germany), with an engine speed of 110 rev/min. The aeration system consisted of blowers and aquarium filters mounted at the bottom of the reactor. Continuous measurements of DO concentration and temperature were conducted by an optical sensor Oxymax COS61D (Endress + Hauser, Weil am Rhein, Germany) connected to a converter Liquiline CM442 (Endress + Hauser, Weil am Rhein, Germany), which modified and relayed the acquired signals to the control box and then to the computer. An important element of the model was the automated control system, SCADA Wonderware InTouch (version 2017-Update 2), cooperating with an oxygen probe, which was responsible for maintaining the assumed oxygen concentration and conducting the remaining technological operations through switching on a pump dosing synthetic wastewater, as well as a mixer and decanting valve.

The schedule of the system's cycle of operation in particular stages and series of the experiment is presented in Figure 3. One complete reactor's operation cycle spanned 8 h and consisted of two unaerated phases (first: 50 min; second: 30 min), two aerated phases (first: 190 min; second: 200 min), and a 10-min decantation phase. Depending on the experimental series, continuous (S.I.1.) or intermittent (S.I.2.–S.II.4.) aeration was applied during the aerated phases. Intermittent aeration was implemented during the aerobic phases by introducing sequences consisting of subphases with ($t_1$) and without ($t_2$) aeration. The duration of the subphases with aeration differed between the series, while those without aeration were constant, i.e., 10 min. Each IA strategy was characterized by the ratio between the times of non-aerated and aerated subphases ($R = t_2/t_1$). A detailed description of the aeration strategies employed is presented in Table 1.

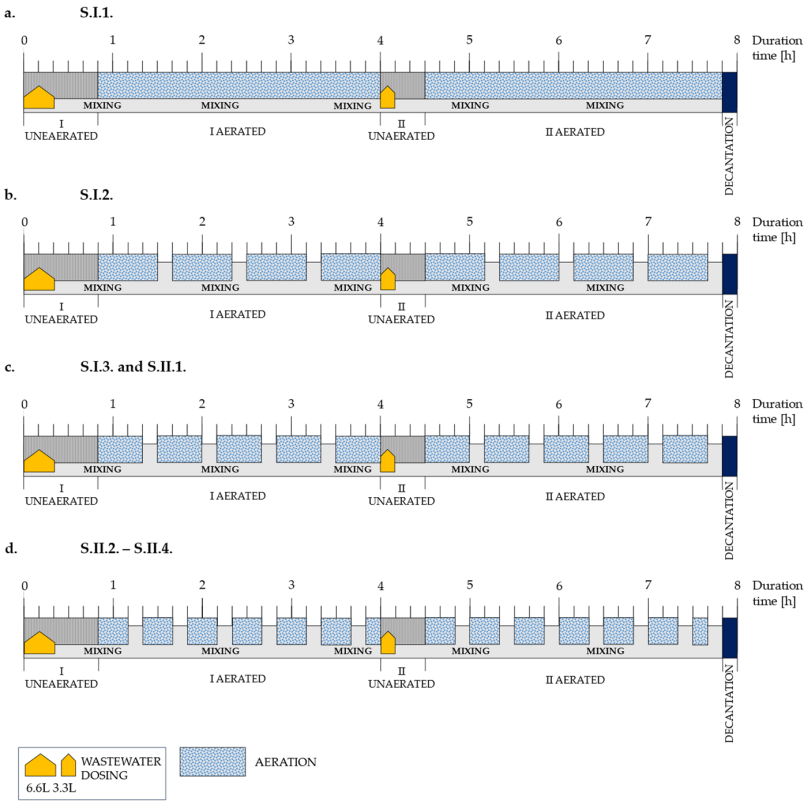


**Figure 3.** Schedule of the reactor operation cycle: (**a**) S.I.1.; (**b**) S.I.2.; (**c**) S.I.3.; S.II.1.; (**d**) S.II.2-4.

**Table 1.** Reactor operation stages.

| Stage | Series | Duration | Oxygen Concentration during Aerated Subphases (DO) | Ratio between Times of Non-Aerated and Aerated Subphases (R) | Organic Loading Rate (OLR) | Nitrogen Loading Rate (NLR) |
|---|---|---|---|---|---|---|
| **Unit** | | **[d]** | **[$mgO_2/L$]** | **[-]** | **[$gCOD/m^3 \cdot d$]** | **[$gN/m^3 \cdot d$]** |
| I | S.I.1. | 1–40 | 6 | 0 | 537 | 64 |
| | S.I.2. | 41–79 | | 1/4 | | |
| | S.I.3. | 80–100 | | 1/3 | | |
| II | S.II.1. | 101–196 | 3.5 | 1/3 | 537 | 64 |
| | S.II.2. | 197–330 | | 1/2 | 537 | 64 |
| | S.II.3. | 331–430 | | 1/2 | 402 | 48 |
| | S.II.4. | 431–445 | | 1/2 | 537 | 64 |

Employing a peristaltic pump, Masterflex® Ismatec® Ecoline (Masterflex Ismatec, Cole-Parmer, Chicago, IL, USA), at the beginning of each non-aerated phase, the reactor was supplied with synthetic wastewater with a composition simulating that of municipal wastewater in a volume of 10 L per cycle (S.I.; S.II.1,2,4) and 6.6 L per cycle (S.II.3.). The detailed list of the compounds used to prepare synthetic wastewater is presented in Supplementary Material (Table S1). The use of synthetic wastewater was necessary to maintain identical loading conditions in each series. The characteristics of synthetic sewage were the same throughout the experiment: COD: 512.35 ± 9.08 $mgO_2/L$, TN: 61.99 ± 2.69 mgN/L, TKN: 60.24 ± 2.76 mgN/L, N-$NH_4^+$: 39.06 ± 1.21 mgN-$NH_4^+$/L; N-$NO_3^-$: 1.75 ± 0.41 mgN-$NO_3^-$/L, TP: 7.52 ± 0.50 mgP/L, pH: 7.6–7.9. Synthetic wastewater was prepared daily, with the exception of the pandemic period, when it was prepared every six days.

The dissolved oxygen concentration during the aerated subphases was maintained at the level assumed for a given stage of the experiment. For stage I, it was 6.0 $mgO_2/L$, and for stage II, it was 3.5 $mgO_2/L$ (Table 1). The DO set-point was selected based on literature reports and the authors' previous studies to ensure that this parameter does not act as a limiting factor for the efficiency of the nitrification process in biofilm-based systems [19,20]. The temperature in the reactor was maintained at 20 °C throughout the study using external air conditioning.

### 2.2. Operating Conditions

The study started on a previously operated MBSBBR system for integrated carbon and nitrogen compound removal with biomass developed in the form of biofilm overgrowing moving carriers (22.4278 gVSS). The nitrification, denitrification, and organic compound removal efficiencies were 99.37%, 79.53%, and 94.97%, respectively (N-$NH_4^+$, TN, and COD in the effluent were: 0.18 mgN-$NH_4^+$/L, 13.30 mgN/L, and 27.60 $mgO_2/L$).

Two research stages were designated in the experiment lasting 445 days (S.I.–S.II.). They differed in the adopted level of oxygen concentration maintained in the reactor during the subphases of aeration. During each of the stages, the assumed changes were successively introduced by designating a particular series. Detailed characteristics of the basic parameters of the reactor's operation during particular stages and series are presented in Table 1.

Stage I involved three research series (S.I.1.–S.I.3.) differing in the applied aeration strategy, each time with DO maintained at a level of 6.0 $mgO_2/L$. The experiment began with a series in which the system operated with continuous aeration—R = 0 (S.I.1.), followed by intermittent aeration (IA), with the introduction of changes in R values through a reduction of the duration of the aerated subphases. In series 2, the duration of aerated subphases was 40 min—R = 1/4 (S.I.2.), followed by its reduction to 30 min in series 3—R = 1/3 (S.I.3.).

At stage II, DO concentration was decreased to 3.5 $mgO_2/L$, and the aeration strategy remained the same—R = 1/3 (S.II.1.). In the following series, the duration of aerated subphases was reduced to 20 min—R = 1/2 (S.II.2.). As a result of the outbreak of the COVID-19 pandemic, changes were introduced in the methodology of operation of MBSBBR. They included a decrease in the reactor's organic and nitrogen loading rates (OLR = 402 $gCOD/m^3 \cdot d$, NLR = 48 $gN/m^3 \cdot d$) through lowering the volume of wastewater dosed to the reactor. The introduced changes permitted the designation of series 3 (S.II.3.). Along with the elimination of some of the restrictions, the initially assumed reactor's organic and nitrogen loading rates were restored (S.II.4.).

Throughout the experiment, the amount of biomass in the form of biofilm averaged 23.2514 ± 5.3619 gVSS. To confirm that the reactor was operated as a pure moving bed technology, volatile suspended solids of biomass present in the reactor in suspended form were checked in parallel. It remained on average at the level of 0.3507 ± 0.0408 gMLVSS, which was less than 1.65% of the biomass in the form of biofilm (see Supplementary Material–Table S2). This confirms that the reactor was operated as a pure moving bed technology throughout the experiment, and this small amount of biomass in suspended form was primarily detached biofilm.

The following was conducted in all series:

- daily control of ammonia nitrogen concentration in the effluent (except the pandemic period—S.II.3.);
- analysis of the influent and effluent, in the following scope: COD, TN, N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$, TP, alkalinity, pH—twice a week (once a week in the pandemic period);
- determination of the quantity of biomass developed in the form of biofilm;
- batch tests of the ammonia utilization rate (AUR) and nitrite utilization rate (NitUR);
- microbiological analyses: quantification of nitrifying bacteria using the absolute qPCR method; description of microbial communities in biofilm based on new generation sequencing data (NGS).

Batch tests and quantitative PCR analysis were conducted at the end of each series. NGS was conducted only in selected series.

### *2.3. Ammonia and Nitrite Utilization Rate Batch Test*

All batch tests were conducted under the same conditions, allowing for tracking changes in the activity of bacteria capable of oxidizing ammonia and nitrite nitrogen.

The tests were carried out for the carriers taken from the MBSBBR in a continuously aerated and mixed 2 L batch test reactor, maintaining the proportions of carriers as in the MBSBBR (25% of active volume—0.25 L), under DO concentration near saturation levels at a temperature of 20 °C (DO concentration was determined based on the oxygen solubility in water at a temperature of 20 °C and reached approx. 9 $mgO_2/L$). Depending on the type of test, the solution of $NH_4Cl$ (AUR test) or $KNO_2$ (NitUR test) was added to the test reactor (the initial concentration of $NNH_4^+$ or $NNO_2^-$ was 15 mg/L), and the loss of $NNH_4^+$ and $NNO_2^-$ was tracked, respectively. Samples with a volume of 30 mL were collected from the reactor every 30 min and immediately filtered through filters with a mesh of 0.45 µm. In the filtrate, the concentration of N-$NH_4^+$, N-$NO_2^-$, and N-$NO_3^-$ (AUR test) or N-$NO_2^-$ and N-$NO_3^-$ (NitUR test) was measured. The test lasted until the concentrations of ammonia (AUR test) or nitrite (NitUR test) reached values approximately equal to 0 mg/L. The slope of the linear regression for the decrease in N-$NH_4^+$ and N-$NO_2^-$ with $R > 0.9$ was utilized to determine the ammonia oxidation rate (AOR) or nitrite oxidation rate (NitOR). The determined AOR and NitOR values indicate changes in the activity of specific groups of nitrifying microorganisms. A detailed methodology of AUR and NitUR batch tests was presented in our previous paper [17], with the difference that in this work, tests were performed only for biomass immobilized on moving carriers (because the reactor worked as a pure moving bed technology).

### *2.4. Quantity of Biofilm*

At the end of each series, the amount of biofilm immobilized on moving carriers was measured using gravimetric methods by calculation of weight loss. Five carriers were randomly collected from the reactor. They were rinsed with demineralized water to remove loosely attached biomass. Then, the biofilm was mechanically removed from the carriers. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined in biofilm in accordance with the Polish Standard PN-EN 872:2007 [21].

### *2.5. Microbiological Analysis*

#### 2.5.1. DNA Extraction

DNA was extracted from the biofilm samples by means of a FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) in accordance with the manufacturer's instructions. The amount of extracted DNA was measured using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The isolated DNA was stored at −18 °C until further analysis.

#### 2.5.2. qPCR

The PCR reaction was performed in an ABI7500 real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA) in MicroAmp TM Optical 96-well reaction plates using Mix

SYBR® A RT PCR reagents (A and A Biotechnology, Gdynia, Poland). AOB were detected with primers targeting the ammonia monooxygenase gene: A189 (5′-GGHGACTGGGAYTTCTGG-3′) [22] and amoA-2R (5′-CCTCKGSAAAGCCTTCTTC-3′) [23]. NOB was detected with primers corresponding to the 16S rRNA gene: NSR1113F (5′-CCTGCTTTCAGTTGCTACCG-3′) and NSR1264R (5′-GTTTGCAGCGCTTTGTACCG-3′) [24]. The abundance of Comammox bacteria was estimated using primers designed by Fowler et al. (2018): NTSP-amoA 162F (5′-GGATTTCTGGNTSGATTGGA-3′), NTSP-amoA 359R (5′-WAGTTNGACCACCASTACCA-3′) [25]. The final volume of the reaction mixture was 20 µL. The following temperature profile was applied: incubation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s. Each sample was analyzed in triplicate. In addition, the melting curves were generated for each primer pair to exclude non-specific amplifications.

The results of qPCR experiments were calculated using absolute quantification with the application of the standard curve method. The copy numbers of the studied genes were calculated using the software of the ABI 7500 real-time PCR system (version 2.3).

Standard curve preparation (see Supplementary Material–Figure S1): The *AmoA* gene fragment was amplified for AOB and Comammox bacteria, whereas the 16S rRNA gene fragment was used for NOB with the application of the aforementioned primers. The quality of the obtained products was verified electrophoretically on a 1% agarose gel and using a molecular mass marker (FastGene100bp DNA Ladder, Nippon Genetics Europe GmbH, Düren, Germany). PCR products were cleaned by means of a Monarch PCR and DNA Cleanup Kit (New England Biolabs, Ipswich, MA, USA) and ligated into the pTZ57R/T vector. Competent *E. coli* cells were transformed with recombinant vectors using an InsTAclone PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA). Clones were verified with Luria-Bertani (LB) agar medium, supplemented with ampicillin, X-Gal, and IPTG. The plates were incubated overnight at 37 °C. Several white colonies were collected from each plate, transferred to a liquid LB medium supplemented with ampicillin, and incubated overnight at 37 °C. The combined plasmids were extracted using a Plasmid mini kit (A and A Biotechnology, Gdynia, Poland) and sequenced to confirm the presence of the correct DNA insert. Standard curves for each primer pair were constructed using tenfold serial dilutions of the recombinant plasmids, used as standard templates for qPCR amplifications.

#### 2.5.3. 16S rRNA Amplicon Sequencing

The taxonomic composition of samples S.I.1., S.I.3., S.II.1., S.II.2., and S.II.4. was determined by sequencing of the V3–V4 hypervariable regions of the 16S rRNA gene. High-throughput Illumina sequencing was performed with S-d-Bact-0341-b-S-17 and S-d-Bact-0785-a-A-21 primers [26] and NEBNext®High-Fidelity 2X PCR Master Mix (Bio Labs Inc., Boston, MA, USA) following the manufacturer's manual. The sequencing reaction was performed on a Miseq sequencer with a MiSeq Reagent Kit V2 (Illumina, San Diego, CA, USA), applying pair-end technology with a read length of 250 base pairs.

Raw sequencing data were analyzed using the QIIME 2 package (version 2022.4) [27]. Pairs of sequences were merged using the fast-join algorithm. Unmerged sequences were excluded from further analysis. Low quality sequences (under 20) were filtered by the Cutadapt algorithm [28]. Chimeric sequences were detected and excluded from analyses using USEARCH [29]. 16S rRNA OTUs were picked from the Illumina reads using a closed-reference OTU picking protocol against the SILVA_V_138 database [30]. Sequences were clustered at 97% identity and trimmed to span only the 16S rRNA V4 region flanked by the sequencing primers. Taxonomy assignments were associated with OTUs based on the taxonomy associated with the SILVA_V_138 reference sequence defining each OTU.

### *2.6. Analytical Methods*

Concentrations of COD, N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$, TN, and TP were analyzed spectrometrically using cuvette tests (Hach Lange GmbH, Dusseldorf, Germany) and DR

3900 spectrophotometer (Hach Lange, GmbH, Loveland, CO, USA) according to APHA Standard Methods [31]. All chemical analyses were performed in duplicate.

### *2.7. Statistical Analysis*

The statistical analysis of the obtained results in the scope of the quality of treated wastewater and the efficiency of the removal of particular pollutants employed program Statistica 13.3PL. A RiR Tukey test was applied for the determination of the significance of differences between the analyzed variables ($p$-value smaller than 0.05 pointed to a statistically significant difference).

## 3. Results and Discussion

### *3.1. Nutrient Removal Performance*

According to the results presented in Table 2, a highly effective nitrification process ($E_{Nit.}$ = 93.36 ± 2.13%) was achieved in all series ($p > 0.277031$), pointing to the lack of effect of IA, DO decrease, and reduction of OLR and NLR on the efficiency of the analyzed process. Nonetheless, the RiR Tukey test showed significant differences between $N\text{-}NH_4^+$ for S.II.1.–S.II.2. ($p$ = 0.005225) and S.II.1.–S.II.3. ($p$ = 0.000803). Despite the recorded differences, the concentrations of $N\text{-}NH_4^+$ were at a low level and remained in a range of 0.10–2.68 mg/L throughout the experiment.

**Table 2.** Effluent characteristics and efficiencies of nitrification, denitrification, and organic compound removal.

| Parameter | Unit | Stage I | | | Stage II | | | |
|---|---|---|---|---|---|---|---|---|
| | | S.I.1. | S.I.2. | S.I.3. | S.II.1. | S.II.2. | S.II.3. | S.II.4. |
| COD | $mgO_2/L$ | 21.42 ± 2.68 | 17.53 ± 2.16 | 18.25 ± 3.96 | 18.39 ± 4.79 | 21.20 ± 2.52 | 21.99 ± 3.23 | 18.84 ± 2.88 |
| TN | mgN/L | 13.98 ± 2.26 | 17.71 ± 2.50 | 18.70 ± 1.02 | 14.10 ± 2.27 | 12.94 ± 1.06 | 18.77 ± 2.01 | 15.43 ± 2.93 |
| TKN | mgN/L | 3.99 ± 1.92 | 3.20 ± 0.75 | 3.39 ± 1.35 | 3.80 ± 1.23 | 4.26 ± 1.16 | 4.34 ± 0.95 | 4.28 ± 0.29 |
| $N\text{-}NH_4^+$ | $mgNH_4^+/L$ | 0.77 ± 0.52 | 0.77 ± 0.40 | 0.60 ± 0.33 | 0.61 ± 0.48 | 0.87 ± 0.44 | 0.99 ± 0.46 | 1.07 ± 0.49 |
| $N\text{-}NO_2^-$ | $mgN\text{-}NO_2^-/L$ | 0.24 ± 0.16 | 0.29 ± 0.37 | 0.03 ± 0.02 | 0.15 ± 0.17 | 0.13 ± 0.13 | 0.30 ± 0.22 | 0.22 ± 0.24 |
| $N\text{-}NO_3^-$ | $mgN\text{-}NO_3^-/L$ | 9.74 ± 3.45 | 14.22 ± 2.85 | 16.14 ± 2.40 | 10.13 ± 2.34 | 8.55 ± 1.17 | 14.14 ± 1.97 | 10.93 ± 2.75 |
| $E_{COD}$ * | % | 95.80 ± 0.55 | 96.59 ± 0.41 | 96.57 ± 0.72 | 96.42 ± 0.95 | 95.84 ± 0.48 | 95.69 ± 0.63 | 96.32 ± 0.57 |
| $E_{Nit}$ * | % | 93.86 ± 2.96 | 94.64 ± 1.68 | 94.52 ± 2.22 | 93.56 ± 2.04 | 93.15 ± 2.29 | 92.68 ± 1.56 | 92.64 ± 0.67 |
| $E_{Denitr.}$ * | % | 79.02 ± 3.42 | 72.19 ± 3.68 | 70.56 ± 3.69 | 76.77 ± 3.69 | 78.79 ± 1.57 | 69.10 ± 3.49 | 74.05 ± 4.71 |

Note: * Calculated in accordance with the methodology provided by Podedworna and Zubrowska-Sudol [32].

The transition from continuous (R = 0) to intermittent aeration (R = ¼) resulted in a certain decrease in the efficiency of the denitrification process ($E_{Denitr.}$) ($p$ = 0.000450). The average $E_{Denitr.}$ decreased from 79.02 ± 3.42% (S.I.1.) to 71.56 ± 3.53% (S.I.2.), accompanied by a 1.27-fold increase in TN concentration in the effluent. An almost 50% increase in the concentration of nitrate nitrogen was simultaneously observed in treated wastewater, with $N\text{-}NO_3^-$ constituting 80% of TN in the effluent (S.I.2.). Another substantial ($p$ = 0.003520) change in the efficiency of the denitrification process was recorded after the reduction of oxygen concentration to 3.5 $mgO_2/L$. It caused an increase in the $E_{Denitr.}$ from 70.56 ± 3.69% (S.I.3.) to 76.77 ± 3.69% (S.II.1.), and the mean TN concentration in the effluent decreased by 1/3. Due to the reduction of the reactor's organic and nitrogen loading rates, the average $E_{Denitr.}$ decreased from 78.79 ± 1.57% (S.II.2.) to 69.10 ± 3.50% (S.II.3.), therefore reaching the lowest level among all average values recorded throughout the experiment. Simultaneously with a decrease in the analyzed efficiency, a 1.47-fold increase in TN concentration in the effluent was recorded in comparison with S.II.2. A certain increase in the efficiency of the denitrification process occurred in the last series (S.II.4.), where the average $E_{Denitr.}$ reached a level of 74.05 ± 4.71%, although the RiR Tukey test showed that the differences were not statistically significant ($p$ = 0.214883).

No accumulation of nitrite nitrogen in treated wastewater was recorded in any of the series, either at stage I or II.

The obtained results also suggest that irrespective of changes introduced in particular series, the effectiveness of COD removal ($E_{COD}$) was maintained at a high and comparable

level—96.07 ± 0.74% ($p > 0.065506$). The COD did not exceed 27 $mgO_2/L$. The RiR Tukey test also showed no statistically significant difference between COD in the effluent for any of the series ($p > 0.085551$).

The nitrification, denitrification, and organic compound removal efficiencies obtained in this study are similar to those reported in full-scale MBBRs described in the work by Phanwilai et al. (2020) [33]. This indicates a high likelihood of success in wastewater treatment using the MBSBBR with the analyzed in this paper also on a full-scale (i.e., DO of 3.5 or 6.0 mg $O_2/L$ and IA with R equal to 0, 1/4, 1/3, or 1/2). Feng et al. (2018), conducting research in lab-scale sequencing batch biofilm reactors, demonstrated slightly higher values of the analyzed processes with respect to nitrification—96.6–98.9% and denitrification—88.1–89.2%, while $E_{COD}$ was at the level of 77.1–89.2% [34]. The observed discrepancies may arise from the strategy of IA that they employed, which could positively influence the denitrification process (R = 2, the duration of the subphase without aeration was longer).

### 3.2. Analysis of the Microbial Community Composition

The microbial composition of biofilm was characterized by Illumina MiSeq paired-end sequencing of the V3–V4 regions of the 16S rRNA gene. The alpha and beta diversity of the analyzed samples was estimated using the EzBioCloud platform [35]. The data were normalized by the number of reads (60,000). The number of OTUs increased in the last series of the first stage, when the aeration phase was the shortest (see Supplementary Material–Table S3). At the beginning of the second stage, it decreased to its initial value and then gradually increased until the end of the experiment. Species richness (based on the CHAO1 index) increased in series S.I.3. and was the highest of all the studied series. A sharp decrease in this value occurred after lowering DO, followed by an increase in the next series. A different trend was observed in community diversity (based on the Shannon index). Its value gradually increased across all series, reaching the highest value in series S.II.4., with the lowest DO and shortest aeration phase (Figure 4).

*Proteobacteria* were the most abundant phylum in all samples, with the highest abundance (47.8%) in the sample from the first series of the experiment, although their proportion decreased after shortening the aeration phase (see Supplementary Material–Figure S2a). In most experimental series, *Bacteroidota* and *Actinobacteriota* were second and third in abundance, respectively. The only exceptions were series S.II.2. and S.II.4., where the R value was the highest. In sample S.II.2, the number of *Actinobacteriota* decreased to 6.3%. This sample also had the highest percentage of *Bacteroidota* (20.8%) and *Nitrospirota* (7.1%). Sample S.II.4. had the highest proportion of *Chloroflexi* (14.9%) compared to the other series.

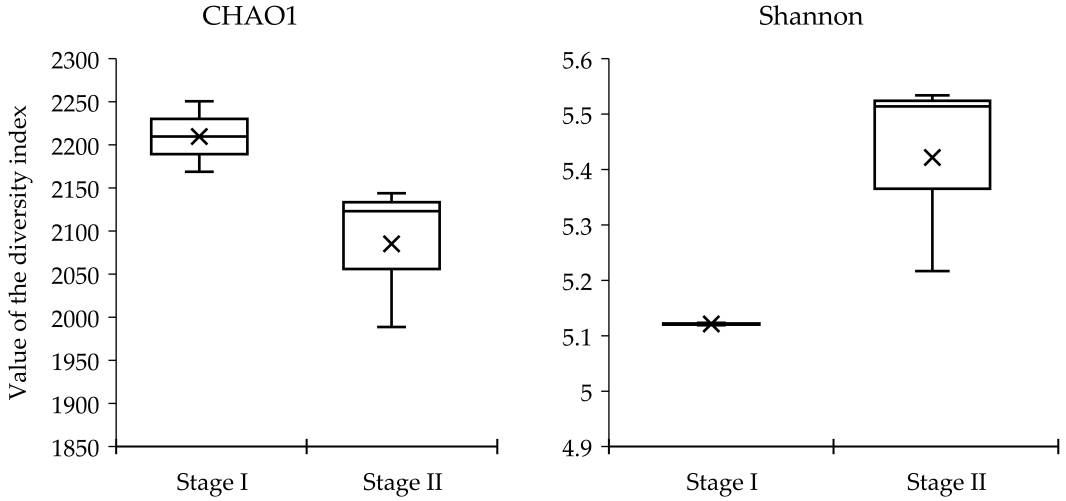


**Figure 4.** Comparison of species richness and diversity indices in two stages of the experiment.

At the genus level, a large percentage of the bacterial population consisted of less abundant and unidentified taxa (see Supplementary Material–Figure S2b). At the first stage of the experiment, *Ornithinibacter* and *Rhodobacter* were the most abundant genera. *Ornithinibacter* was particularly abundant in series S.I.3. (19.5%). At the next stage, with lower DO and higher R, the abundance of these two bacterial groups decreased, reaching the lowest values in sample S.II.2. Series S.II.2. was characterized by a high percentage of *Nitrospira* (7.1%). A gradual increase throughout the experiment occurred in *Kouleothrix*, whose abundance increased with decreasing R, from 0.1% in the sample from series S.I.1. to 5.23% in the sample from series S.II.4. In all series, the abundance of NOB bacteria was higher than that of AOB (see Supplementary Material–Figure S3). Moreover, the abundance of NOB increased steadily from series to series. Only in the last series, after reducing the loading rate of the reactor, there was a significant decrease in their abundance.

### *3.3. Nitrification Kinetics and Nitrifier Diversity in the Moving Bed Sequencing Batch Biofilm Reactor*

This study combined kinetics batch tests and microbiological analysis to assess the influence of different intermittent aeration strategies, dissolved oxygen concentration, and the reactor's organic and nitrogen loading rate on the nitrification process and nitrifying microorganism diversity in a pure moving bed sequencing batch biofilm reactor—MBSBBR with Nitrification/Denitrification. The values of AOR and NitOR determined in a particular series are presented in Figure 5, whereas changes in the abundance of nitrifying bacteria are shown in Figure 6 (charts with results of batch tests providing the basis for the determination of AOR and NitOR are presented in the Supplementary Materials—Figures S4–S10).

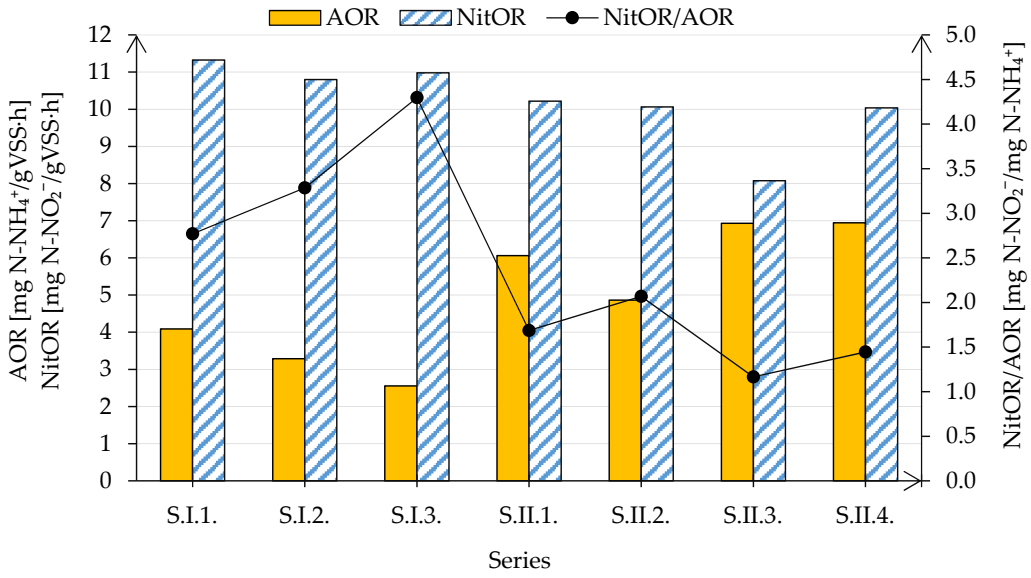


**Figure 5.** Ammonia Oxidation Rate (AOR) and Nitrite Oxidation Rate (NitOR) determined in AUR and NitUR batch tests and NitOR/AOR ratio.

The analysis of the data collected from batch tests shows that changes introduced in MBSBBR operation parameters had a greater effect on the activity of ammonia-oxidizing bacteria. Previous studies also reported that the activity of ammonia oxidizers was more susceptible to changes in system operation than those capable of oxidizing nitrite [17,18]. It is worth emphasizing that in that case, the biofilm came from a hybrid reactor, where, next to this form of biomass, also activated sludge developed. This study investigated the system with pure moving bed technology. Nevertheless, a similar trend was observed. Furthermore, the batch test results showed that the activity of bacteria capable of oxidizing ammonia nitrogen changed in a different way than that of those capable of oxidizing nitrite. However, the results of qPCR showed a similar direction of change in the amount

of AOB and NOB bacteria except S.II.4. For example, after changing the aeration strategy from continuous to intermittent (R = 1/4) (S.I.2.), the abundance of all studied bacteria increased by 6.9-fold, 8.1-fold, and 2.7-fold, for AOB, NOB, and Comammox bacteria, respectively. The same trend was observed in the biofilm from the aforementioned hybrid reactor, where, upon introducing intermittent aeration in the same regime, the abundance of these bacteria increased, despite the DO concentration being half as low as that in the mentioned study [17].

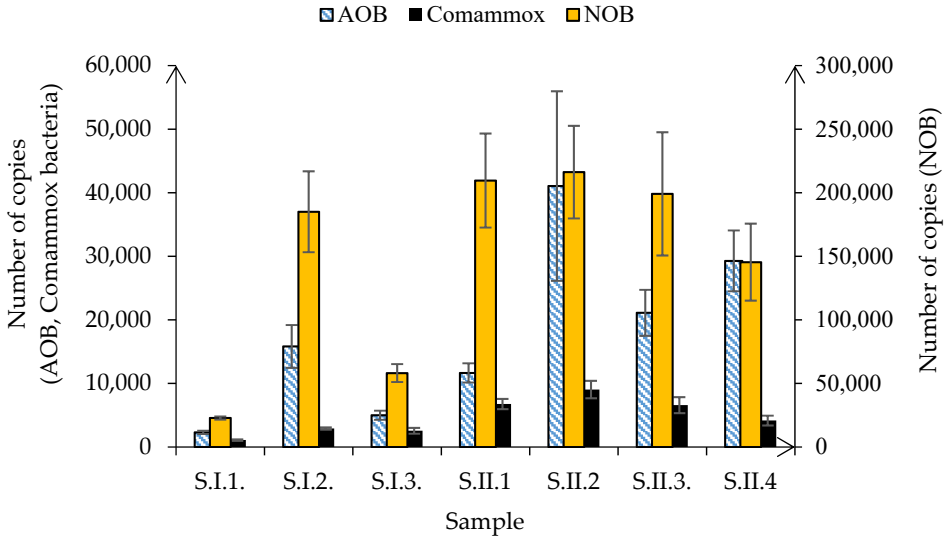


**Figure 6.** Absolute quantification of the AOB, NOB, and Comammox bacteria.

Each time, shortening the duration of subphases with aeration (increase in R) led to a drop in the activity of bacteria capable of oxidizing ammonia nitrogen by approximately 20% (from S.I.1. to S.I.2., from S.I.2. to S.I.3., from S.II.1. to S.II.2.), whereas reducing the OLR and NRL and a decrease in DO concentration followed in an increase in their activity. The greatest difference between AOR values was recorded after a decrease in DO (S.I.3. vs. S.II.1.). This aligns with the observations made for the biofilm from hybrid systems [17,18].

Data presented in Figure 5 also suggest that irrespective of the research stage, nitrite-oxidizing bacteria showed greater activity, which is consistent with qPCR results, where NOB were the predominant bacterial group in all samples. Interestingly, in the biofilm developing within the hybrid reactor, despite the markedly higher NitOR than AOR, the detected abundance of nitrite oxidizers was lower than that of AOB [17].

In the further series of stage I, with an increase in R, the difference between NitOR and AOR increased. The NitOR values were then 2.769 (S.I.1.), 3.284 (S.I.2.), and 4.296 (S.I.3.) fold higher than those of AOR. At stage II, the differences between the analyzed values decreased, and the lowest NitOR/AOR ratio was recorded after a decrease in the OLR and NLR (S.II.3.). The values of the analyzed rates differed only 1.17-fold. Undoubtedly, all these observations can be attributed to the fact that in the second stage of the experiment, changes in the reactor conditions had a much greater effect on the AOB bacteria, whose abundance fluctuated significantly compared to the other groups. In studies conducted in a laboratory-scale novel rotating self-aerated biofilm reactor by Luan et al. (2023), a higher activity of nitrite oxidizers was also demonstrated [36]. In the literature, there are also studies that describe this phenomenon in full-scale WWTPs [37,38]. For example, Regmi et al. (2011), conducting batch tests with carriers taken from a hybrid reactor, demonstrated that NitOR was 1.52 times higher than AOR [37]. Interestingly, they did not observe similar relationships in parallel tests conducted on activated sludge from the same system. Meanwhile, Yao and Pang (2017) investigated nitrification activity in 10 full-scale activated sludge biological nutrient removal WWTPs [38]. In batch tests conducted for activated sludge samples from WWTPs, the cited authors demonstrated that in each case,

the NitOR was higher than the AOR. This was explained by the fact that, stoichiometrically, six electrons are needed for oxidizing one mole of ammonia to nitrite, but only two for nitrite to nitrate. It is also interesting that the rates they obtained were, in most cases, lower than those determined in this study. The average AOR value from all 10 WWTPs was 1.53 times lower than that obtained in this work, while the difference between the average NitOR values was as much as 2.25 times. This can be considered evidence that systems based on biofilm provide a better environment for the development of nitrifiers, promoting a higher nitrification rate.

The comparison of the NitOR values obtained in subsequent series showed that the activity of nitrite-oxidizing bacteria was only influenced by the reduction of the OLR and NRL, suggesting greater stability of these microorganisms. It led to a 1.25-fold drop in the NitOR in comparison with that from S.II.2. (from 10.063 mgN-$NO_2^-$/gVSS·h to 8.076 mgN-$NO_2^-$/gVSS·h). The observed decline in NitOR value can be primarily attributed to a decrease in the activity of NOB due to no significant change in their number (less than 8%). After the change in the OLR and NLR, in the case of the biofilm from the hybrid system, a decrease in the rate of nitrite nitrogen oxidation was also observed [18]. In contrast, Yang and Yang (2011), conducting research in a moving bed membrane bioreactor with SND, observed a decrease in the nitrite oxidation rate after increasing the R value [39]. The cited authors, however, did not shorten the duration of the subphases with aeration such as in this work but extended the one during which aeration was not taking place.

As mentioned before, the reduction of the reactor's organic and nitrogen loading rates also caused an increase in AOR, although the number of AOB bacteria decreased two-fold. It is worth emphasizing that a 1.4-fold decrease in the amount of Comammox bacteria was recorded in parallel. These observations provide the basis for the conclusion that the nitrifiers capable of oxidizing ammonia, remaining in the biofilm at that time, showed much greater activity than those before the reduction of the OLR and NLR values. It is also possible that microorganisms capable of oxidizing ammonia nitrogen that were not detected with the used primers, due to their high phylogenetic diversity, appeared in MBSBBR. Shao et al. (2017) also observed an increase in the AOR after reducing the OLR. The cited authors conducted tests for biomass taken from a hybrid reactor [40]. After a two-fold reduction in OLR, they recorded a 1.51-fold increase in AOR, and after reducing it again by 1.66 times, the AOR value rose from 7.99 mgN-$NH_4^+$/gVSS·h to 18.25 mgN-$NH_4^+$/gVSS·h.

Lowering the concentration of DO by almost half increased the activity of microorganisms oxidizing ammonia nitrogen almost 2.4 times. In parallel, qPCR results provided the basis for recording an increase in the frequency of occurrence of AOB and Comammox bacteria, 2.3-fold and 2.7-fold, respectively. The abundance of NOB also increased (3.6-fold), although no significant changes were recorded in the NitOR value. The observations suggest that despite reducing DO, its concentration was sufficiently high for diffusion within biofilm not to be of significant importance. These conditions were also conducive to the growth of types of nitrifying microorganisms that prefer lower concentrations of DO and are more active at such concentrations. According to the literature, such organisms include the oligotropha lineages of *Nitrosospira* and *Nitrosomonas* [41]. Based on the relative abundance of the most prevalent phyla in the studied samples, an increase in *Proteobacteria* and *Nitrospirota* was observed. *Proteobacteria* are widely known to include various microorganisms responsible for removing nitrogen, among them AOB and NOB [42]. On the other hand, *Nitrospirota* was recognized as a microorganism responsible for the removal of ammonia and nitrite during biological treatment under aerobic conditions [43]. At the genus level, an increase in the abundance of *Nitrospira*, *Thaurea*, *Zoogloa*, and *Paracoccous* was detected. According to Luan et al. (2022), *Nitrospira* is widely known as the dominant NOB in biofilm reactors [44]. *Paracoccus* is a genus commonly recognized as a heterotrophic AOB [45].

It is also worth looking at the results presented in S.II.2. and S.II.4. MBSBBR operated under identical technological conditions during both series. Although the effectiveness of the nitrification process during these two series was comparable, some differences were

observed in the activity of individual groups of nitrifying microorganisms as well as their abundance. There were no significant changes in the NitOR values, but the AOR in S.II.4. was almost 1.5 times higher than that before OLR and NLR reduction (S.II.2.). This suggests that the number of bacteria capable of oxidizing ammonia nitrogen in the sample from series S.II.4. will be higher than in series S.II.2., and the abundance of NOB will be at a comparable level. The qPCR results showed, however, that contrary to expectations, despite identical operating conditions of the MBSBBR in S.II.2. and S.II.4., biomass was then poorer in all the analyzed groups of nitrifiers. AOB and NOB abundance decreased more than 1.40 times, and the amount of Comammox bacteria was 2.2 times lower than in S.II.2. Changes were also evident at the phyla and genera level. The relative abundance of *Nitrospira* decreased 2.67 times. This suggests that changes in the activity and abundance of individual groups of nitrifying microorganisms could have resulted from a decrease in the OLR and NLR in series S.II.3. Although the reactor operated for a long time under the same conditions as set in S.II.2., biomass did not return to its previous characteristics.

In addition to the canonical nitrifiers that oxidize ammonia nitrogen to nitrites and nitrites to nitrates, referred to as AOB and NOB, Comammox bacteria were detected in the MBSBBR. These microorganisms are believed to be genetically adapted to carry out the full nitrification process because their genome contains a full set of genes enabling the oxidation of both ammonia and nitrite nitrogen [46]. Biofilm is considered a favorable environment for the development of these microorganisms due to the low growth rate of Comammox *Nitrospira* [47]. Comammox *Nitrospira* may even dominate nitrification in biofilm under certain conditions [48,49]. Although it has been 7 years since the discovery of these microorganisms in wastewater treatment systems, the conditions conducive to their development are still not well defined. The results of the qPCR showed that the frequency of occurrence of Comammox bacteria was mostly affected by the transition from continuous to intermittent aeration (SI.1.–S.I.2.), as well as by reducing the DO. Next to the obvious decrease in DO concentration in MBSBBR, the introduction of IA also resulted in the biomass being periodically subjected to lower oxygen concentrations. According to Roots et al. (2019), lower DO is one of the factors considered favorable for Comammox bacteria [50]. Other studies have shown, however, a lack of significance in the relationship between DO and the occurrence of Comammox bacteria [51]. For example, in analogous research conducted for a hybrid system, a reduction in DO did not yield any discernible impact on the abundance of Comammox bacteria in the biofilm [17]. Similarly, to this study, a significant increase in this group of microorganisms occurred when continuous aeration was switched to intermittent aeration, despite markedly different reactor operating conditions (DO = 3 $mgO_2/L$ + presence of activated sludge). Zhao et al. (2022), conducting research in the MBBR reactor, observed selective enrichment of Comammox bacteria while the system operated at a DO concentration above 6 $mgO_2/L$ [15]. In contrast to the findings of Zhao et al. (2022) [15], this study determined the highest abundance of Comammox bacteria during the series, with the highest number of subphases without aeration during the aerobic phase (R = 1/2) and the lowest concentration of DO (3.5 $mgO_2/L$). The observed differences, could among, others result from continuous feeding of the MBBR in the cited paper with synthetic wastewater containing only ammonia and no organic carbon. Moreover, unlike this work, they did not use IA. These discrepancies indicate the need for further research on different systems that may, in the future, clearly determine how the concentration of DO affects Comammox bacteria.

Based on this study, the intermittently aerated pure biofilm reactor (MBSBBR) may be considered a promising technological solution applicable to biological wastewater treatment. The analysis of the quality of treated wastewater showed high efficiency in the removal of pollutants, regardless of the modifications applied to the operation of the reactor.

Future research should focus on further investigating the effect of the operational conditions of MBSBBR as a long-term gradual decrease of the reactor's organic and nitrogen loading rate and DO lowering to improve the operation of full-scale MBSBBRs as much

as possible. Reducing the costs associated with aeration will help reduce the energy expenditure of sewage treatment plants, which has benefits for the environment. Such research will also be promising in terms of the analysis of microorganisms inhabiting biofilm developing on moving carriers, with a particular focus on Comammox bacteria, to better understand the environmental conditions in which these poorly characterized nitrifiers can exist.

## 4. Conclusions

The findings from this long-term study have provided valuable information on the use of intermittently aerated pure biofilm reactors, such as the MBSBBR, for municipal wastewater treatment. In this research, particular emphasis was placed on the nitrification process, which represents one of the primary links in the nitrogen cycle in nature. Shortly, the main conclusions are summarized in the following points:

- The analysis of the quality of treated wastewater showed high efficiency in COD ($96.07 \pm 0.74\%$), N-$NH_4^+$ ($93.36 \pm 2.13\%$), and TN ($75.77 \pm 4.57$) removal across a wide range of system operating parameters, indicating its high versatility and adaptability to prevailing technological conditions.
- Despite no significant shifts in nitrification efficiency, disparities were observed in the activities and abundance of particular nitrifying microbial populations.
- The activity of bacteria capable of oxidizing ammonia nitrogen changed differently from that of bacteria capable of oxidizing nitrite in response to modifications in the MBSBBR operation, while the abundance of AOB, NOB, and Comammox bacteria shifted in the same direction.
- The oxygen concentration had a significant influence on the diversity of the bacterial community. The reduction of DO in the second stage led to a decrease in the number of taxa (based on the Chao1 index) and an increase in the homogeneity of the bacterial community (based on the Shannon index). This indicates that not all taxa present at the beginning of the experiment were able to adapt to the low DO concentration.
- The primary factor causing the reduction of the activity of ammonia oxidizers was shortening the duration of subphases with aeration during intermittent aeration. The change in the activity of the NOB was only affected by the reduction of the reactor's organic and nitrogen loading rates.
- The most significant shifts in the abundance of both AOB and NOB were noted after the change in aeration strategy from continuous to intermittent.
- The frequency of occurrence of Comammox bacteria was mostly affected by the transition from continuous to intermittent aeration, as well as by reducing the DO from 6 $mgO_2/L$ to 3.5 $mgO_2/L$.
- Despite long-term stable MBSBBR operation under the same conditions in two separate series, the characteristics of microorganism communities were different.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16040534/s1, Figure S1: qPCR standard curves created for amoA gene fragments of canonical AOB, 16S gene fragments of NOB, and amoA of Comammox bacteria; Figure S2: Relative abundance of the most prevalent (a) phyla and (b) genera in the studied samples. The graphs show only taxa which contributed more than 1.0% to the total bacterial community. The abundance of the remaining taxa was summed and labeled as "ETC"; Figure S3: Relative abundance of the nitrifying guilds; Table S1: Compounds of the synthetic wastewater; Table S2: Volatile suspended solids of biomass present in the MBSBBR; Table S3: Estimates of microbial diversity and richness indices in the studied series; Figure S4: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.I.1.; Figure S5: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.I.2.; Figure S6: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.I.3.; Figure S7: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.II.1.; Figure S8: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.II.2.; Figure S9: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.II.3.; Figure S10: N-$NH_4^+$, N-$NO_2^-$, N-$NO_3^-$ profiles during test (a) AUR (b) NitUR—S.II.4.

**Author Contributions:** Conceptualization, O.Z. and M.Z.-S.; Methodology, O.Z., M.Z.-S., M.G. and S.C.; Formal analysis, O.Z. and M.G.; Investigation, O.Z. and M.G.; Writing—Original Draft Preparation, O.Z. (1.; 2.1.; 2.2.; 2.3.; 2.4.; 2.6.; 2.7.; 3.1.; 3.3.; 4) and M.G. (2.5.; 3.2.); Writing—review and editing, M.Z.-S. and S.C.; Visualization, O.Z. and M.G.; Supervision, M.Z.-S.; Project Administration, M.Z.-S. All authors have read and agreed to the published version of the manuscript.

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