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*Case Report*

# Characteristics of Ammonia Removal and Nitrifying Microbial Communities in a Hybrid Biofloc-RAS for Intensive *Litopenaeus vannamei* Culture: A Pilot-Scale Study

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**Abstract:** Ammonia is the main pollution factor of the aquatic environment in marine shrimp culture systems. In order to demonstrate the feasibility of the combination of biofloc technology and nitrifying biofilter for the ammonia removal, a 70-day production trial was conducted in a simplified pilot-scale hybrid biofloc-based recirculating aquaculture system (biofloc-RAS) with the intensive culture of *Litopenaeus vannamei*. Nitrogen dynamics and nitrifying microbial communities were investigated in three replicated systems simultaneously under the conditions of high feed loading and zero water exchange. Along with biofloc development in the culture tank and biofilm formation in the nitrifying biofilter during the trial, nitrification could be fastly and effectively established in the system, which was indicated by the dynamics of total ammonia nitrogen (TAN), $NO_2^-$-N, $NO_3^-$-N, and total nitrogen (TN) concentrations. Meanwhile, similar nitrifying microorganisms could be found between biofloc and biofilm, despite some differences in abundance, diversity, and composition of ammonia-oxidizing archaea and bacteria and nitrite-oxidizing bacteria. High TAN removal rate could be achieved and was significantly and positively correlated with abundances of these nitrifying microbial communities in both biofloc and biofilm, further indicating that both biofloc and biofilm could contribute highly to nitrification performance of the biofloc-RAS. The results of this study indicate a potential application of the biofloc-RAS in coastal intensive aquaculture.

**Keywords:** shrimp culture; recirculating aquaculture system; ammonia removal; nitrifying microbial community; biofloc; biofilm

## 1. Introduction

Shrimp culture represents one of the fastest-growing and most widely production activities in the global seafood industry, with a global production of 6.0 million tons and a value of 38.4 billion dollars in 2018 [1]. In the past two decades, the increasing demand for food shrimp has pushed the rapid expansion and intensification of shrimp culture over the world [2,3]. However, the water environment of intensive systems can rapidly deteriorate as a result of the continued input of plenty of high protein

feed [4]. In particular, endogenous nitrogenous wastes, such as ammonia, rapidly accumulate in the water column, which is harmful to cultured shrimp, even at very low concentrations [5,6]. In practice, high-frequency and a large quantity of water is commonly exchanged to maintain the good water quality of the aquaculture systems [4,7]. This traditional operation has often caused several environmental and economic problems, such as overuse of water sources, pollution of surrounding waters, destruction of coastal ecosystems, significant crop losses from disease outbreaks [8–11]. Therefore, there is an increasing demand for efficient and environmentally-friendly culturing systems and operating technologies to achieve sustainable development of the modern shrimp culture industry [12–14].

The land-based recirculating aquaculture system (RAS) is a sustainable alternative to traditional aquaculture systems and has been developed for the intensive culture of marine shrimp [13,15]. The biofilter is an indispensable component in a traditional RAS for the removal of ammonia, and its running directly influences the recycling of culture water in the system [15–17]. The establishment of nitrifying biofilm in external biofilters of RAS has been demonstrated feasible for the maintenance of water quality in shrimp intensive culture [18–20]. However, the water in RAS is usually operated under oligotrophic conditions for recirculation, which is probably not an optimal water environment for the culture of detritivorous species, such as marine shrimps [21,22]. More importantly, the rapid establishment and persistent stability of nitrifying biofilm and associated nitrification in the biofilters are still a technical challenge for the running of a marine RAS [23,24]. The technological difficulty, together with high investment and operation costs, impedes the commercial application of traditional RAS for shrimp culture at present.

In recent years, biofloc technology (BFT), as a new in situ water treatment technology, has demonstrated great potential in the maintenance of water quality (especially for the removal of ammonia) for shrimp culture [14,25,26]. In intensive feeding systems, the biofloc can naturally form from active microorganisms and accumulated organic materials and then suspend in the culture water to act as tiny biofilters in an extremely large number [25]. The biofloc can remove ammonia by both the nitrification of chemoautotrophic bacteria and the assimilation of heterotrophic bacteria, which process dominates depends on water condition and applied management [14,27,28]. However, under zero water exchange conditions, an increasing amount of biofloc will produce in the culture water, resulting in not only a raise of water respiration rate and decline of dissolved oxygen but also environmental stress to cultured shrimp [25,29,30]. Moreover, large fluctuations of water quality indicators (such as ammonia, nitrite, etc.) may happen because of excess bioflocs or suspended solids accumulating in the system [26,31]. Previous studies have suggested that controlling moderate concentrations of biofloc benefit water quality and shrimp performance in BFT systems [26,29,30].

The idea of constructing a hybrid RAS to combine the advantages of in situ BFT and ex situ biofilter has been proposed by previous studies [21,32], but practical research and application are very scarce, especially in coastal aquaculture. Therefore, in this study, we attempted to develop a simplified hybrid biofloc-based RAS at a pilot scale for intensive culture of marine shrimp and tested the feasibility and effectiveness of combined nitrification performed by biofloc and biofilm for the removal of total ammonia nitrogen (TAN) under the condition of high feeding rates and zero water exchange. We chose a globally important culture species of marine shrimp, *Litopenaeus vannamei* (Boone, 1931), for an intensive feeding trial in the system. Inorganic nitrogen dynamics, ammonia removal, and shrimp performance were analyzed to evaluate the operating performance of the system. It was hypothesized that nitrification mediated by both biofloc and biofilm was the main microbial conversion pathway for ammonia removal in the system. Nitrification comprises two main reactions and is generally carried out by different microorganisms, where ammonia is first oxidized to nitrite by the ammonia-oxidizing archaea and bacteria (AOA and AOB) and subsequently to nitrate by nitrite-oxidizing bacteria (NOB) [17]. Thereby, a comparison analysis of the abundance, diversity, and community composition of AOA, AOB, and NOB between biofloc and biofilm samples was further performed to provide microbial community structure associated with nitrification. The results of this study would expand our knowledge on the microbial ecology of nitrification for ammonia removal in the hybrid biofloc-RAS and

provide a reference for further application and management of water resources and the environment in coastal intensive aquaculture.

## 2. Materials and Methods

### *2.1. Design and Setup of a Hybrid RAS*

A pilot-scale hybrid biofloc-RAS was designed for the intensive culture of *L. vannamei*, as shown in Figure 1. The biofloc-RAS was improved from the biofloc-based system described by Xu et al. [33]. The biofloc-RAS consisted of a cement culture tank (6 m/length × 6 m/width × 1 m/height, the water volume of 30 $m^3$), a settling clarifier (working volume of 0.12 $m^3$), and an aerated nitrifying biofilter (working volume of 0.28 $m^3$). The system was driven by a 750 W circulating pump (SBP100, Guangdong Lingxiao Pump Industry Co., Ltd., Yangjiang, Guangdong, China) to provide continuous aeration and mixing and circulation of tank water. Eight water injectors (Yangjiang Shrimp Bio-Tech Co., Ltd., Yangjiang, Guangdong, China) were installed evenly along the bottom of the tank and connected to the pump through the main loop on the pump discharge pipe. The clarifier and biofilter were connected, in turn, to the pump through the side loop on the pump discharge pipe. The biofilter was a cylinder with 1.0 m in height and 0.6 m in diameter and filled with 0.15 $m^3$ of suspended bio-media. A disc aerator was installed near the bottom of the biofilter to supply dissolved oxygen with an air-to-liquid ratio of 1:2. The bio-medium was made of high-density polyethylene and had a size of 25 mm in diameter and 4 mm in height and a specific surface area of approximately 1200 $m^2$ $m^{-3}$ (Tongxiang Small Boss Special Plastic Products Ltd., Tong Xiang, Zhejiang, China). Water from the culture tank was recirculated through the main loop at a rate of 6-12 $m^3$ $h^{-1}$ and the side loop at a rate of 0.4–2.0 $m^3$ $h^{-1}$.

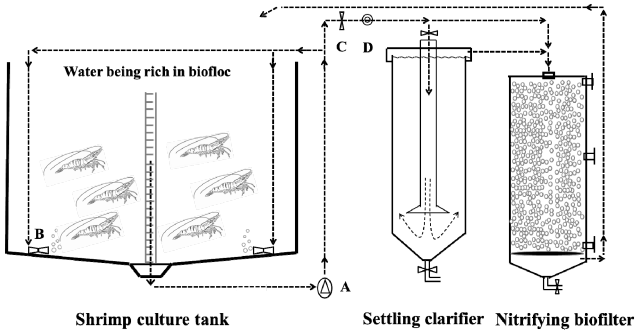


**Figure 1.** Schematic diagram of a hybrid biofloc-based recirculating aquaculture system (biofloc-RAS) combining in situ biofloc technology and ex situ biofilter for the intensive culture of *L. vannamei*. Dotted arrows indicate the water flow through the system. Water was pumped from the culture tank by running a circulating pump; most of the water circulated to the culture tank through water injectors; the rest of the water in-flowed into a settling clarifier and then a nitrifying biofilter and lastly returned to the culture tank by gravity. The water entering the clarifier and biofilter was adjusted by regulating valves at water flow rates of 0.42-1.68 $m^3$ $h^{-1}$. (**A**) circulating pump, (**B**) water injector, (**C**) regulating valve, (**D**) flow-meter. RAS, recirculating aquaculture system.

### *2.2. Shrimp Production Trial and System Operation Condition*

The study was conducted during the autumn months (September to November) of 2017 at the facilities in Shrimp Aquaculture Station (N 22°43′36″, E 115°35′2″) of South China Sea Fisheries Research Institute, located in the city of Shanwei, Guangdong province, China. Three independent and identical systems were established in an indoor insulated building and then prepared for the trial. A production trial of *L. vannamei* intensive culture was conducted simultaneously in the three systems. The culture tank of each system was filled with 27 $m^3$ of sand-filtered and chlorinated natural seawater and 3 $m^3$ of biofloc-rich water from the shrimp nursery pond. Previously, eight-day-old postlarvae of

*L. vannamei* were stocked into the nursery pond at a density of 3200 shrimps $m^{-3}$ and reared for 32 days under limited water exchange. During the nursery period, molasses were added to the culture water daily to achieve an input C/N ratio of 12:1 based on the carbon-nitrogen contents of the applied feed and the carbon content of the molasses [33]. The main water quality characteristics of the nursery pond on day 32 are provided in Table S1. The biofloc-rich water served as nitrifying seeding to accelerate the start-up of the nitrification process in the three systems. The circulating pump was started to run the system. Juvenile shrimps (1.92 ± 0.43 g) from the same nursery pond were stocked into each culture tank at a density of 300 shrimps $m^{-3}$ and a biomass of 576 g $m^{-3}$. Juvenile shrimps were cultured for a period of 70 days to harvest size. The shrimps were fed a commercial feed (40.0% protein, 8.0% lipid, 3.5% fiber, and 14.0% ash) by continuous automatic feeders. The feed loading rate was calculated based on the assumption of a growth rate of 2.0 g $wk^{-1}$, an initial feed conversion ratio (FCR) of 1.2, and an initial survival rate of 100% [26]. The feed loading rate was adjusted weekly based on an increase of FCR at 0.03 per week and a decrease of survival rate at 0.1% per week over the 70-day culture period.

During the 70-day trial period, the three systems were operated under the following physical-chemical conditions: natural photoperiod with a light intensity of 500-900 lx, the salinity of 26–28 g $L^{-1}$, the water temperature of 28.0-30.0°C, the dissolved oxygen concentration of 4.5–6.2 mg $L^{-1}$, pH of 7.0–7.8, and total alkalinity of 150–300 mg $L^{-1}$ as $CaCO_3$. Light intensity over tank water surface was monitored daily by a hand-held ZDS-10 W-2D illumination photometer (Shanghai Qiuhe Instruments Inc., Shanghai, China); salinity, water temperature, dissolved oxygen concentration, and pH of tank water were monitored daily by a hand-held YSI-650 multi-parameter (Yellow Springs Instruments Inc., Yellow Springs, OH, USA). Total alkalinity was measured once a week following the American Public Health Association (APHA) method [34]. Sodium bicarbonate was added to compensate for alkalinity loss and meanwhile ensure pH above 7.0 in the water of the tanks. The biofilter was operated at a water flow rate of 1.68 $m^3 \cdot h^{-1}$ at start-up phase (from day 0 to 14, characterized by the gradual increase of TAN concentration), reducing to 0.84 $m^3$ $h^{-1}$ at transition phase (from day 14 to 35, characterized by the gradual decrease of TAN concentration) and further to 0.42 $m^3$ $h^{-1}$ at stabilization phase (from day 35 to 70, characterized by the relative stability of TAN concentration). The corresponding hydraulic retention time in the biofilter was 10, 20, 40 min, respectively. The clarifier was started and operated during the transition and stabilization phases. The clarifier and biofilter were purged as needed for the removal of settled and accumulated biofloc solids. No water was exchanged for the three systems during the 70-day trial period, and the only freshwater was added to compensate for evaporation losses and waste discharge as needed.

### *2.3. Determination of Nitrogen Dynamics, TAN Removal, and Shrimp Performance*

Tank water of the three systems was sampled weekly for the analysis of TAN, nitrite nitrogen ($NO_2^-$-N), nitrate nitrogen ($NO_3^-$-N), total nitrogen (TN) by following the APHA methods [34].

Since the system was in the complete recirculating mode without water exchange, the source of TAN production in the system was only feed input and shrimp excretion. The daily TAN production rate ($PR_{TAN}$; mg·$L^{-1}$ $d^{-1}$) based upon the feed loading rate was taken as the TAN into the system and calculated using equation [27]:

$$PR_{TAN} = F \times PC \times 0.144 \quad (1)$$

where $F$ is the loading rate of feed in mg·$L^{-1}$ $d^{-1}$, $PC$ is the protein content in feed in percentage.

The TAN removal rate ($RR_{TAN}$; mg·$L^{-1}$·$d^{-1}$) and TAN removal efficiency ($RE_{TAN}$; %) were estimated using following equations:

$$RR_{TAN} = P_{i-1} + M_{i-1} - M_i \quad (2)$$

$$RE_{TAN} = RR_{TAN}/PR_{TAN} \times 100 \quad (3)$$

where $P_{i-1}$ is the produced concentration of TAN in mg $L^{-1}$ on the day (i−1), $M_{i-1}$ is the measured concentration of TAN in mg $L^{-1}$ on the day (i−1), $M_i$ is the measured concentration of TAN in mg $L^{-1}$ on the day i.

After 70 days, the shrimps were harvested from each tank for counting and weighing. Growth rate (GR; g·$wk^{-1}$), survival rate (SR; %), yield (Y; kg $m^{-3}$), and FCR were calculated using the following equations:

$$GR = (W_t - W_0)/t \quad (4)$$

$$SR = (N_t - N_0) \times 100 \quad (5)$$

$$Y = TW_t/V \quad (6)$$

$$FCR = FW/(TW_t - TW_0) \quad (7)$$

where $W_t$ is the individual weight of harvest shrimp in g, $W_0$ is the individual weight of stocking shrimp in g, $t$ is the duration of the trial period in a week, $N_t$ is the count of harvest shrimp in number, $N_0$ is the count of stocking shrimp in number, $TW_t$ is the total weight of harvest shrimp in kg, $TW_0$ is the total weight of stocking shrimp in kg, $V$ is the total volume of used water in $m^3$, $FW$ is the total weight of offered feed in kg.

### *2.4. A Sampling of Biofloc and Biofilm and Determination of Their Biomass*

The biofloc and biofilm were sampled weekly for the determination of their biomass during the 70-day trial. In addition, the biofloc and biofilm were sampled for microbial analysis on day 0, 21, and 42. The inoculated biofloc-rich water was sampled as an initial sample (day 0) for both biofloc and biofilm. For the biofloc sample, water-containing biofloc was taken out from the culture tank by a sterile water sampler. For the biofilm sample, water-containing biomedia was taken out from the biofilter by a flexible 10-cm-diameter tube, and then the attached biofilm was removed from biomedia by shaking, rinsing, and then suspension. For both collected biofilm and biofloc samples, their volatile suspended solids (VSS) were measured following the APHA methods [34]. The biomass of biofilm and biofloc samples was estimated in terms of VSS.

### *2.5. Microbial DNA Extraction and qPCR Analysis*

Both biofloc and biofilm samples (100 mL) were firstly filtered using 0.2-μm pore size polycarbonate membranes (Millipore Corporation, Billerica, MA, USA) to obtain microbial samples. Then, total genomic DNA was extracted by protease K lysis, followed by phenol-chloroform extraction. The concentration and purity of extracted DNA were checked by using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The qualified DNA was stored at −80 °C for subsequent analyses.

Quantitative real-time polymerase chain reaction (qPCR) was applied to identify the abundances of AOA, AOB, and NOB in the qualified DNA of samples using FTC-3000™ real-time PCR systems (Funglyn Biotech Inc., Toronto, ON, Canada). To identify AOA, archaeal *amo*A gene was targeted; to identify AOB, bacterial *amo*A gene and 16S rRNA gene were targeted, respectively; to identify NOB, bacterial *nxr*A gene and 16S rRNA gene were targeted, respectively. The primer and qPCR protocol for each target gene are listed in Table 1 [35–39]. The standard curve was set up by 10-fold serially diluting plasmid of a pMD18-T vector with the corresponding amplicon for each target gene from $10^2$ to $10^7$ copies $\mu L^{-1}$. The qPCR reaction was performed in 8-strip tubes (Axygen, Union, CA, USA) using 25 μL reaction mixture that contained 12.5 μL SYBR Premix Ex Taq™ (2×) (Takara, Otsu, Shiga, Japan), 1 μL (10 μM) of each forward and reverse primers, 5 μL of template DNA, and 5.5 μL sterilized deionized water. Melting curve analyses were performed from 60 °C to 96 °C with increments of 0.1 °C per cycle. The number of target gene copy was calculated by cycle threshold (Ct) value and the standard curve. Each primer corresponding to qPCR assays' standard curve was considered to be effective as amplification efficiency in 91%–94% (except for the 16S rRNA gene of NOB in 86.8%) and

$R^2 > 0.99$. All the standard DNA and test samples were run in triplicate. The abundances of AOA, AOB, and NOB of both biofloc and biofilm were normalized gene copy numbers to the biomass of samples (copy numbers per mg VSS).

**Table 1.** Primers and target genes used for qPCR and PCR amplification in this study.

| Primer Name and Sequence | Target Gene | qPCR Amplification Procedure | Reference |
|---|---|---|---|
| Arch-*amo*AF 5′-STAATGGTCTGGCTTAGACG-3′<br>Arch-*amo*AR 5′-GCGGCCATCCATCTGTATGT-3′ | AOA-*amo*A | 95 °C for 30 s; 40 cycles (95 °C for 10 s, 50 °C for 30 s), and 72 °C for 45 s | [35] |
| *amo*A-1F 5′-GGGGTTTCTACTGGTGGT-3′<br>*amo*A-2R 5′-CCCCTCKGSAAAGCCTTCTTC-3′ | AOB-*amo*A | 95 °C for 30 s; 40 cycles (95 °C for 10 s, 55 °C for 30 s), and 72 °C for 30 s | [36] |
| CTO-189F 5′-GGAGRAAAGYAGGGGATCG-3′<br>CTO-654R 5′-CTAGCYTTGTAGTTTCAAACGC-3′ | AOB-16S rRNA | 95 °C for 30 s, 40 cycles (95 °C for 10 s, 55 °C for 30 s), and 72 °C for 30 s | [37] |
| *nxr*AF 5′-CAGACCGACGTGTGCGAAAG-3′<br>*nxr*AR 5′-TCYACAAGGAACGGAAGGTC-3′ | NOB-*nxr*A | 95 °C for 30 s, 40 cycles (95 °C for 10 s, 55 °C for 30 s), and 72 °C for 30 s | [38] |
| FGPS-872 5′-TTTTTTGAGATTTGCTAG-3′<br>FGPS-1269 5′-CTAAAACTCAAAGGAATTGA-3′ | NOB-16S rRNA | 95 °C for 30 s, 40 cycles (95 °C for 10 s, 50 °C for 30 s), and 72 °C for 30 s | [39] |

AOA-*amo*A represents ammonia-oxidizing archaea based on the archaeal *amo*A gene, AOB-*amo*A represents ammonia-oxidizing bacteria based on bacterial *amo*A gene, AOB-16S rRNA represents ammonia-oxidizing bacteria based on 16S rRNA gene, NOB-*nxr*A represents nitrite-oxidizing bacteria based on *nxr*A gene, NOB-16S rRNA represents nitrite-oxidizing bacteria based on 16S rRNA gene.

### *2.6. Miseq Sequencing and Bioinformatics Analysis*

The qualified DNA of samples was also subjected to Miseq high-throughput sequencing at Tiny Gene Bio-Tech (Shanghai) Co., Ltd. (Shanghai, China). Archaeal *amo*A gene, *amo*A gene, and 16S rRNA gene of AOB and *nxr*A gene and 16S rRNA gene of NOB were targeted for PCR amplification, gene library construction, and sequencing. PCR amplification of each sample was carried out in triplicate. The primers are given in Table 1 [35–39]. After purification by AxyPrep DNA gel extraction kits (Axygen, Union, CA, USA), the barcoded PCR products were pooled and quantified by the FTC-3000$^{TM}$ real-time PCR and then normalized to equimolar amounts. Finally, the libraries were sequenced on the Illumina Miseq platform using MiSeq v3 reagent kit (Illumina, San Diego, CA, USA). All the raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession number: PRJNA664244).

The raw fastq files were demultiplexed based on the barcode. PE reads for all samples were run through Trimmomatic (version 0.35) [40] to remove low-quality base pairs using these parameters (SLIDINGWINDOW: 50:20 MINLEN: 50). Trimmed reads were then further merged using the FLASH program (version 1.2.11) [41] with default parameters. The low-quality contigs were removed based on screen.seqs command using the following filtering parameters: maxambig = 0, minlength = 200, maxlength = 580, maxhomop = 8. For the archaeal *amo*A gene, the sequence length was 635 bp, and the single-end sequencing results were used for analysis. The sequences were analyzed using a combination of software mothur (version 1.33.3), UPARSE (usearch version v8.1.1756, http://drive5.com/uparse/), and R (version 3.2.3). The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse-cmds.html). The OTU representative sequences were assigned for taxonomy against Silva 128 database with a confidence score of ≥0.8 by the classify.seqs command in mothur. OTU taxonomies were determined based on NCBI. For the archaeal *amo*A gene, *amo*A gene of AOB, *nxr*A gene of NOB, homology searches were carried out by the Basic Local Alignment Search Tool (BLAST). Chao richness and Shannon diversity indices were estimated to indicate the community alpha-diversity using Mothur

(version 1.34.0) and plotted by R. It should be pointed out that as targeted functional genes could not be detected by Miseq sequencing for some samples from day 0 and day 21, the alpha-diversity between biofloc and biofilm and their community composition for AOA, AOB, and NOB based on *amo*A and *nxr*A genes were only given at day 42.

### *2.7. Statistical Analysis*

All data were presented as means (±S.D.) of three replicates. Data of VSS, TAN, $NO_2^-$-N, $NO_3^-$-N, TN, TAN removal rate, and TAN removal efficiency were analyzed using repeated measures one-way ANOVA (General Linear Model) after conducting Mauchly's test of sphericity. Comparison of parameters for abundance, alpha-diversity, and percentage of nitrifying microbial communities between biofloc and biofilm was analyzed using independent-samples *t*-test after Levene's test of homogeneity of variance. Differences were considered significant at $p < 0.05$. The relationships between levels of TAN, $NO_2^-$-N, $NO_3^-$-N, TN, TAN removal rate and abundances of AOA, AOB, NOB were assessed by Pearson's correlation analyses. All statistical analyses were performed using IBM SPSS Statistics 20.0 software for Windows (IBM Corporation, NY, USA).

## 3. Results and Discussion

### *3.1. Operating Mode, Nitrogen Dynamics, and Nitrification Establishment*

The ammonia removal in the biofloc-RAS for shrimp intensive production was targeted to perform by the combination of in situ suspended biofloc and ex situ attached biofilm in this study. From the aspect of nitrogen dynamics and nitrification establishment, the operational process of the system passed through three phases in the trial: start-up phase (0-14 days), transition phase (14-35 days), and stabilization phase (35-70 days). All the monitored water quality parameters were maintained within acceptable ranges for *L. vannamei* culture throughout the 70-day trial (Table 2). Under these conditions, the shrimp growth rate of 2.15 g week$^{-1}$, survival rate of 93.11%, the yield of 4.98 kg·m$^{-3}$, and FCR of 1.60 were achieved in this study (Table 3). These results of production indicators were comparable to those previously published reports on the commercial-scale intensive culture of *L. vannamei* in the biofloc-based system [26,33], RAS [19], and hybrid RAS [21]. The results of this study demonstrated that it was feasible to remove continuously produced ammonia of culture water timely and effectively in our specially designed hybrid biofloc-RAS under the condition of high feed loading and zero water exchange.

**Table 2.** Mean and range values of the monitored water environmental parameters in the shrimp culture tanks of the biofloc-based recirculating aquaculture system (biofloc-RAS) during a 70-day trial (mean ± S.D., N = 3).

| Parameter | Mean ± S.D. | Min-Max |
|---|---|---|
| **Light intensity (lx)** | 628 ± 11 | 475–942 |
| **Salinity (g L$^{-1}$)** | 27.2 ± 0.1 | 25.9–28.5 |
| **Water temperature (°C)** | 28.9 ± 0.1 | 27.7–30.2 |
| **Dissolved oxygen (mg L$^{-1}$)** | 5.2 ± 0 | 4.6–6.2 |
| **pH** | 7.3 ± 0 | 7.0–7.8 |
| **Alkalinity (mg·L$^{-1}$ as $CaCO_3$)** | 232 ± 9 | 155–330.0 |
| **TAN (mg L$^{-1}$)** | 0.6 ± 0.1 | 0.21–1.81 |
| **$NO_2^-$-N (mg L$^{-1}$)** | 0.6 ± 0.1 | 0.16–2.41 |
| **$NO_3^-$-N (mg L$^{-1}$)** | 76.5 ± 2.7 | 2.7–146.3 |
| **TN (mg L$^{-1}$)** | 89.9 ± 0.8 | 5.7–154.3 |
| **VSS (mg L$^{-1}$)** | 116.1 ± 3.6 | 30.2–170.9 |

TAN: total ammonia nitrogen; TN: total nitrogen; VSS: volatile suspended solids.

**Table 3.** Production performance of *L. vannamei* cultured in the biofloc-RAS for a 70-day trial (mean ± S.D., N = 3).

| Initial Weight (g) | Final Weight (g) | Growth Rate (g $wk^{-1}$) | Survival Rate (%) | Yield (kg $m^{-3}$) | Feed Conversion Rate |
|---|---|---|---|---|---|
| 1.92 ± 0.43 | 17.85 ± 0.56 | 2.15 ± 0.18 | 93.11 ± 1.66 | 4.98 ± 0.10 | 1.60 ± 0.03 |

Similar to the sound practice of biofloc-based shrimp culture systems reported in other studies, a 10% volume of biofloc-enriched water was inoculated into culture water to accelerate the development of biofloc in the shrimp culture tank of each system [26,42]. The development of biofloc and biofilm in terms of VSS is shown in Figure 2, which corresponded to the continuous input of the feed and regular operation of the system. The VSS of biofloc increased with continuous input of feed nutrients and dense proliferation of various microorganisms during the start-up and transition phases [26,42] and then was maintained at relatively low levels during the stabilization phase by joint operations of clarifier and biofilter. Meanwhile, biofloc-rich water from shrimp culture tanks continuously in-flowed into the biofilter by the adjustment of water flow rate at different phases, and the trapped biofloc together with organic substrates accelerated the formation and development of nitrifying biofilm attached to moving bio-media [32,43]. The VSS of biofilm increased gradually from zero at the initial stage to 2998 mg $L^{-1}$ at the end of the trial, indicating the fast and good formation of nitrifying biofilm in the biofilter.

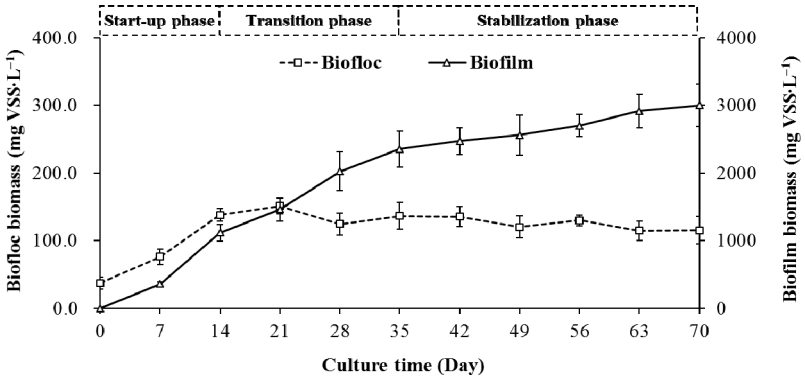


**Figure 2.** Biomass variation of biofloc in shrimp culture tank and biofilm in external aerated biofilter in the biofloc-RAS during a 70-day production trial (mean ± S.D., N = 3).

Along with biofloc development in shrimp culture tanks and biofilm formation in nitrifying biofilters in the trial, the changes of TAN, $NO_2^-$-N, $NO_3^-$-N, and TN concentrations over the 70-day trial are shown in Figure 3. These nitrogen dynamics indicated that nitrification could be instantly started up and performed stably for ammonia removal during the running of the system in this study [20,44]. It is certain that nitrifying microorganisms in the inoculated biofloc-rich water acted as nitrifying seeding and accelerated the start-up and establishment of total nitrification mediated by biofloc and biofilm in the system [20,26,42]. Certainly, a short-term time lag was still observed in the establishment of nitrification, as indicated by the slight increases in TAN and $NO_2^-$-N concentrations during the start-up and transition phases in the trial. On the one hand, the nitrifying microorganisms associated with biofloc needed two to three weeks to grow to sufficient quantities in order to cope with the increased production of ammonia. As observed in the trial, not only the biofloc level (in terms of VSS) but also the nitrifying microbial abundance significantly increased from the start-up phase to the transition phase. On the other hand, the newly started biofilter also needed a certain time to acclimate to the biofloc-rich water [23], especially for that the growth condition of nitrifying microorganisms transferred from biofloc suspension to biofilm attachment. The nitrifying biofilm gradually formed and attached to the blank biomedia in the biofilter, as observed in the trial. Nonetheless, the peaks of both TAN and $NO_2^-$-N were within the safe ranges for *L. vannamei* culture [5,26], and then their

concentrations decreased quickly in one to two weeks, remaining at stably low levels. As for the industrial practice, the inoculation and acclimatization method in this study could be a good choice for shrimp farmers to start a hybrid biofloc-RAS, in particular, to cope with slow-growing nitrifying microorganisms and a highly time-consuming process [44]. Moreover, relatively fast and stable nitrification could be achieved in the hybrid biofloc-RAS resulting from the combined effects of biofloc and biofilm, even under the conditions of high feeding rates and zero water exchange for shrimp intensive culture.

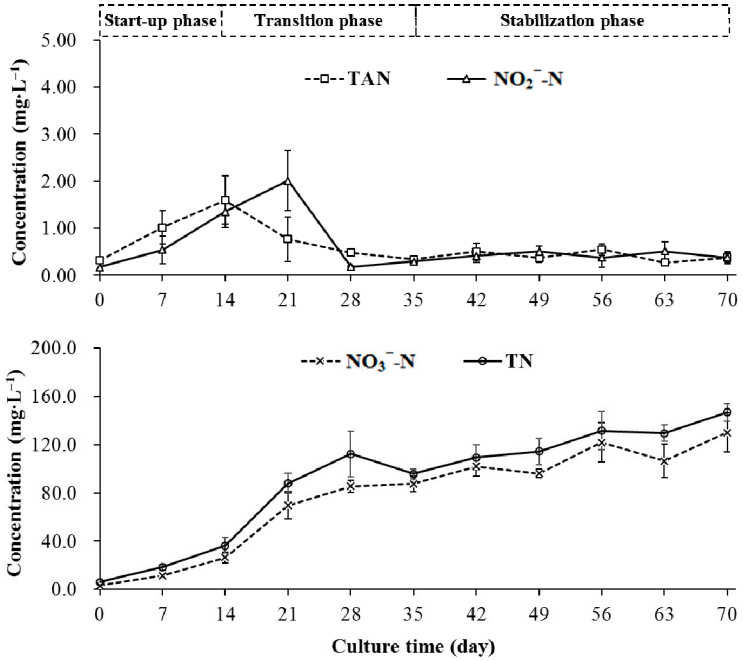


**Figure 3.** Concentration variation of TAN, $NO_2^-$-N, $NO_3^-$-N, and TN in shrimp culture tanks of the biofloc-RAS during a 70-day production trial (mean ± S.D., N = 3).

### *3.2. Comparison of Nitrifying Microbial Communities Between Biofloc and Biofilm*

In this study, the nitrifying microbial communities (AOA, AOB, and NOB) in both biofloc and biofilm should together be responsible for total nitrification in the hybrid biofloc-RAS. Some differences in abundance, diversity, and composition of AOA, AOB, and NOB could be found between biofloc and biofilm, although both of them were shaped from the same seeding of biofloc-rich water during the 70-day trial. This supported the hypothesis that the nitrifying microbial communities responded differently to the distinct conditions, such as water condition and microbial growth nature [19,45]. Due to the effect of settling clarifier, the nitrifying microorganisms grew in the form of suspended biofloc in the eutrophic condition of culture tanks, while they grew in the form of attached biofilm in the oligotrophic condition of the aerated biofilter.

The abundances of AOA, AOB, and NOB all increased in both biofloc and biofilm as the trial proceeded (Figure 4), indicating that these nitrifying microorganisms could grow and reproduce in their respective existing forms in the different components of the system. Not only that, these nitrifying microorganisms tended to reproduce more in the biofilm than in the biofloc, as indicated by the result that higher abundances of AOA, AOB, and NOB were detected in biofilm than those in biofloc after the system stabilized (Figure 4). It can be deduced that the eutrophic water in the culture tank was more favorable for the fast growth of heterotrophs, thereby restraining the enrichment of slow-growing and autotrophic-dominated nitrifying microorganisms in the suspended biofloc [43]. Moreover, although some differences in Chao richness of AOA, AOB, and NOB between biofilm and biofloc were found, there was no significant difference in the Shannon diversity of AOA, AOB, and NOB between biofilm and biofloc after the system stabilized in this study (Figure 5). Taken together,

these results suggested that the stable and efficient nitrification performance in the hybrid biofloc-RAS could be attributed to the abundance rather than the alpha-diversity of AOA, AOB, and NOB of both biofloc and biofilm. This speculation was confirmed by the further analysis that positive and significant correlations were found between the abundance of the detected nitrifying functional communities in both biofloc and biofilm and nitrification performance of the system.

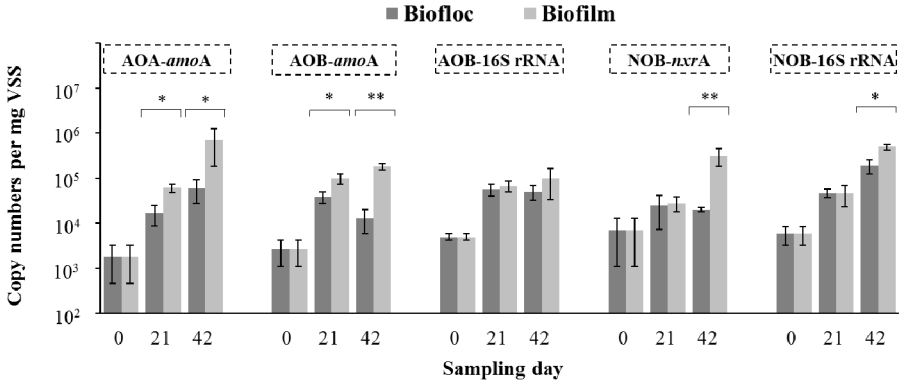


**Figure 4.** Abundance comparison of nitrifying microbial communities based on qPCR of 16S rRNA and functional genes between biofloc and biofilm from the biofloc-RAS for shrimp intensive culture in a 70-day trial (mean ± S.D., N = 3; * $p < 0.05$; ** $p < 0.01$). AOA-*amo*A represents ammonia-oxidizing archaea based on the archaeal *amo*A gene, AOB-*amo*A represents ammonia-oxidizing bacteria based on bacterial *amo*A gene, AOB-16S rRNA represents ammonia-oxidizing bacteria based on 16S rRNA gene, NOB-*nxr*A represents nitrite-oxidizing bacteria based on *nxr*A gene, NOB-16S rRNA represents nitrite-oxidizing bacteria based on 16S rRNA gene.

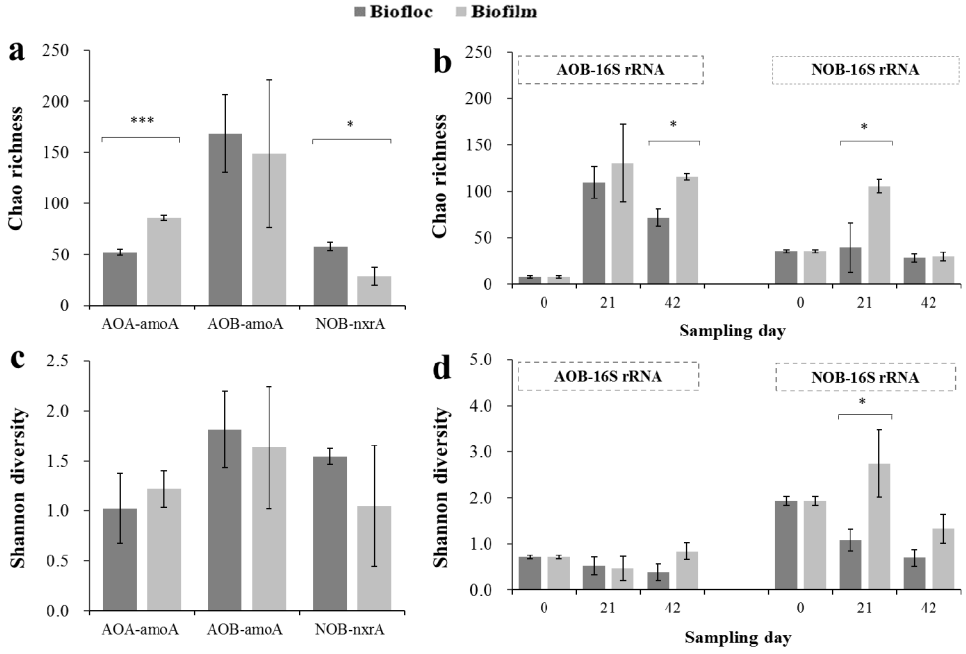


**Figure 5.** Alpha-diversity comparison of nitrifying microbial communities based on Miseq sequencing of 16S rRNA and functional genes between biofloc and biofilm from the hybrid biofloc-RAS for shrimp intensive culture in a 70-day trial (mean ± S.D., N = 3; * $p < 0.05$; *** $p < 0.001$). (**a**) Chao richness of AOA-*amo*A, AOB-*amo*A and NOB-*nxr*A at day 42; (**b**) Chao richness of AOB-16S rRNA and NOB-16S rRNA at day 0, 21 and 42; (**c**) Shannon diversity of AOA-*amo*A, AOB-*amo*A and NOB-*nxr*A at day 42; (**d**) Shannon diversity of AOB-16S rRNA and NOB-16S rRNA at day 0, 21 and 42.

As expected, most of the detected nitrifying functional communities could be found in both biofloc and biofilm. The dominant genera of AOA, AOB, and NOB were similar between biofilm and biofloc, but differences could be found in their percentages (Figure 6). The results were similar to the previous study, which hypothesized that each RAS should have a unique microbial community composition shaped by operational controls and components implemented in the system [46]. In this study, the community composition of AOA, AOB, and NOB shaped from the same inoculation of biofloc-rich water, which indicated that the initial seeding might play a fundamental role in the shaping and developing of the composition of nitrifying functional communities [47,48].

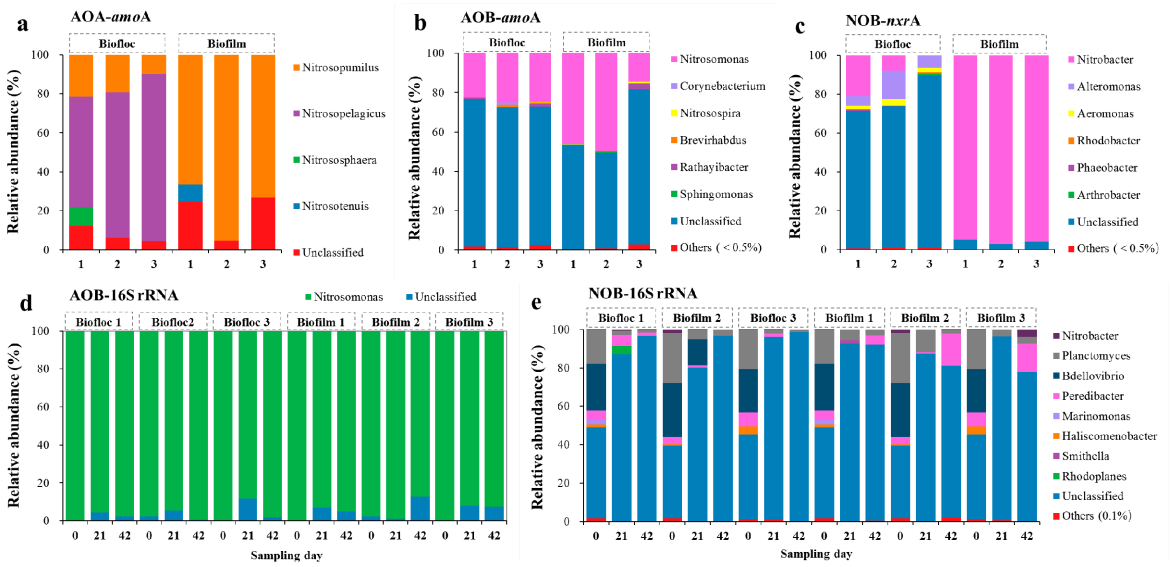


**Figure 6.** Genus-level composition of nitrifying microbial communities based on Miseq sequencing of 16S rRNA and functional genes in biofloc and biofilm from the biofloc-RAS for shrimp intensive culture in a 70-day trial. (**a**) Relative abundance of AOA-*amo*A at day 42; (**b**) Relative abundance of AOB-*amo*A at day 42; (**c**) Relative abundance of NOB-*nxr*A at day 42; (**d**) Relative abundance of AOB-16S rRNA at day 0, 21 and 42; (**e**) Relative abundance of NOB-16S rRNA at day 0, 21 and 42.

For those dominant genera of AOA, AOB, and NOB involving corresponding nitrogen conversion processes [49], significant different percentages were found between biofloc and biofilm ($p < 0.05$) (Table 4). The genera of *Nitrosopumilus* and *Nitrosopelagicus* composed the most abundant AOA groups in biofloc and biofilm, respectively. It is acknowledged that members of both *Nitrosopumilus* (*N. maritimus*) and *Nitrosopelagicus* (*Candidatus N. brevis*) genera are ubiquitously found in oligotrophic marine environments and grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite [50–52]. Previous studies have also found that the *Nitrosopumilus* sp. are dominant in biofilters' biofilm of a marine shrimp RAS [20] and different marine fish RASs [53,54].

**Table 4.** Percentage and nitrogen conversion process of involved main nitrifying microbial communities between biofloc and biofilm from the biofloc-RAS for shrimp intensive culture in a 70-day trial.

| Target Community | Dominant Genus | Percentage (%) | | Nitrogen Conversion Process |
|---|---|---|---|---|
| | | Biofloc | Biofilm | |
| AOA-*amo*A | *Nitrosopumilus* | 16.7 ± 4.9 [b] | 77.0 ± 12.4 [a] | Ammonium oxidization |
| | *Nitrosopelagicus* | 71.5 ± 12.1 [b] | 0 ± 0 [a] | Ammonium oxidization |
| AOB-*amo*A | *Nitrosomonas* | 23.9 ± 1.2 [a] | 36.6 ± 15.8 [a] | Ammonium oxidization |
| AOB-16S rRNA | *Nitrosomonas* | 96.8 ± 1.0 [a] | 95.2 ± 0.6 [a] | Ammonium oxidization |
| NOB-*nxr*A | *Nitrobacter* | 9.7 ± 8.7 [b] | 96.0 ± 0.9 [a] | Nitrite oxidization |
| NOB-16S rRNA | *Nitrobacter* | 0 ± 0 [b] | 1.5 ± 1.9 [a] | Nitrite oxidization |
| | *Planctomyces* | 1.9 ± 0.6 [a] | 2.8 ± 0.5 [a] | Anammox (possible) |

Each value represents the mean ± S.D. (N = 3). Values in the same row with different superscripts are significantly different ($p < 0.05$).

It is interesting to note that the *Nitrosopelagicus* was a predominant AOA group in biofloc but did not exist in biofilm. The genus *Nitrosomonas* was the dominant AOB group in both biofloc and biofilm. The *Nitrosomonas* species are commonly found to be the most dominant AOB in the biofilm of marine RAS biofilters [18,53–55]. The percentage of the genus *Nitrosomonas* in AOB was similar between biofloc and biofilm, suggesting that the genus *Nitrosomonas* could adapt to different microbial aggregates and nutrient environments in this study [56]. The genus *Nitrobacter* was the dominant NOB group in biofilm, while it showed low abundance in biofloc. This was different from the previous studies that reported the genus *Nitrospira* to be the dominant NOB group in the biofilm of marine RAS biofilters [17,18,55,57,58]. In addition, the genus *Planctomycetes* was detected at relatively high percentages in both biofloc and biofilm, indicating that the anammox reaction probably occurred in the system [49].

### *3.3. Correlation between TAN Removal and Nitrifying Functional Communities of Biofloc and Biofilm*

During the 70-day trial, along with feed loading rate increasing from 102.9 mg·$L^{-1}$ $d^{-1}$ to 127.6 mg· $L^{-1}$ $d^{-1}$ in the system, the estimated TAN production rate increased linearly from 5.94 mg·$L^{-1}$ $d^{-1}$ to 7.35 mg·$L^{-1}$ $d^{-1}$ (Figure 7). Meanwhile, TAN removal rate and efficiency first decreased significantly and then recovered quickly during the start-up and transition phases, and then remained at stable high levels above 6.28 mg·$L^{-1}$ $d^{-1}$ and 92.2%, respectively, during the stabilization phase (Figure 7). Overall, the high rate and efficiency of TAN removal could be achieved under conditions of continuous TAN production and zero water exchange in the system with shrimp intensive production. The results indicated that the hybrid system combining suspended biofloc and attached biofilm was feasible and effective to remove TAN to ensure a safe water environment for shrimp intensive culture.

Correlation analysis showed that the TAN removal rate was significantly and positively correlated with abundances of AOA and NOB in biofloc ($p < 0.05$), while it was significantly and positively correlated with abundances of AOB and NOB in biofilm ($p < 0.05$) (Figure 8). The results indicated that nitrifying microbial communities in both biofloc and biofilm should contribute to TAN removal in the hybrid system, and moreover, the TAN removal rate increased as the abundances of nitrifying microbial communities increased in biofloc and biofilm.

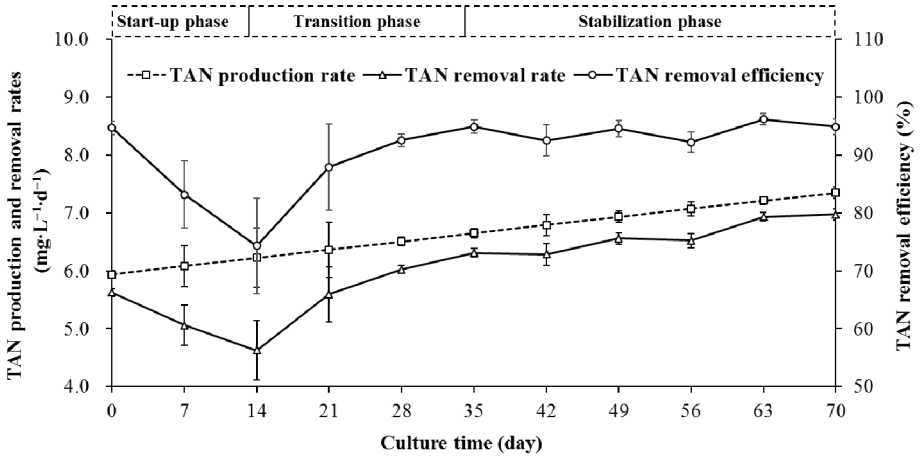


**Figure 7.** Variation in the production rate, removal rate, and removal efficiency of TAN in shrimp culture tanks of the biofloc-RAS in a 70-day production trial (mean ± S.D., N = 3).

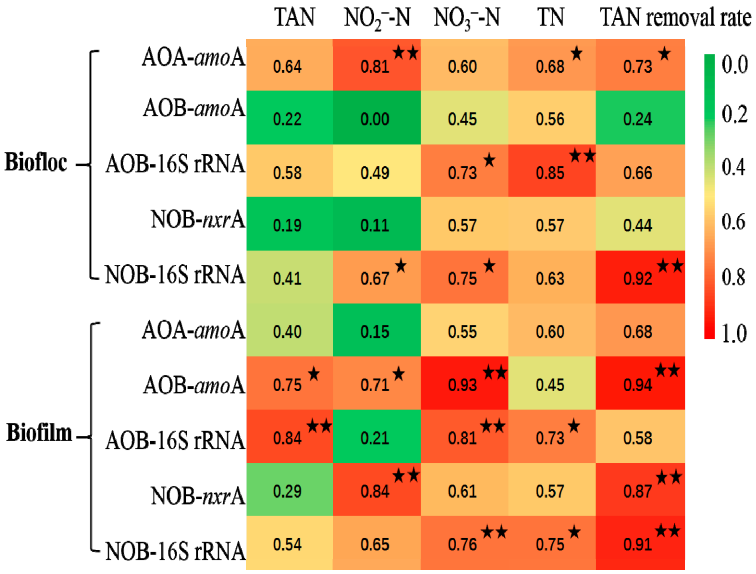


**Figure 8.** The heatmap of Pearson correlation coefficients (r-value) between the levels of TAN, $NO_2^-$-N, $NO_3^-$-N, TN, TAN removal rate and abundances of AOA, AOB, NOB in biofloc and biofilm from the biofloc-RAS for shrimp intensive culture in a 70-day trial. Significance: * $p < 0.05$; ** $p < 0.01$.

The total abundances of nitrifying microbial communities depended on the total biomass of biofloc and biofilm in the whole system. In the trial, the biofloc existed in the whole water of the culture tank (working volume of 30 $m^3$), while the biofilm limited to the moving bio-media of nitrifying biofilter (working volume of 0.28 $m^3$). According to the detected biomasses of biofloc (≈110 mg VSS·$L^{-1}$) and biofilm (2300-3000 mg VSS $L^{-1}$) after the system stabilized (Figure 2), the estimated total biomass of biofloc was higher than that of biofilm in less than one order of magnitude. Meanwhile, the abundances of nitrifying microbial communities in biofloc were about one order of magnitude lower than those of biofilm (Figure 4). As a result, the total abundances of nitrifying microbial communities in biofloc and biofilm in the whole system were estimated to be of the same order of magnitude after the system stabilized. This highlighted that both biofloc and biofilm could contribute highly to the nitrification in the hybrid biofloc-RAS. The in situ suspended biofloc and ex situ attached biofilm played complementary and coordinate roles in nitrification performance and system stability for ammonia removal in the hybrid biofloc-RAS.

## 4. Conclusions

This study demonstrated that high efficiency and stability of ammonia removal through nitrification could be achieved by the combined effects of biofloc and biofilm in a simplified pilot-scale hybrid biofloc-RAS for *L. vannamei* intensive culture with zero water exchange. Inoculating biofloc-rich water into the hybrid system could accelerate the development of suspended biofloc in in situ culture tanks and attached biofilm in ex situ nitrifying biofilters, thereby contributing to a fast and effective establishment of nitrification during practical operation even with ever-increasing feeding rates. Similar nitrifying microorganisms could be found between biofloc and biofilm, despite some differences in the abundance, diversity, and composition of AOA, AOB, and NOB. Meanwhile, a high TAN removal rate could be achieved and was significantly and positively correlated with abundances of nitrifying microbial communities in both biofloc and biofilm, further indicating that both biofloc and biofilm could contribute highly to the nitrification in the hybrid RAS. The information presented could contribute to the understanding of microbial ecology of ammonia removal in the hybrid biofloc-biofilm RAS and indicate the potential application of the system and related technologies in coastal intensive aquaculture for saving water resources and reducing environmental impacts.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4441/12/11/3000/s1, Table S1: The main water quality characteristics of the shrimp nursery pond.

**Author Contributions:** Conceptualization, W.X. and Y.C.; Methodology, W.X. and Y.X.; Software, W.X., H.S., and X.H.; Validation, G.W. and Y.C.; Formal analysis, W.X. and Y.C.; Investigation, Y.X. and K.Y.; Resources, G.W. and Y.C.; Data curation, W.X., G.W., and Y.C.; Writing—Original draft preparation, W.X.; Writing—review and editing, W.X., G.W., and Y.C.; Visualization, W.X., H.S., and X.H.; Supervision, G.W. and Y.C.; Project administration, W.X., G.W., and Y.C.; Funding acquisition, W.X., G.W., and Y.C. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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