Removal of High-Strength Ammonia Nitrogen in Biofilters: Nitrifying Bacterial Community Compositions and Their Effects on Nitrogen Transformation

Fuquan Peng [1],[†], Yuexiang Gao [1],[†], Xiang Zhu [1], Qingqing Pang [1], Longmian Wang [1],*, Wenwen Xu [2], Jianghua Yu [2], Pengcheng Gao [1],[3], Jingxian Huang [1],[3] and Yibin Cui [1],*

[1] Nanjing Institute of Environmental Sciences, Ministry of Ecology and Environment, Nanjing 210042, China; pfq@nies.org (F.P.); gyx@nies.org (Y.G.); zx1420775396@163.com (X.Z.); pqq18375335636@163.com (Q.P.); gpc1150@163.com (P.G.); huangjx2020@163.com (J.H.)

[2] Collaborative Innovation Center of Atmospheric Environment and Equipment Technology, Jiangsu Key Laboratory of Atmospheric Environment Monitoring and Pollution Control, School of Environmental Science and Engineering, Nanjing University of Information Science & Technology, Nanjing 210044, China; xww_developing@163.com (W.X.); yujh@nuist.edu.cn (J.Y.)

[3] College of Environment, Hohai University, Nanjing 210098, China

* Correspondence: wlm@nies.org (L.W.); cyb@nies.org (Y.C.)

† Co-first authors.

check for updates

Abstract: Increasing attention has been given to the treatment of livestock and poultry wastewater because of its high ammonium nitrogen (NH_4^+-N) content and low carbon/nitrogen ratio (C/N). Ceramic filter medium (CFM) and dewatered aluminum sludge (DAS), which are products from cast-off materials, are used as small-scale combined biological filters (CFM-DAS) for wastewater treatment. The high and stale removal efficiency of chemical oxygen demand (COD), NH_4^+-N, and total nitrogen (TN) in the DAS filter indicate that DAS plays a major role in pollutant removal. Although significant differences are found between the composition of nitrifying bacteria in CFM and DAS, the structures of nitrifying communities are evenly distributed in each layer of CFM or DAS irrespective of the running time. Microbial compositions are attributed to the comprehensive effect of various environmental factors such as pH and TN at effluents. In the DAS, *Nitrosospira* shows significant negative correlation with the concentrations of NH_4^+-N in effluents, whereas it has positive correlation with NO_3^--N, and *Nitrososphaera* has a significant negative correlation with NO_3^--N in effluents. Pearson correlation test reveals that certain genera may be used in estimating or predicting NH_4^+-N consumption and NO_3^--N accumulation in CFM-DAS for treating sewage with a high NH_4^+-N content.

Keywords: ammonia-oxidizing archaea; ammonia-oxidizing bacteria; ammonium nitrogen removal; biological filter; ceramic filter medium; dewatered aluminum sludge; nitrite-oxidizing bacteria; wastewater treatment

1. Introduction

In light of continuous economic developments, livestock and poultry wastewater with high concentrations of organic substances and most farms without pollution control facilities has become one of major pollution sources, after industrial wastewater and domestic sewage [1]. Discharging high levels of ammonium nitrogen (NH_4^+-N) from agricultural drainage to rivers, sewer systems, or soil causes severe pollution problems [2]. Therefore, economical and eco-nitrogen (N) removal

technologies for treating piggery wastewater with low carbon/nitrogen (C/N) ratio and high NH_4^+-N concentrations have attracted significant attention. These technologies ensure the security of water resources and human health.

Livestock and poultry wastewater containing high NH_4^+-N concentrations is usually treated using biological treatment systems, which are the most popular systems in terms of degree of treatment [3,4]. Compared with using biofilters, which are energy intensive and have high technique requirement, adsorption is a high-efficient and well-established process. The filter medium and feeding mode used affect the performance of biofilters [5]. Conventional combinations of soil, sand, and gravel are used as wetland substrates. In this study, a combination of ceramsite filter material and dewatered alum sludge (CFM-DAS), which is a novel industrial waste-based biofilter, was used. Wang et al. [6] combined neutralized used acid (NUA) and DAS to remove high-N concentration from synthetic livestock wastewater. However, this system caused device blockage problems and decreased removal effect. To solve these issues, we used CFM to replace NUA.

CFM is commonly used in reducing N concentrations in sewage for its specific adsorption properties and long working cycle [7]. Wang et al. [8] used sediments as a resource for developing a novel ceramsite presenting the characteristic of solidity, which suggests the potential application as bioretention medium for purifying storm water. Cao et al. [9] developed a floating constructed wetlands by using light ceramsite as a substrate for microorganisms and macrophytes, where NH_4^+-N concentration decreased from 0.50–1.32 mg/L to 0–0.55 mg/L, whereas TN concentration decreased from 1.32–2.97 mg/L to 0.05–1.32 mg/L, which shows constructed wetlands using CFM for bioremediation of wastewater is feasible under low water temperature conditions. Wu et al. [10] applied a novel sludge–ceramsite as the main substrate in constructed wetlands with intermittent aerated subsurface flow and high COD (97.2%), NH_4^+-N (98.9%), and TN (85.8%) removal efficiency (97.2%, 98.9%, and 85.8%, respectively). DAS is an industrial by-product used as chemical agent for coagulation–flocculation process in water treatment [11]. Therefore, this method reuses DAS to save resources and protect the environment at the same time. DAS is extensively utilized as a constructed wetland (CW) medium to remove N from animal farm sewage effectively [12]. Hu et al. [13] showed that a single-bed tidal flow constructed wetland with DAS achieve robust TN elimination performance (85% on average under N loading rate of 28 g N m^{-2} day^{-1}), among which NH_4^+-N removal was recorded as 95%, which makes DAS an adequate option to provide advanced wastewater treatment for peri-urban communities and rural areas. Alternatively, DAS is a mode of treating wastes with another type of waste [14] that solves the problem of sludge treatment and simultaneously process polluted water. Combined CFM and DAS rationally and strategic operations were employed, another new alternative cost-effective system for livestock wastewater treatment might be developed for the reduction of N loadings.

Commonly nitrification is performed in two steps [15,16]:

(1)　NH_4^+-N $+ 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$, transformed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).
(2)　$NO_2^- + 0.5O_2 \rightarrow NO_3^-$, transformed by nitrite-oxidizing bacteria (NOB).

These two reactions are rate-limiting steps in the removal of biological N from wastewater, in which microbial community structure and diversity are key factors [4,17]. AOA, AOB, and NOB distribution have been analyzed in recent single- and multi-stage CWs [15,18]. As mentioned earlier, some studies were conducted to discuss AOA, AOB, and NOB compositions in CFM [9,10], but their compositions in combined CFM-DAS remain unknown where CFM and DAS possibly act synergistically in the CFM-DAS system, and the microbial community structures obtained from the substrates were possibly more complex than those in a system with only one type of medium.

Considering the possibility of using a novel combination of biofilters for the removal of NH_4^+-N from livestock and poultry wastewater and the mechanism of nitrifying bacteria in its system, this study focus on the following aspects: (I) the NH_4^+-N removal efficiency of CFM-DAS on the simulated

livestock and poultry wastewater with high nitrogen, (II) the relative abundance characteristics of AOA, AOB, and NOB in the temporal and spatial distributions of the system, (III) the influence of environmental factors on the nitrifying bacterial community compositions, and (IV) the relationship between the dominant genera and N concentrations at effluents. This research aims to provide an in-depth theoretical understanding on the biological mechanism of nitrogen removal and transformation in biofilter and provide an alternative biofilter technology for the removal of high NH_4^+-N in wastewater.

2. Materials and Methods

2.1. Bioreactor Construction and Basic Physicochemical Properties of Filter Media

The schematic of the laboratory-scale experimental system (Figure 1) was constructed at the Nanjing Institute of Environmental Sciences, Ministry of Ecological Environment, Nanjing, China, during April to September in 2016. The main devices were two identical rectangular-shaped Plexiglas columns. One was filled with CFM, and the other with DAS. CFM (particle size <0.25 mm) was obtained from a ceramic production plant in Anhui Province, and DAS (particle size <0.25 mm) was obtained from the by-product of coagulants, such as aluminum salt in the water purification system of a waterworks in Nanjing. Each device had a height of 1 m, width of 0.2 m, and effective volume 40 L. Containers were connected with a 0.25 m height difference for drop aeration oxygenation. The top of the CFM filter box was sealed to provide an anoxic environment suitable for denitrification, and the top of the DAS was left open to feed and oxidize. From top to bottom, the following measurements were ensured: a 0.1 m water distribution area, a 0.7 m reaction layer, and a 0.2 m cobblestone supporting layer, in which a sampling port for every 0.2 m was set up. Two drainage outlets were installed on the top of the CFM (outlets 1 and 2) to release the treated water. One reflux outlet and one outlet hole (total outlet) were installed at the bottom of DAS to reflow some of the water to the backflow reservoir and discharge the final purified water, respectively.
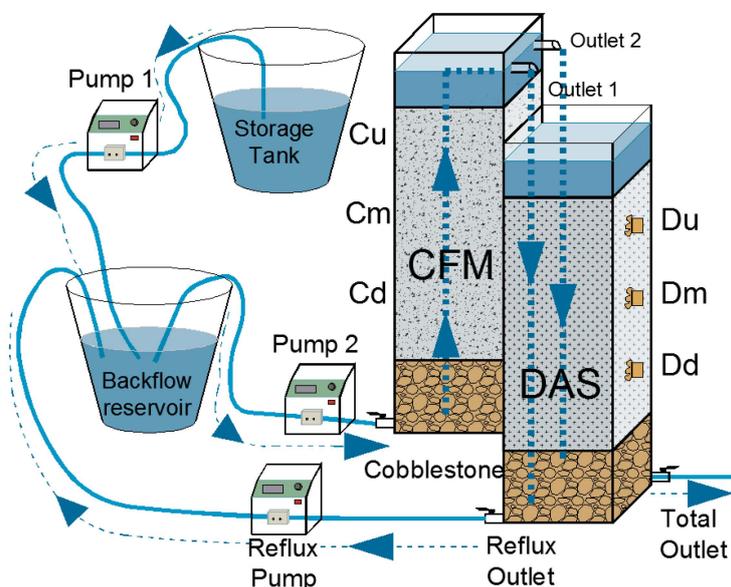

Figure 1. Schematic diagram of ceramic filter medium-dewatered aluminum sludge (CFM-DAS) experimental apparatus and operating.

The pH of the filter material was measured in a 10% (w/v) aqueous solution with a digital pH meter. The specific surface area of the filter materials was measured at room temperature and atmospheric pressure through the Brunauer-Emmett-Teller method with N. NOVA 3000e surface area and pore size analyzer (Quantachrome Instruments, Boynton Beach, Florida, USA) were used. Bulk density and porosity were determined using standard soil science methods [6]. The chemical compositions of the

media were analyzed using an ARL-9800 X-ray fluorescence spectrometer (ARL Corporation, Beijing, China). The result of physicochemical characteristics are listed in Tables S1 and S2.

2.2. Bioreactor Operation and Sampling

The synthetic wastewater was applied in this experiment throughout the entire operation period. Before the test cycle, CFM and DAS columns were filled with synthetic wastewater. Reflux outlet and total outlet were closed, whereas peristaltic pumps 1 and 2 were opened. Initial synthetic wastewater was introduced into the first stage filter at 2.0 $m^3 \cdot (m^2 \cdot d)^{-1}$ flow rate with pump 2, in which wastewater flowed into the CFM layer from bottom to top. Treated synthetic wastewater by CFM overflowed from outlets 1 and 2, completed self-oxygenation process for altitude differences, and entered the second-stage filter material device. Subsequently, wastewater coming from CFM flowed from top to bottom in sequence. The DAS device was full after 12 h, and the reflux outlet was subsequently opened. Half of the reaction volume treated sewage in the DAS device entered the backflow reservoir through the reflux pump and mixed with the initial synthetic wastewater. The combined sewage was pumped into the CFM device with pump 2. The flow rates of the three pumps in the above process were consistent at 1.0 $m^3 \cdot (m^2 \cdot d)^{-1}$. Subsequently, the system entered a state of formal dynamic cycle. The complete hydraulic retention of this system including recirculation was 48 h. The reflux and total outlets were normally opened for continuous backflow and water overflow prevention. The full effective volumes in the CFM and DAS devices were ensured by adjusting the total effluent valve. The system was ran in this dynamic cyclic state for 140 days.

2.3. Synthetic Wastewater Composition

The suspended solid-free synthetic wastewater samples were composed of NH_4Cl, KH_2PO_4, $C_6H_{12}O_6$, $NaNO_3$, and $NaNO_2$. We used magnesium, calcium, and iron salts as trace elements for microbial growth and adjusted the pH with HCl and NaOH after mixing evenly. The properties of synthetic wastewater for simulating diluted piggery sewage [19] are shown in Table 1.

Table 1. Qualities of synthetic livestock wastewater in experiment.

Index	Values
chemical oxygen demand (COD)	174.4 ± 59.9 (mg·L^{-1})
ammonia nitrogen (NH$_4^+$-N)	117.0 ± 28.9 (mg·L^{-1})
nitrate nitrogen (NO$_3^-$-N)	1.5 ± 0.7 (mg·L^{-1})
Total nitrogen (TN)	131.1 ± 26.9 (mg·L^{-1})
pH	8.0 ± 0.2
Air temperature	25.1 ± 7.7 °C
Water temperature	23.5 ± 6.3 °C
total phosphorus (TP)	22.8 ± 4.0 (mg·L^{-1})

2.4. Analyses

2.4.1. Water Sample Collection and Water Quality Analysis

The influents and effluents from CFM and DAS columns were sampled every week for the measurement of COD, TN, NH$_4^+$-N, and NO$_3^-$-N concentrations according to the methods described in Examination of Water and Wastewater [20]. Temperature (T), pH, and dissolved oxygen (DO) were measured in situ with a multi-parameter water quality meter (YSI Model No.550A, YSI Corporation, OH, USA). All samples were analyzed in triplicate.

2.4.2. Filter Material Extraction and High-Throughput Sequencing (HTS) Analysis

Filters approximately 100 g each from initial substrates (C0, D0), upper-layer substrates (Cu1/2/3, Du1/2/3), middle-layer substrates (Cm1/2/3, Dm1/2/3), and down-layer substrates (Cd1/2/3, Dd1/2/3)

collected in CFM and DAS columns were obtained (C and D represent CFM and DAS, respectively, 1/2/3 indicates 30th/90th/140th day). The prepared DNA were stored at −80 °C until analysis through polymerase chain reaction (PCR).

A total of 20 samples using CFM-DAS system were prepared by Miseq library, including genomic deoxyribo nucleic acid (DNA) extraction, PCR amplification, high throughput Illumina Miseq 2 × 300 bp sequencing, and bioinformatics analysis by Tinygene Company (Shanghai, China). Based on sample type, we selected the best extraction method, using an Ultra Clean Soil DNA isolation kit (MO BIO Laboratories, Loker Ave West, Carlsbad, CA, USA), in genomic DNA extraction from 250 mg of sediment. Then, two-way sequencing was performed according to the requirements of Illumina HiSeq. The target region and fusion primer with "5′miseq connector-barcode- sequencing primer-specific primer-3′" were designed [21]. We constructed a library through two-step PCR amplification. The primers utilized for amplification and cycle condition are shown in Table S3. Subsequently, specific primers were used to amplify the target fragments, and the target fragments were recovered and used as templates for secondary PCR amplification with eight cycles. Finally, we sequenced the necessary connectors for Illumina platform, sequenced the primers, and added barcodes to both ends of the target fragment.

The PCR amplification of Cu1, Cu2, Cu3, Cm1, Cm2, Cm3, Cd1, Cd2, and Cd3 with AOA *amo*A primers failed possibly because of PCR primer specificity or representative sampling. The sequences generated in this study were deposited to the National Centre for Biotechnology Information under accession numbers SRP126015 (AOA), SRP126023 (AOB), and SRP126023 (NOB).

2.4.3. Statistical Analyses

The calculations were as follows:

$$\text{CFM removal efficiency} = (C_{in} - C_{CFM}) / C_{in} \tag{1}$$

$$\text{Total removal efficiency} = (C_{in} - C_{eff}) / C_{in} \tag{2}$$

where C_{in} is the influent concentration, C_{CFM} is the CFM effluent concentration, and C_{eff} is the total system effluent concentration.

The ratio between nitrate production and ammonium consumption ($\Delta NO_3^- \text{-N}/\Delta NH_4^+ \text{-N}$) was calculated according to Equation (3) [22].

$$\Delta NO_3^- \text{-N} / \Delta NH_4^+ \text{-N} = (NO_3^- \text{-N}_{eff} - NO_3^- \text{-N}_{in}) / (NH_4^+ \text{-N}_{in} - NH_4^+ \text{-N}_{eff}) \tag{3}$$

The concentration curve and removal efficiency were analyzed using the Origin software (Origin 2018, OriginLab Corporation, MA, USA). Valid sequences were then distributed to operational taxonomic units (OTUs) with a cutoff value of >97%, and raw sequencing data were processed in Mothur v.1.33.3 (The University of Michigan, MI, USA). A weighted uniFrac statistical analysis was performed to provide the index of community distances between each pair of samples based on β diversity. The R heat map package (R 3.0.2, University of Auckland, Auckland, New Zealand) was used. For the categorization of the samples at the genus level, the relative abundance was computed using the R programming language. Redundancy analysis were conducted using Canoco (version 4.5, Wageningen University & Research on Social Media, Wageningen, Holland) for the analysis of the effect of correlation between environment factors and on nitrifying bacteria structures and characteristics in different media. Statistical analyses were conducted using SPSS (version 20.0, IBM Corporation, New York, NY, USA) for the determination of the Pearson correlation between the relative abundance of the major genera and water quality index of the effluents. All values were presented at $p < 0.05$ levels.

3. Results

3.1. Wastewater Treatment Performance

As shown in Figure 2, the removal efficiency of COD, TN, and NH_4^+-N in the CFM filter and the total system started to stabilize after 5 weeks. Under the condition of influent COD concentration of 173.3 mg L^{-1}, the removal efficiency of COD for the CFM filter medium and integral system was stable at approximately 23.8% and 91.3%, respectively, after 5 weeks (Figure 2a). TN removal efficiency of CFM and CFM-DAS system in Figure 2b shows that when the initial concentration of influent was 131.1 mg·L^{-1}, the outflow was approximately 113 mg·L^{-1} by CFM and 26 mg·L^{-1} by total steady, with removal efficiency of 13.2% and 75.1%, respectively. The removal efficiency of NH_4^+-N by CFM and CFM-DAS system were between 6.7% and 40.2% and between 89.5% and 98.5%, respectively, as shown in Figure 2c. After stabilization, the average NH_4^+-N removal efficiency of CFM reached 13.9%, and the total system was approximately 95.8%. The NO_3^--N had a certain degree of accumulation in the effluent of CFM and the total effluent in Figure 2d. The stable average effluent concentrations of CFM and total effluent were approximately 8 and 20 mg/L, respectively.
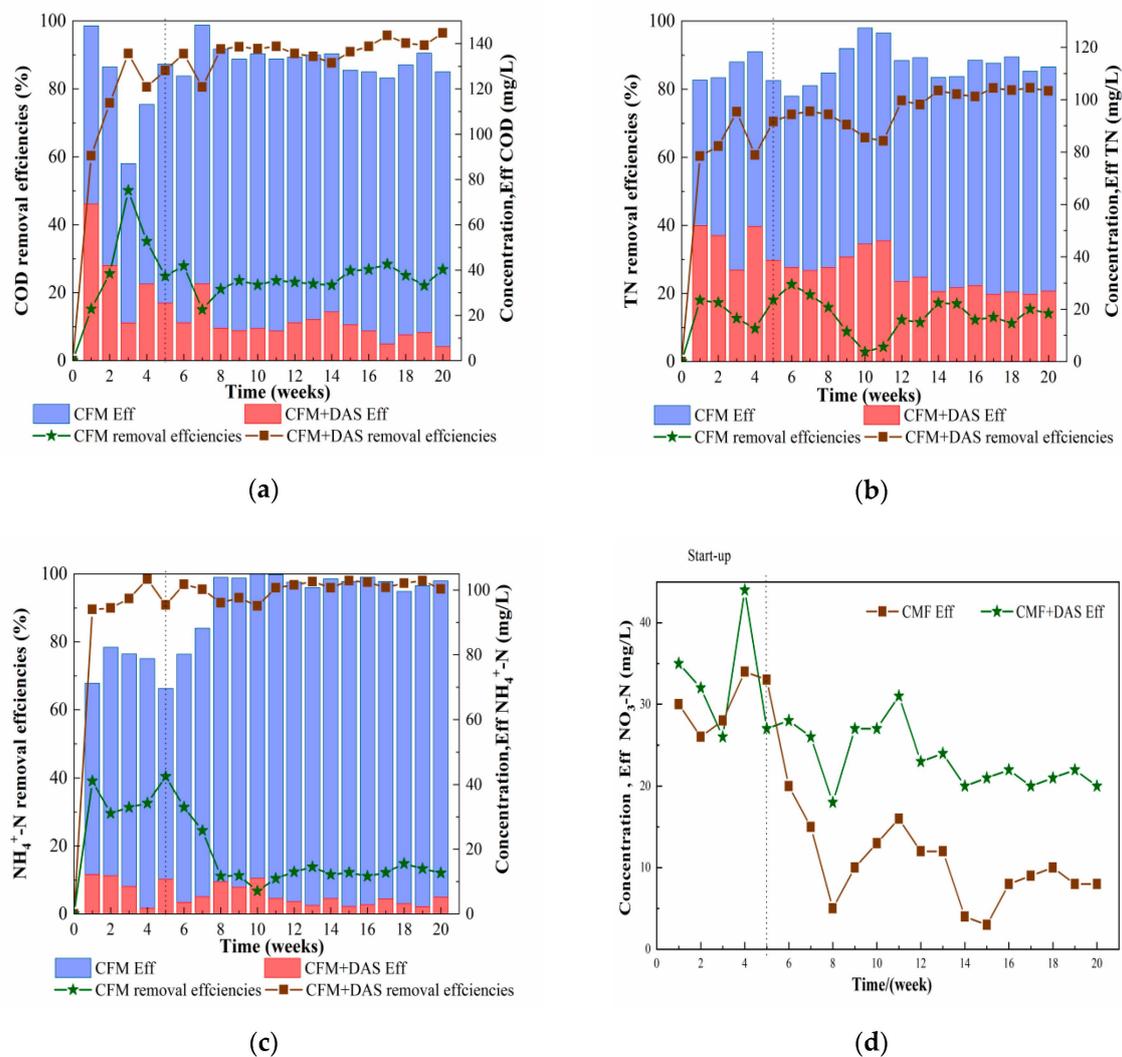

(a)

(b)

(c)

(d)

Figure 2. Mean concentration of the effluent and COD, total nitrogen (TN), and NH_4^+-N removal efficiencies using the CFM-DAS biofilter over time. (**a**) Removal of chemical oxygen demand (COD). (**b**) Removal of total nitrogen (TN). (**c**) Removal of ammonium nitrogen (NH_4^+-N). (**d**) Effluent of nitrate nitrogen concentration (NO_3^--N).

In this study, the ratio of $\Delta NO_3^--N/\Delta NH_4^+-N$ was used to roughly predict the growth status of AOA/AOB and NOB. Its ratio in the DAS was low and stable (0.1), indicating high AOA/AOB or low NOB growth (Figure 3). Conversely, the values in the CFM had large fluctuations, which were more than 0.5 and reached a maximum of 1.5, and the forecasting growth of nitrifying bacteria in the CFM was contrary to that in the DAS.
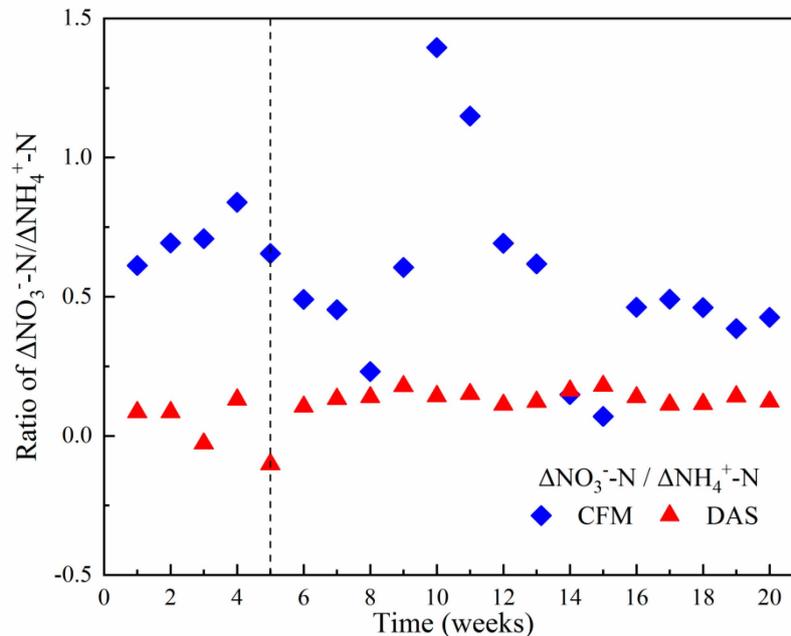

Figure 3. Ratio of $\Delta NO_3^--N/\Delta NH_4^+-N$ in CFM and DAS systems.

3.2. Nitrifying Bacteria Community Composition Distributions

In general, except individual and initial samples, the structures of nitrifying communities were evenly distributed in each layer of CFM and DAS irrespective of the running time. However, differences between the compositions of the nitrifying bacteria in CFM and DAS were observed. The matrix thermal diagram of AOA, AOB, and NOB are represented in Figure 4, which shows that the differences observed in NOB was smaller than those in AOA and AOB. For AOA (Figure 4a), primary genera of C0 and D0 dramatically differed from those of other samples, and middle layer of DAS at 30 days (Dm1) was different for the middle layers from the other samples. The β diversity of AOB from Figure 4b illustrates that CFM is different than the DAS filter, except the initial sample of DAS. After 30 days of operation, the difference between the filler samples was generally small, but after 90 and 140 days, the difference between the samples distinctly increased, especially in the lower layer of the CFM filter. A similar conclusion was confirmed for NOB in Figure 4c. No significant difference between the DAS systems during operation was observed, but the difference between the CFM samples in down layer increased with time.
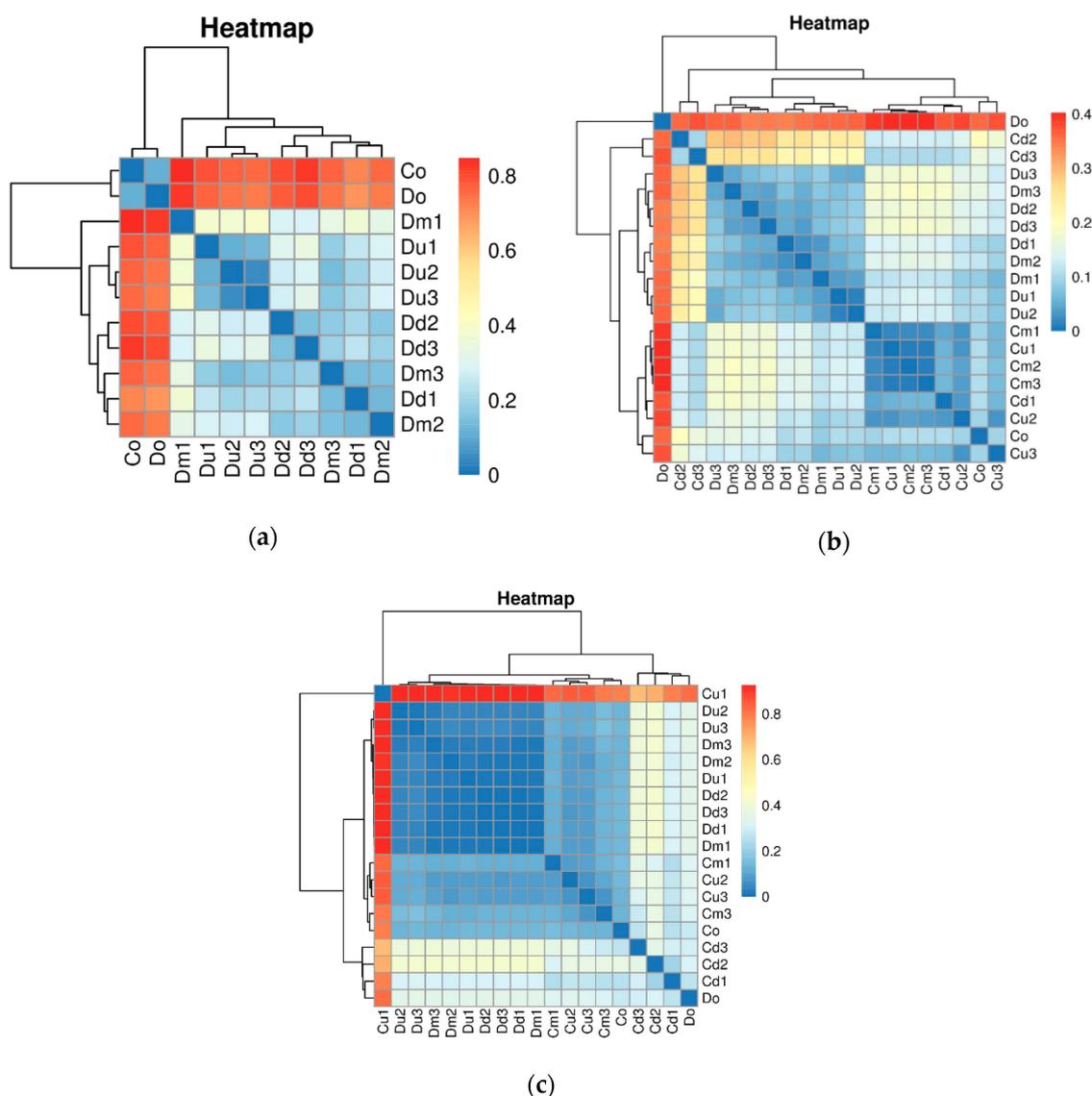
(a)

(b)

(c)

Figure 4. Heat map of weighted UniFrac dissimilarity matrix based on the bacterial community at different heights and times of (**a**) ammonia-oxidizing archaea (AOA), (**b**) ammonia-oxidizing bacteria (AOB), and (**c**) nitrite-oxidizing bacteria (NOB) in the CFM-DAS system.

To further clarify the differences between AOA, AOB, and NOB compositions with the samples, the relative abundances in CFM and DAS biofilter media were investigated (Figure 5). In Figure 5a, 17 dominant genera of AOA are detected in the DAS samples, which is relatively more than AOB (9 genera) and NOB (7 genera). The relative abundance of *Nitrosopumilus* had the highest level in C0 (69.0%) and D0 (27.5%). The rest of the DAS samples were dominated by *Nitrososphaera* (over 80%), and its dominant position did not change with time and space. From Figure 5b, *Nitrosomonas* was the main genus in CFM (average 82.9%), although its relative abundance in Cu3 slightly decreased. Similarly, *Nitrosomonas* was one of the dominant genera, with average relative abundance of 45.0%, in DAS. Another main genus, *Nitrosospira*, which was 6.7% in C0, was mainly concentrated on the upper layer of DAS (approximately 32.3 to 51.0%), and its relative abundance in the three layers increased slightly with time. The number of NOB dominant genera in CFM (i.e., 3) was higher than that in DAS (i.e., only 1; Figure 5c). *Nitrobacter* had an absolute dominance in CFM and DAS, especially in DAS (over 99.5%) except in Cu1 (6.0%). *Deinococcus* and *Lambdalikevirus* were observed in CFM and the

highest in Cu1 of 29.9% and 15.8%, respectively. *Deinococcus* increased with running time in down and middle layer, whereas *Lambdalikevirus* decreased. Simultaneously, *Devosia* occurred in Cd3.

Figure 5. Microbial community composition of (**a**) AOA, (**b**) AOB, and (**c**) NOB at genus-level from different heights and times in CFM-DAS.

3.3. Correlation between Environmental Variables and Nitrifying Bacteria Structures

The relationship between environmental factors and structures of different bacteria showed different correlations and degrees of influence. As shown in Figure 6a, most indices corresponded with AOA on the first axis. The most observed were T, DO, TN, and NO_3^--N for −0.470, 0.438, 0.430, and 0.416, respectively. As shown in Figure 6b, TN, NH_4^+-N, COD, and pH are relatively important environmental factors, and their correlation coefficients were −0.673, −0.648, −0.619, and 0.591, respectively. The correlation coefficients of DO and T on the second axis were 0.566 and −0.479, respectively. NOB was significant with TN, NH_4^+-N, COD, and pH on the first axis, and the correlation coefficients were −0.808, −0.636, −0.540 and 0.654, respectively, whereas COD and NH_4^+-N on the second main axis had correlation coefficients of 0.441 and 0.305, respectively (Figure 6c). Overall, pH and TN concentrations in the effluent were the main characteristics influencing the nitrifying bacteria structures. Furthermore, DO and T had relatively important effects on the compositions of AOA and

AOB, whereas COD and NH_4^+-N had important effects on AOB and NOB. NO_3^--N was an essential key index affecting AOA distribution.
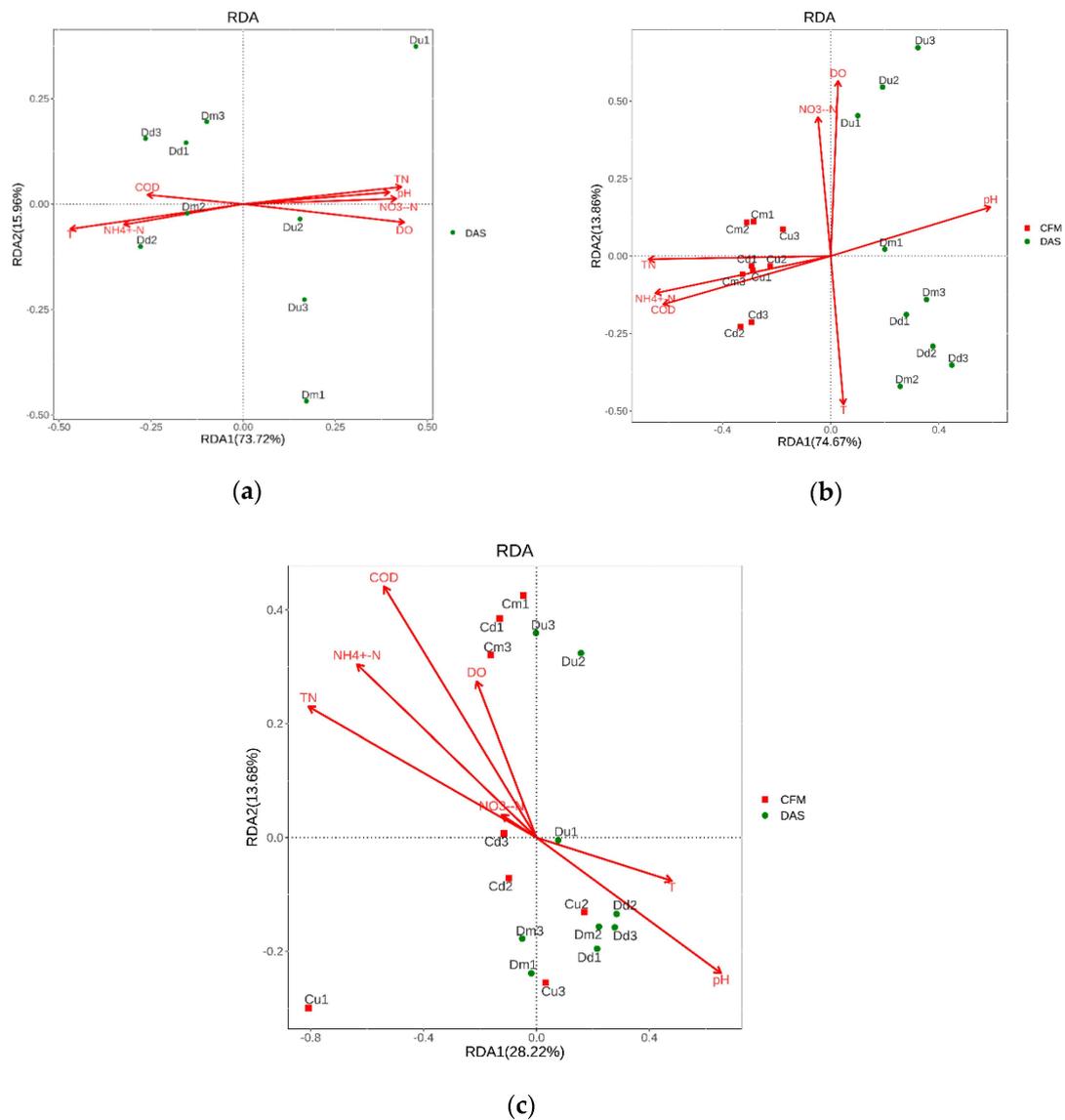

Figure 6. Ordination plots generated by redundancy analysis of (**a**) AOA, (**b**) AOB, and (**c**) NOB.

3.4. Pearson Relationship between the Nitrifying Bacterial Genera and Nitrogen Removal

The effect of microorganisms on nitrogen removal in CFM and DAS was evaluated by analyzing the relative abundances of the dominant genera (Table 2) and effluent wastewater concentrations through Pearson correlation. Interestingly, the relative abundances of the four main genera in CFM were not significantly correlated with effluent concentration. Notably, in DAS, *Nitrosospira* plays an important indicative role and was negatively correlated with NH_4^+-N concentration (R is −0.807, $p < 0.01$) and positively correlated with NO_3^--N (R is 0.808, $p < 0.01$). In addition, *Nitrososphaera* negatively corresponded with NO_3^--N in the effluents of DAS (R is −0.672, $p < 0.05$).

Table 2. Pearson correlation coefficients (R) between the relative abundances of the dominant genera and effluent wastewater concentrations factors in CFM and DAS.

Filter	Genus		Eff. COD	Eff. TN	Eff. NH_4^+-N	Eff. NO_3^--N
CFM	*Nitrosomonas*	R	0.066	−0.111	−0.065	−0.285
		p-Value	0.866	0.776	0.867	0.458
	Nitrobacter	R	0.328	0.139	0.485	0.115
		p-Value	0.427	0.742	0.224	0.786
	Lambdalikevirus	R	−0.638	−0.097	−0.188	−0.250
		p-Value	0.088	0.819	0.656	0.551
	Deinococcus	R	−0.443	0.222	−0.687	0.294
		p-Value	0.271	0.598	0.060	0.480
DAS	*Nitrososphaera*	R	0.439	−0.441	0.569	−0.672 *
		p-Value	0.237	0.234	0.110	0.048
	Nitrosomonas	R	0.538	0.336	0.435	−0.135
		p-Value	0.135	0.377	0.242	0.729
	Nitrosospira	R	−0.764 *	0.384	−0.807 **	0.808 **
		p-Value	0.016	0.307	0.009	0.008
	Nitrobacter	R	0.144	−0.032	0.092	−0.069
		p-Value	0.711	0.935	0.814	0.860

* and ** indicate significant (2-tailed) effects at the $0.01 < p < 0.05$ and $p < 0.01$ levels, respectively.

4. Discussion

4.1. Effective COD and Nitrogen Removal

The removal of organic matter from the system was mainly through physical, chemical, and microbial interactions [4,23]. The removal efficiencies of pollutants in the CFM–DAS filter fluctuated remarkably during the first five weeks of the system operation and gradually stabilized after five weeks mainly because of the biofilm stage in the first five weeks. At the startup period, the fluctuations in the removal efficiency may be due to the biofilm on the media that did not accumulate to form a sufficient biomass and support high organic matter removal.

Compared with the average removal efficiency in a previous study [6], average removal efficiency increased by 4.2% for TN and 6.6% for COD after the operation mode of the device was improved and optimized. The attached biofilm decomposed the organic matter intercepted in the filter materials, which in turn reduced COD concentration [6]. As NH_4^+-N was the primary N compound present in the synthetic livestock wastewater, it was removed from the two columns through rapid adsorption by organic matter and biomass during the contact period. In this system, the retention at the substrate was the major contributor to NH_4^+-N adsorption. Given that the specific surface area of DAS was twice that of CFM, the removal efficiency was high. In addition, the high pH and DO in DAS were conducive to the removal of NH_4^+-N, and DAS played a major role in this system. Although a certain amount of TN was removed from the influent, notable NO_3^--N buildup was observed in the effluents at the DAS column. As NO_3^--N was mainly converted through NH_4^+-N oxidation in the DAS system, NO_3^--N concentrations increased concomitantly with the decrease in NH_4^+-N concentration. The certain degree of NO_3^--N accumulation in CFM and DAS suggests that although NOB plays an important role in the oxidation of NO_2^--N to NO_3^--N, the rate of nitration was probably faster than the rate of denitrification by NO_2^--N in the CFM-DAS biofilter, as observed also in CWs with intermittent aeration using DAS as filter material by Hu et al. [12].

4.2. Compositions of Nitrifying Bacterial Communities and Its Relationship with Environment Factors

In this study, AOA could not be detected in the first biofilter. This finding indicated that the CFM medium was no longer suitable for AOA growth. It is possibly due to the fact that the co-existing microorganisms might provide some substances necessary for AOA growth in DAS but not in CFM.

The biological oxidation of NH_4^+-N and NO_2^--N, and the activity of nitrifiers decreased when the pH was below 6.4 and above 9.0 [16]. As AOA is mesophilic and neutrophilic, the optimum pH was 7.5 for the growth of AOA in DAS (Table S4). Similarly, the certain different pH between CFM and DAS had a strong influence on the structures of AOB and NOB (Figure 6). As reported, AOB showed high diverse communities and densities in slightly alkaline environments. *Nitrosospira* and *Nitrosomonas* strains of AOB are sensitive to low pH [18,24]. *Nitrosospira* is commonly dominant under low pH conditions [18,22]. In this study, pH could be considered neutral. Thus, *Nitrosospira* was not the major component. Alternatively, TN concentration is another important factor (Figure 6) because of complex N-form transformation [4,25].

AOA are considered the dominant ammonia oxidizers in a variety of habitats where the NH_4^+-N levels are relatively low, whereas AOB grow best in NH_4^+-N-rich environments [26,27]. The NH_4^+-N influent concentration was approximately 174 mg/L in CFM, which was higher than that in natural systems. Thus, AOB is more advantageous and competitive than AOA. The relative abundance of *Nitrosopumilus* was at the highest level at C0 and D0, which was reported to be dominant in natural freshwater wetland and at extremely low NH_4^+-N concentrations [15,26,28]. A previous study revealed that *Nitrosopumilus* and *Nitrososphaera* are the dominant AOA genera in natural freshwater wetlands [29]. *Nitrososphaera* was the most abundant genus in the present study, which found that the AOA obtained from WWTPs belong to the *Nitrososphaera* cluster [30]. These results led us to conclude that the AOA in C0 and D0 were similar to the ones from natural environments. By contrast, the AOA from treated DAS were similar to those from wastewater treatment systems. Moreover, the RDA analysis results showed that AOA and AOB possessed a higher affinity for T and DO than NOB (Figure 6) and were in agreement with literature [4,31]. Increased DO concentrations promoted NH_4^+-N and COD degradation. Nsenga and Meng [18] and Yan et al. [32] reported that as an electron acceptor, the DO concentration is important for AOA and AOB. Hence, the CFM that shows no or few AOA because the DO was lower than 2.2 mg/L and the DAS had DO of 3.1 mg/L (Table S4) may be the appropriate condition for the growth of AOA and AOB.

The work-assessing AOB and NOB in this system with different NH_4^+-N and COD concentrations through CFM and DAS demonstrated variation in their composition and abundance. The available carbon source decreased with the wastewater flow. Therefore, the diversity in CFM was higher than that in DAS. This result indicated that the biofilters or CWs suffered shortage in carbon source [4,16,33]. Wells et al. [34] determined that *Nitrosomonas* is the dominant AOB in various bioreactors, whereas Terada et al. [35] identified that *Nitrosospira* is predominant in the completely stirred tank reactor with high DO and low NH_4^+-N concentrations. Compared with the samples from CFM, those from DAS had higher relative abundance of *Nitrosospira*. This finding may be associated with the NH_4^+-N concentration of the synthetic wastewater in addition to the effect of the pH and DO discussed earlier. In terms of NOB, the abundance of the phylum *Proteobacteria* was reduced because of the relatively low COD/NH_4^+-N level in the CFM system. Several studies have demonstrated that *Nitrospira* is a more prevalent nitrite oxidizer in activated sludge bioreactors and CWs [4,22,36], and this finding was inconsistent with that in CFM–DAS. *Nitrobacter* was the dominant NOB in the system because the low NO_2^--N concentration at the inflow provided a selective advantage for *Nitrobacter*. This finding, which was similar to that reported in previous studies [6,37], revealed that *Nitrobacter* was the dominant NOB in activated sludge treated with high-N wastewater. Furthermore, in this CFM–DAS system from April to September, AOA and AOB were more sensitive to temperature than NOB.

Results showed that several factors, such as pH and NH_4^+-N concentration in effluents, can affect the growth of AOA, AOB, and NOB microorganisms (Figure 6). *Nitrososphaera*, *Nitrosomonas*, *Nitrosospira*, and *Nitrobacter* were involved in nitritation and nitratation reactions and dominant in the CFM–DAS system.

4.3. Response of Dominant Genera to Nitrogen Removal and Transformation

The significant correlation between dominant genera and N concentration in the effluents showed that some genera can be used as indicators of N transformations. Interestingly, the four genera in CFM (*Nitrosomonas*, *Nitrobacter*, *Lambdalikevirus*, and *Deinococcus*) had no relationship with N concentration in the effluent (Table 2) possibly because of the unique characteristics of the filter media of CFM, where the growth of bacteria was not affected by the concentrations of pollutants that produced relatively stable relationship between genera in AOB and NOB. By contrast, structures in CFM that were not subjected to space–time changes supported the relatively stable nitrifying bacterial compositions irrespective of changes in N concentration. Similarly, two of the four genera (*Nitrosomonas* and *Nitrobacter*) in DAS were not significantly related with N concentration in the effluent (Table 2). The only major AOA genus, *Nitrososphaera*, existed in the DAS recorded in Table 2, which correspond with NO_3^--N concentration in the effluent negatively. The low $\Delta NO_3^--N/\Delta NH_4^+-N$ ratio in the DAS caused by high AOA growth in Figure 3 confirmed this result. This finding revealed that NO_3^--N, rather than COD, TN, or NH_4^+-N, had positive effects on the relative abundance of this genus, and can be used in predicting the degree of NO_3^--N accumulation from another point. In terms of AOB, two main genera were identified, *Nitrosomonas* and *Nitrosospira*. Only *Nitrosospira* was present in DAS and was not the dominant genus in CFM and DAS. Thus, underrepresentation was observed. However, the results in Table 2 show the most important one that works in COD, NH_4^+-N, and NO_3^--N removal, confirming that *Nitrosomonas*, which had the highest abundance, did not necessarily act as a crucial indicator of N variation. Compared with no significant correlation between genera and NH_4^+-N in CFM, *Nitrosospira* in DAS had a better prediction of NH_4^+-N variation because of a significant relationship between its relative abundance and NH_4^+-N concentration was determined. Similarly, *Nitrosospira* showed an inverse relationship with NH_4^+-N and NO_3^--N, and the overgrowth of this genus facilitated the decrease in NH_4^+-N concentration at effluents via nitration, thereby resulting in low $\Delta NO_3^--N/\Delta NH_4^+-N$ ratio in the DAS (Figure 3). Furthermore, the decrease in DO along the depth gradient (Table S4) in accordance with the apparent increase in the relative abundance of *Nitrosospira* from the lower to the upper layer (Figure 5) caused NH_4^+-N elimination and NO_3^--N accumulation. This result was in agreement with a previous finding [38]. Given that the rate of AOA was not significantly higher than that of AOB in N-rich environments [16], AOA was weaker than AOB in terms of the correlation between genus and COD, NH_4^+-N, and NO_3^--N concentrations in the CFM–DAS system. Unexpectedly, no correlation between *Nitrobacter* and COD, TN, NH_4^+-N, and NO_3^--N concentrations in the effluent was observed unlike in a previous study [6]. Thus, the relative abundance of *Nitrobacter*, which was dominant in NOB, cannot be used as an indicator of N transformation.

5. Conclusions

The average removal efficiency of COD, NH_4^+-N, and TN in CFM-DAS reached 91.3%, 95.8%, and 75.1%, respectively, in the stabilization stage. High-throughput sequencing analysis confirmed that the compositions of nitrifying bacteria of CFM and DAS are distinctly different, although their structures for spatio-temporal CFM or DAS slightly changed. Multiple environmental variables collaboratively contributed to nitrifying bacteria compositions. Notably, pH and TN concentrations in the effluents potentially affected the similarities and differentiations of nitrifying bacteria compositions in the system. Further results revealed that N concentration in effluents can be adjusted by changing the relative abundances of dominant genera, especially close connection between *Nitrosospira* and NH_4^+-N removal in DAS. The results of this study present a potent tool using waste materials to treat wastewater containing high NH_4^+-N, and provides a theoretical basis to promote nitrogen removal capacity by regulating dominant genera.

References

1. Kizito, S.; Wu, S.; Kipkemoi Kirui, W.; Lei, M.; Lu, Q.; Bah, H.; Dong, R. Evaluation of slow pyrolyzed wood and rice husks biochar for adsorption of ammonium nitrogen from piggery manure anaerobic digestate slurry. *Sci. Total Environ.* **2015**, *505*, 102–112. [CrossRef] [PubMed]

2. Eryuruk, K.; Tezcan Un, U.; Bakır Ogutveren, U. Electrochemical treatment of wastewaters from poultry slaughtering and processing by using iron electrodes. *J. Clean. Prod.* **2018**, *172*, 1089–1095. [CrossRef]

3. Dias, D.F.C.; Passos, R.G.; Rodrigues, V.A.J.; de Matos, M.P.; Santos, C.R.S.; von Sperling, M. Performance evaluation of a natural treatment system for small communities, composed of a UASB reactor, maturation ponds (baffled and unbaffled) and a granular rock filter in series. *Environ. Technol.* **2018**, *39*, 490–502. [CrossRef] [PubMed]

4. Winkler, M.K.; Straka, L. New directions in biological nitrogen removal and recovery from wastewater. *Curr. Opin. Biotechnol.* **2019**, *57*, 50–55. [CrossRef] [PubMed]

5. Callery, O.; Healy, M.G.; Rognard, F.; Barthelemy, L.; Brennan, R.B. Evaluating the long-term performance of low-cost adsorbents using small-scale adsorption column experiments. *Water Res.* **2016**, *101*, 429–440. [CrossRef]

6. Wang, L.; Wang, X.; Yang, F.; Kong, M.; Peng, F.; Chao, J.; Gao, Y.; Wu, D.; Zhu, Y.; Zhang, Y. Nitrogen removal performance and ammonia- and nitrite-oxidizing bacterial community analysis of a novel industrial waste-based biofilter. *Chem. Eng. J.* **2016**, *299*, 156–166. [CrossRef]

7. Al Nakouzi, N.; Le Moulec, S.; Albigès, L.; Wang, C.; Beuzeboc, P.; Gross-Goupil, M.; de La Motte Rouge, T.; Guillot, A.; Gajda, D.; Massard, C.; et al. Cabazitaxel Remains Active in Patients Progressing After Docetaxel Followed by Novel Androgen Receptor Pathway Targeted Therapies. *Eur. Urol.* **2015**, *68*, 228–235. [CrossRef]

8. Wang, J.; Zhao, Y.; Zhang, P.; Yang, L.; Xu, H.A.; Xi, G. Adsorption characteristics of a novel ceramsite for heavy metal removal from stormwater runoff. *Chin. J. Chem. Eng.* **2018**, *26*, 96–103. [CrossRef]

9. Cao, W.; Wang, Y.; Sun, L.; Jiang, J.; Zhang, Y. Removal of nitrogenous compounds from polluted river water by floating constructed wetlands using rice straw and ceramsite as substrates under low temperature conditions. *Ecol. Eng.* **2016**, *88*, 77–81. [CrossRef]

10. Wu, H.; Fan, J.; Zhang, J.; Ngo, H.H.; Guo, W.; Liang, S.; Lv, J.; Lu, S.; Wu, W.; Wu, S. Intensified organics and nitrogen removal in the intermittent-aerated constructed wetland using a novel sludge-ceramsite as substrate. *Bioresour. Technol.* **2016**, *210*, 101–107. [CrossRef]

11. Wang, L.; Zou, F.; Fang, X.; Tsang, D.C.W.; Poon, C.S.; Leng, Z.; Baek, K. A novel type of controlled low strength material derived from alum sludge and green materials. *Constr. Build. Mater.* **2018**, *165*, 792–800. [CrossRef]

12. Hu, Y.; Zhao, Y.; Zhao, X.; Kumar, J.L. High rate nitrogen removal in an alum sludge-based intermittent aeration constructed wetland. *Environ. Sci. Technol.* **2012**, *46*, 4583–4590. [CrossRef] [PubMed]

13. Hu, Y.; Zhao, Y.; Rymszewicz, A. Robust biological nitrogen removal by creating multiple tides in a single bed tidal flow constructed wetland. *Sci. Total Environ.* **2014**, *470*, 1197–1204. [CrossRef] [PubMed]

14. Hu, Y.; Zhao, X.; Zhao, Y. Achieving high-rate autotrophic nitrogen removal via Canon process in a modified single bed tidal flow constructed wetland. *Chem. Eng. J.* **2014**, *237*, 329–335. [CrossRef]

15. Chen, H.; Jin, W.; Liang, Z.; Abomohra, A.E.F.; Zhou, X.; Tu, R.; Han, S. Abundance and diversity of ammonia-oxidizing archaea in a biological aerated filter process. *Ann. Microbiol.* **2017**, *67*, 405–416. [CrossRef]

16. Wongkiew, S.; Hu, Z.; Chandran, K.; Lee, J.W.; Khanal, S.K. Nitrogen transformations in aquaponic systems: A review. *Aquacult. Eng.* **2017**, *76*, 9–19. [CrossRef]

17. Tian, J.; Yu, C.; Liu, J.; Ye, C.; Zhou, X.; Chen, L. Performance of an Ultraviolet Mutagenetic Polyphosphate-Accumulating Bacterium PZ2 and Its Application for Wastewater Treatment in a Newly Designed Constructed Wetland. *Appl. Biochem. Biotechnol.* **2017**, *181*, 735–747. [CrossRef]

18. Nsenga Kumwimba, M.; Meng, F. Roles of ammonia-oxidizing bacteria in improving metabolism and cometabolism of trace organic chemicals in biological wastewater treatment processes: A review. *Sci. Total Environ.* **2019**, *659*, 419–441. [CrossRef]

19. Meng, J.; Li, J.; Li, J.; Antwi, P.; Deng, K.; Wang, C.; Buelna, G. Nitrogen removal from low COD/TN ratio manure-free piggery wastewater within an upflow microaerobic sludge reactor. *Bioresour. Technol.* **2015**, *198*, 884–890. [CrossRef]

20. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Waste Water*; American Public Health Association: Washington, DC, USA, 2005.

21. Ma, J.; Wu, H.; Wang, Y.; Qiu, G.; Fu, B.; Wu, C.; Wei, C. Material inter-recycling for advanced nitrogen and residual COD removal from bio-treated coking wastewater through autotrophic denitrification. *Bioresour. Technol.* **2019**, *289*, 121616. [CrossRef]

22. Yue, X.; Yu, G.; Lu, Y.; Liu, Z.; Li, Q.; Tang, J.; Liu, J. Effect of dissolved oxygen on nitrogen removal and the microbial community of the completely autotrophic nitrogen removal over nitrite process in a submerged aerated biological filter. *Bioresour. Technol.* **2018**, *254*, 67–74. [CrossRef] [PubMed]

23. Zhou, X.; Wang, X.; Zhang, H.; Wu, H. Enhanced nitrogen removal of low C/N domestic wastewater using a biochar-amended aerated vertical flow constructed wetland. *Bioresour. Technol.* **2017**, *241*, 269–275. [CrossRef] [PubMed]

24. Meng, H.; Katayama, Y.; Gu, J.D. More wide occurrence and dominance of ammonia-oxidizing archaea than bacteria at three Angkor sandstone temples of Bayon, Phnom Krom and Wat Athvea in Cambodia. *Int. Biodeterior. Biodegrad.* **2017**, *117*, 78–88. [CrossRef]

25. Nsenga Kumwimba, M.; Lotti, T.; Senel, E.; Li, X.; Suanon, F. Anammox-based processes: How far have we come and what work remains? A review by bibliometric analysis. *Chemosphere* **2020**, *238*, 124627. [CrossRef] [PubMed]

26. Martens-Habbena, W.; Berube, P.M.; Urakawa, H.; de la Torre, J.R.; Stahl, D.A. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* **2009**, *461*, 976–979. [CrossRef] [PubMed]

27. Ouyang, Y.; Norton, J.M.; Stark, J.M.; Reeve, J.R.; Habteselassie, M.Y. Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil. Biol. Biochem.* **2016**, *96*, 4–15. [CrossRef]

28. Roy, D.; McEvoy, J.; Blonigen, M.; Amundson, M.; Khan, E. Seasonal variation and ex-situ nitrification activity of ammonia oxidizing archaea in biofilm based wastewater treatment processes. *Bioresour. Technol.* **2017**, *244*, 850–859. [CrossRef]

29. Liu, Z.; Xie, H.; Hu, Z.; Zhang, J.; Zhang, J.; Sun, H.; Lan, W. Role of Ammonia-Oxidizing Archaea in Ammonia Removal of Wetland Under Low-Temperature Condition. *Water Air Soil Pollut.* **2017**, *228*, 356. [CrossRef]

30. Gao, J.F.; Luo, X.; Wu, G.X.; Li, T.; Peng, Y.Z. Quantitative analyses of the composition and abundance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria in eight full-scale biological wastewater treatment plants. *Bioresour. Technol.* **2013**, *138*, 285–296. [CrossRef]

31. Limpiyakorn, T.; Furhacker, M.; Haberl, R.; Chodanon, T.; Srithep, P.; Sonthiphand, P. amoA-encoding archaea in wastewater treatment plants: A review. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 142–539. [CrossRef]

32. Yan, L.; Li, Z.; Wang, G.; Gao, Y.; Wang, Y.; Gu, J.D.; Wang, W. Diversity of ammonia-oxidizing bacteria and archaea in response to different aeration rates during cattle manure composting. *Ecol. Eng.* **2016**, *93*, 46–54. [CrossRef]

33. Hu, D.; Zhou, Z.; Niu, T.; Wei, H.; Dou, W.; Jiang, L.M.; Lv, Y. Co-treatment of reject water from sludge dewatering and supernatant from sludge lime stabilization process for nutrient removal: A cost-effective approach. *Sep. Purif. Technol.* **2017**, *172*, 357–365. [CrossRef]

34. Wells, G.F.; Park, H.D.; Yeung, C.H.; Eggleston, B.; Francis, C.A.; Criddle, C.S. Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: Betaproteobacterial dynamics and low relative abundance of Crenarchaea. *Environ. Microbiol.* **2009**, *11*, 2310–2328. [CrossRef] [PubMed]

35. Terada, A.; Sugawara, S.; Yamamoto, T.; Zhou, S.; Koba, K.; Hosomi, M. Physiological characteristics of predominant ammonia-oxidizing bacteria enriched from bioreactors with different influent supply regimes. *Biochem. Eng. J.* **2013**, *79*, 153–161. [CrossRef]

36. Vandekerckhove, T.G.L.; Kerckhof, F.M.; De Mulder, C.; Vlaeminck, S.E.; Boon, N. Determining stoichiometry and kinetics of two thermophilic nitrifying communities as a crucial step in the development of thermophilic nitrogen removal. *Water Res.* **2019**, *156*, 34–45. [CrossRef] [PubMed]

37. Pelissari, C.; Guivernau, M.; Vinas, M.; Garcia, J.; Velasco-Galilea, M.; Souza, S.S.; Sezerino, P.H.; Avila, C. Effects of partially saturated conditions on the metabolically active microbiome and on nitrogen removal in vertical subsurface flow constructed wetlands. *Water Res.* **2018**, *141*, 185–195. [CrossRef]

38. Wang, L.K.; Zeng, G.M.; Yang, Z.H.; Luo, L.L.; Xu, H.Y.; Huang, J. Operation of partial nitrification to nitrite of landfill leachate and its performance with respect to different oxygen conditions. *Biochem. Eng. J.* **2014**, *87*, 62–68. [CrossRef]