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Original Research

Research on Nitrate Removal from Recirculating Aquaculture Systems Using Solid-phase Denitrification Process

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Abstract

In order to solve the problem of significant nitrate accumulation during the process of factory-circulating aquaculture, which affects the growth and development of aquaculture species, this project added a solid-phase denitrification (SPD) treatment unit to the recirculating aquaculture system (RAS) to explore the removal effect of the treatment unit on nitrate in the aquaculture water and the change of the microbial community structure of each unit in the RAS before and after the setting of the SPD unit. The results showed that the SPD treatment unit can remove nitrate accumulated by RAS to approximately 40 mg/L after 100 days of operation, with a nitrate removal rate of 0.32 g/(L·d). The analysis of microbial community structure showed that in each unit of the RAS, Proteobacteria, Bacteroidetes, and Cyanobacteria were all dominant phyla before and after the setting of SPD units, although their abundance changed. Experiments have proved that setting up an SPD treatment unit in the RAS can better purify the nitrate pollution accumulated in the system, which provides certain theoretical reference and technical support for its large-scale practical application.

Keywords: recirculating aquaculture system, nitrate removal, solid-phase denitrification; microbial community

Introduction

Recirculating aquaculture system (RAS) has become one of the main directions of the aquaculture industry all over the world, especially for inland areas where land resources and water resources are increasingly scarce; the development of high-density recirculating

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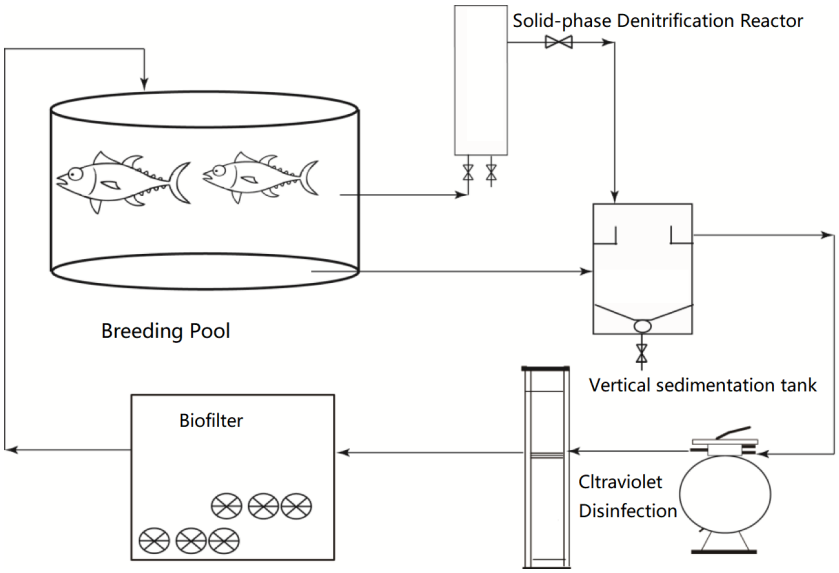

Fig. 1. Schematic diagram of the recirculating aquaculture system.

aquaculture is an inevitable trend in the development of the aquaculture industry [1]. In RAS, water quality purification is the core technology. Through the conventional nitrogen removal treatment process, it can effectively remove organic matter from the aquaculture water and convert ammonia nitrogen into nitrate, so that the water quality can be improved to some extent. However, due to the oxygen-enriched environment and low C/N ratio of RAS, biological denitrification is difficult to carry out properly, often resulting in a large accumulation of nitrate in the system [2]. Although nitrate is much less toxic to aquaculture species than the same concentration of ammonia and nitrite, it can also affect the growth, development, and hormone secretion of aquaculture species [3]. Therefore, the production often relies on water exchange to reduce the nitrate concentration of RAS, which not only causes a huge waste of water and energy, but also the discharge of nitrate-rich effluent, which aggravates the pollution of the environment. Therefore, the development of suitable nitrate removal technology for RAS is important to ensure the health of farming objects, save water resources, and reduce pollutant discharge from the aquaculture industry.

To achieve denitrification of low C/N ratio water bodies such as RAS effluent, conventional denitrification processes usually add soluble organic matter such as methanol and ethanol as supplementary carbon sources [4]. However, there is a risk that the soluble carbon source can be easily overdosed, and the stable operation and maintenance of the system are more difficult. In order to overcome the above disadvantages, investigators try to use non-water-soluble solid organic matter as the carbon source of denitrifying microorganisms and a biofilm carrier to achieve denitrification to remove nitrate from polluted water bodies. This process is called solid-phase denitrification (SPD) [5]. The process does not require a complex carbon source dosing control system, is simple to operate, and stable in operation [6].

In recent years, scholars have used the SPD process to achieve effective removal of high nitrate concentrations from RAS wastewater, with research focusing on process optimization of SPD and analysis of microbial communities in the reactor [7-9]. The main problem with existing studies is that they usually only address end-of-pipe treatment of RAS effluent and do not achieve return flow of treated water to the RAS [10]. The effect of the SPD process on RAS water quality indicators, culture objects, and microbial community structure of the whole culture system is not clear because of the failure to integrate the SPD process into the RAS.

In this paper, a SPD unit with PHBV as the carbon source was added to the RAS. The purpose of this study was to: (1) investigate the changes of water qualities in a pilot-scale RAS connecting with a SPD reactor (RAS-SPD); (2) compare the microbial community structures of different working units in the RAS-SPD. This study provides a theoretical basis for the application of the SPD reactor to remove nitrate in RAS, and illustrates the changes in microbial population during nitrate removal.

Materials and Methods

Recirculating Aquaculture System

The experiment was conducted at the aquaculture unit of Yancheng Normal College from March to the end of May 2022. A pilot-scale RAS was used; the aquaculture system was composed of an aquaculture pond, SPD reactor, moving bed biofilm reactor (MBBR), filter sand tank, temperature controller, and ultraviolet disinfection pipe. The flow chart is shown in Fig. 1. The total volume of the breeding pond was about

1.5 m^3, and the actual breeding water body was 1 m^3. The breeding density was 2.5 kg/m^3. 5% (weight of cultivated fish) of pelleted fodder was daily added into the aquaculture pond with approximately 6.0% crude fat, 25% crude protein, 15.0% ash, 4.0% crude fiber, and 15.0% moisture content to maintain the growth of fish. The SPD unit was positioned after the MBBR reactor in the circulating aquaculture system and operated at up to 100 mg/L.

The MBBR reactor was of a cylindrical type, with a total volume of 0.34 m^3 and an effective volume of 0.2 m^3, supplemented with 50 L of suspended filler. The used filler was a circular, high-density polyethylene material of 0.95 g/cm^3, and had a specific surface area of approximately 500 m^2/m^3. Glucose was added as a carbon source for the nitrification process (COD = 20 mg/L). Furthermore, sodium bicarbonate ($NaHCO_3$) and disodium hydrogen phosphate (Na_2HPO_4) were added to maintain the stability of the environmental pH. The aquaculture pool and MBBR reactor were provided with sufficient dissolved oxygen (DO) by an air pump (HD602, HDOM, Shenzhen, China). The pH value of the RAS was maintained between 7 and 8 with $NaHCO_3$.

The SPD reactor consisted of a cylindrical plexiglass container that had an 80-mm inner diameter and was 850 mm high, which was filled with 1200 g [poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHBV] granules. The PHBV granules were purchased from Ningbo Cheonan Biological Material Co., Ltd., and the diameter and height of the cylindrical particles were 2.5 mm and 3.5 mm, respectively. The specific surface area was 2 m^2/kg, and the molecular weight was 330000. The effective volume of the SPD reactor was 2.0 L. The aquaculture pond wastewater was transported into the bottom of the SPD reactor by a peristaltic pump. Strain SL-205 was used as the inoculum and had been cultured in LB medium before use. The culture medium of strain SL-205 can be inoculated into the PHBV denitrification SPD reactor at the OD600 = 1.8-2.0. The inoculation method was also by transporting the strain from the bottom of the SPD reactor through a peristaltic pump. The inoculum for the upflow fixed-bed PHBV denitrification reactor was 400 mL of strain SL-205. When the nitrate nitrogen in the aquaculture pool increased to 80 mg/L, the SPD reactor was started up.

Analytical Methods for Effluent Quality

Daily samples were gathered from the aquaculture pond and the SPD reactor, filtered through a 0.45 μm filter, and then analyzed using a detector. Nessler's reagent spectrophotometry, N-(1-naphthalene)-diaminoethane spectrophotometry, ultraviolet spectrophotometry, and alkaline potassium persulfate photometry were used to determine the concentrations of NH_4^+-N, NO_2^+-N, NO_3^+-N, and total nitrogen (TN) in the effluent [11]. A TOC analyzer was used to measure the amount of dissolved organic carbon (DOC) (vario TOC cube, Elementar, Germany).

High-Throughput Sequencing

Biofilm samples were obtained from the surface of the MBBR suspended biological filler and the SPD reactor carbon source PHBV. Water samples were filtered from the aquaculture pond to obtain biofilm samples. Biofilm sampling, DNA extraction, and PCR amplification were all done in the same way as in a previous study [12]. The attached biofilm samples were extracted from the PHBV granules using ultrasonic treatment (Shumei, Kunshan, China). A FastDNA Spin Kit for soil was used to extract DNA from biofilm samples (MP Biomedicals, CA, USA). The NanoDrop 2000 UV-vis spectrophotometer was used to evaluate the purity and concentration of the DNA product (Thermo Scientific, Wilmington, USA). Primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3')

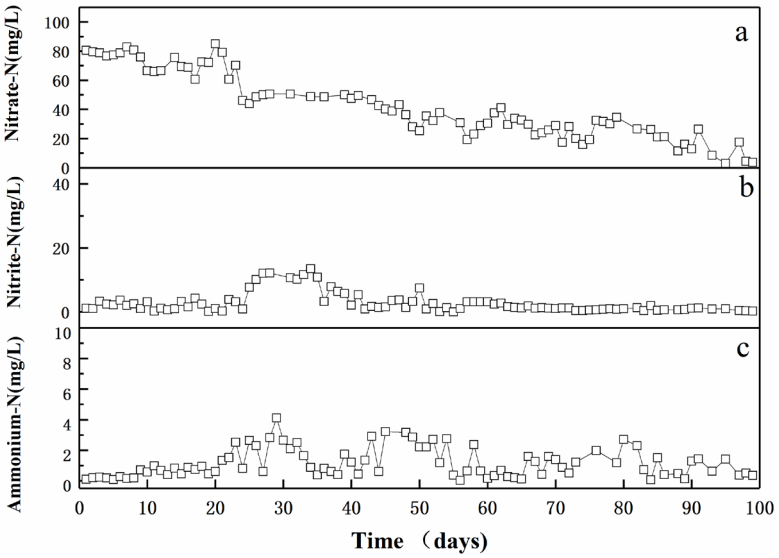

Fig. 2. The concentrations of nitrate, nitrite, and ammonia nitrogen in the effluents of SPD.

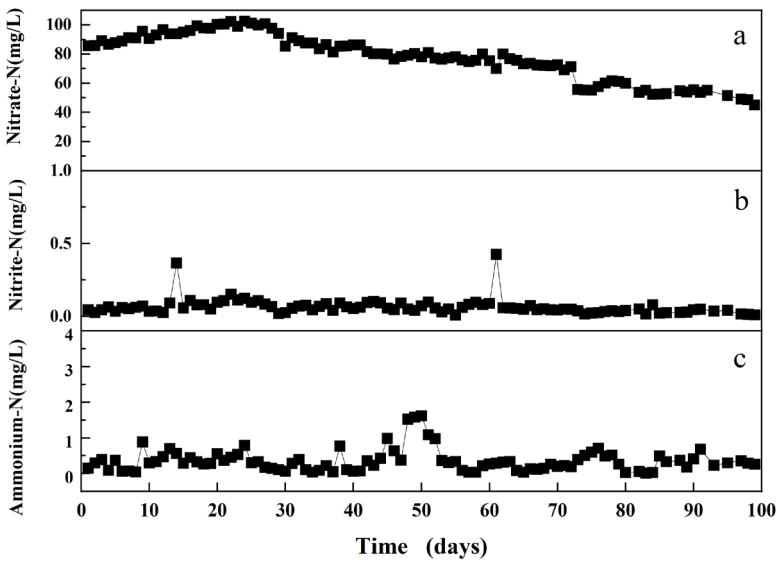

Fig. 3. The concentrations of nitrate, nitrite, and ammonia nitrogen in the aquaculture pool.

and 806R (5'-GGACTACHVGGGTWTCTA AT-3') were used to amplify the V4 hypervariable regions of the 16S rRNA gene. Electrophoresis on a 1% agarose gel was used to evaluate the purity of the extracted DNA.

DNA samples from biofilms were sequenced on the Illumina MiSeq platform (Illumina, San Diego, USA) using standard protocols by Meiji Bio-Pharm Technology Co., Ltd. (Shanghai, China). The completeness of the barcodes and adapters was examined, and sequences shorter than 50 bp were deleted to reduce the impact of random sequencing errors. We spliced the matched readings together into a sequence with a 10 bp overlap length and a maximum mismatch ratio of 0.2 in the overlap region. End readings were assigned to the sample based on their unique barcode and were shortened by removing the barcode and primer sequences. The operational taxonomic units (OTUs) were clustered using a 97% similarity cut-off with UPARSE (version 7.1 http://drive5.com/uparse/), and the chimeric sequences were identified and removed using UCHIME. The RDP Classifier algorithm (http://rdp.cme.msu.edu/) was used to compare the taxonomy of each 16S rRNA gene sequence to the Silva (SSU123) 16S rRNA database, with a confidence level of 70%. DNA library building and data analysis were performed in accordance with the method by Zhang et al. [12]. This study's raw data were placed in the NCBI Sequence Read Archive (SRA) with the accession number "SRP132428".

Results and Discussion

Denitrification Performance of the SPD Reactor in RAS

When the nitrate concentrations in the aquaculture pool were accumulated to 80 mg/L, the SPD unit was added to the RAS system. The SPD reactor was connected in series in the RAS system, and the domestication period lasted 20 days. The water quality data in the SPD reactor are shown in Fig. 2. During the domestication phase, the denitrification efficiency of the SPD reactor was low because the biofilm on the surface of PHBV had not been formed. This led to the nitrate concentration in the aquaculture pond slightly increasing instead of decreasing. Nitrogen concentration in the aquaculture pond increased from an initial 85.2 mg/L to 20 days of 102.6 mg/L (Fig. 3a)). This increase is due to the conversion of ammonia nitrogen to nitrate nitrogen formed by the fed residual feed, fecal accumulation, etc. Starting from day 24, the SPD reactor entered a stable operation phase. In previous studies, PBS was used as a carbon source and carrier for denitrification and inoculated with lake sediment to treat tilapia culture wastewater; it took 60 days to complete the domestication phase [13]. Previous studies have demonstrated that strain SL-205 effectively shortens the domestication time of the PHBV-supported SPD reactor [12]. On the 24th day, the effluent of the SPD reactor was below 50 mg/L (Fig. 2a)), and the removal efficiency was 55%. At the same time, the concentration of nitrate nitrogen in the aquaculture pond began to gradually decrease (Fig. 3a)). The SPD reactor significantly reduced the introduced nitrates from the aquaculture pond after 100 days of stable operation (Fig. 2a)). The average nitrate removal rate was 0.39±0.07 kg NO_3^--N /$m^{-3}d^{-1}$.

In RAS, ammonia nitrogen and nitrite produced by protein feed are converted to nitrate by nitrification, and although nitrate has lower toxicity than ammonia and nitrite, high nitrate concentrations can affect fish growth [14]. The variation of nitrate nitrogen concentration in the aquaculture pond was shown in Fig. 3. During the domestication phase of the SPD reactor, the nitrate concentration in the aquaculture pond continued to

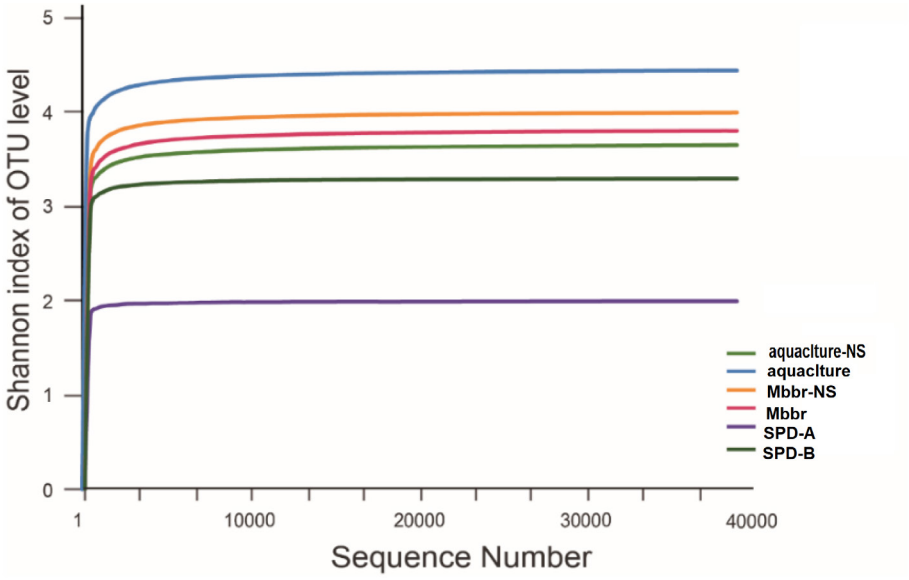

Fig. 4. Rarefaction curves of OTUs clustered at 97% sequence identity across different treatment units of RAS. Aquaculture-NS represents biofilm samples in the aquaculture pool with an unconnected SPD reactor; aquaculture represents the biofilm sample in the aquaculture pool after connecting the SPD reactor; Mbbr-NS represents the biofilm samples in the Mbbr unit with an unconnected SPD reactor; Mbbr represents the biofilm sample in the Mbbr unit after connecting the SPD reactor; SPD-A and SPD-B represent the biofilm samples obtained from the SPD reactor run for 50 days and 100 days, respectively.

rise. This phenomenon was caused by the accumulation of ammonia nitrogen and nitrite produced by the protein feed into nitrate by nitrification [15, 16]. As the denitrification efficiency of the SPD reactor increases, the concentration of nitrate in the aquaculture pond decreases. From the 24th to 100th days, the average nitrate removal efficiency in the aquaculture pond was 58.3%±12.6%. Finally, nitrate concentration in the aquaculture pond was decreased to 45.1 mg/L (Fig. 3a)). Therefore, setting a denitrification unit was an effective way to remove nitrate in recirculating aquaculture systems [7, 17].

There was virtually no accumulation of nitrite (<0.05 mg/L) in the aquaculture pond (Fig. 3b)). There are two main reasons for the production of nitrite: on the one hand, due to the difference in multiplication time between nitrification bacteria (ammonia-oxidizing bacteria and nitrification bacteria) and heterotrophic degrading bacteria, the growth of autotrophic growing nitrification bacterial bacteria tends to lag behind, which can lead to high ammonia-nitrogen concentrations and incomplete reactions throughout the nitrification phase, resulting in the accumulation of nitrite. On the other hand, due to the insufficient oxygenation of RAS, resulting in local hypoxia, which will convert nitrate-nitrogen to nitrite-nitrogen in the water column and will also cause the accumulation of nitrite-nitrogen [18, 19].

In aquaculture, ammonia nitrogen concentration is an important water quality monitoring indicator. Higher ammonia concentrations could inhibit the effluent of ammonia from the gills, raising ionic ammonia in the blood, affecting fish growth, and causing a variety of physiological problems. After the nitrification of the MBBR bioreactor, the concentration of ammonia nitrogen in the aquaculture pond did not exceed 2 mg/L (Fig. 3c)). The maximum concentration of ionic ammonia allowed in conventional culture water was 5 mg/L. Compared with pond culture and cement pond flowing water culture, the factory recirculating water culture system could effectively eliminate the

Table 1. Comparison of sample sequencing statistics and diversity index.

	ACE	Chao1	Coverage	Shannon	Simpson
MBBR-NS	860.0±44.8	872.3±65.4	0.992±0.001	3.9±0.1	0.061±0.01
MBBR	972.6±40.3	998.0±56.0	0.992±0.001	3.8±0.1	0.063±0.008
aquaculture-NS	1139.7±140.9	1151.4±158.5	0.991±0.001	4.0±0.7	0.066±0.036
aquaculture	1113.9±46.9	1130.4±58.0	0.991±0	4.3±0.1	0.042±0.009
SPD-A	182.4±40.6	165.8±17.9	0.998±0	1.7±0.6	0.387±0.258
SPD-B	464.3±50.9	452.5±84.7	0.996±0	3.2±0.1	0.086±0.005

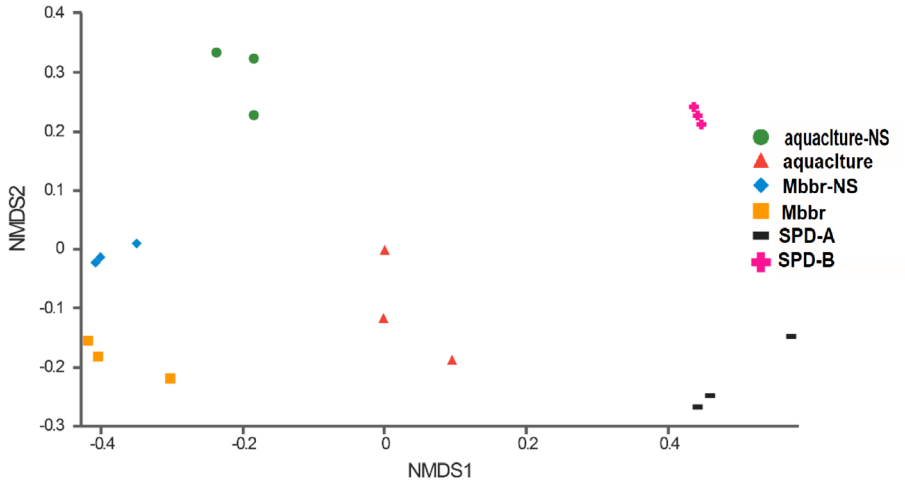

Fig. 5. Principal component analyses of the microbial community structure in MBBR biofilms.

accumulation of ammonia nitrogen in the culture water body [20, 21].

Alpha Diversity

To further understand the effect of the SPD system on the microbial community structure of each treatment unit in the recirculating aquaculture system. 16s rRNA analysis of biofilm samples from MBBR, aquaculture pond, and SPD stages before and after SPD reactor connection was performed using high-throughput sequencing technology. 606469 sequences were obtained, with an average fragment length of 357 bp. The diversity index for each sample was calculated by statistical analysis based on the effective sequence obtained from sequencing, and the results are shown in Table 1. The biofilm samples were all sequenced at over 99% depth. It indicates that this sequencing can contain most of the microbial structural information in the samples. The sparse curves of the samples in Fig. 4 also indicated that the sequencing results of the biofilm samples have reached saturation, which can truly reflect the information of bacterial diversity in the samples. The Shannon Sinon index and Simpson Simpson index, as common microbial diversity indicators, were often used to reflect the richness and evenness of microbial communities. The data in Table 1 show that the connection to the SPD reactor has diametrically opposed effects on the biodiversity of the different treatment units. From the Shannon index, the bacterial diversity in the MBBR biofilter decreased after connecting the SPD reactor, while the bacterial diversity in the culture pond increased.

The differences in microbial community structure can be characterized by NMDS analysis (Fig. 5). The MBBR and MBBR-NS samples are located closer together. It indicates that the SPD reactor has less effect on the bacterial community structure of the MBBR biofilter in the RAS. This phenomenon occurs because the MBBR biofilter carries out biological processes in an aerobic environment, which favors the enrichment of aerobic microorganisms. The addition of the anoxic denitrification process does not change the aerobic environment of the MBBR, so the structure of the MBBR biofilter community does not appear to change significantly.

Effect of SPD Reactor on Microbial Community Structure

Fig. 6a) shows the relative abundance of bacterial communities at the phylum level in RAS units. Proteobacteria and Bacteroidetes were the key groups in all biofilm samples from the RAS units. Proteobacteria are advantageous in denitrification systems employing polymers as the carbon source [22]. Given that the most denitrifying bacteria are subjected to Proteobacteria, its increase in the SPD was as expected. Remarkably, a higher abundance of Proteobacteria was discovered in the SPD unit. The Bacteroidetes phylum plays an important role in the catabolism of macromolecules in wastewater treatment, and their existence in SPD is possibly due to the catabolism of the sole carbon source PHBV [23]. The differences in environmental conditions in the different treatment units accounted for the abundance difference of the Bacteroidetes phylum between the units of the RAS. Proteobacteria, Bacteroidetes were also dominant in high salinity wastewater treatment systems [24]. Cyanobacteria were a dominant bacterium in the MBBR biofilter and culture pond, but were not detected in the SPD unit. Cyanobacteria are a group of microorganisms that have a photosynthetic oxygen release function, along with the capacity of nitrogen fixation and resisting an adverse environment. After the SPD reactor ligation in RAS, Bacteroidetes abundance was significantly reduced in the MBBR biofilter, and Bacteroidetes abundance was slightly decreased in the culture pond. This phenomenon occurs because the connection of the SPD reactor does

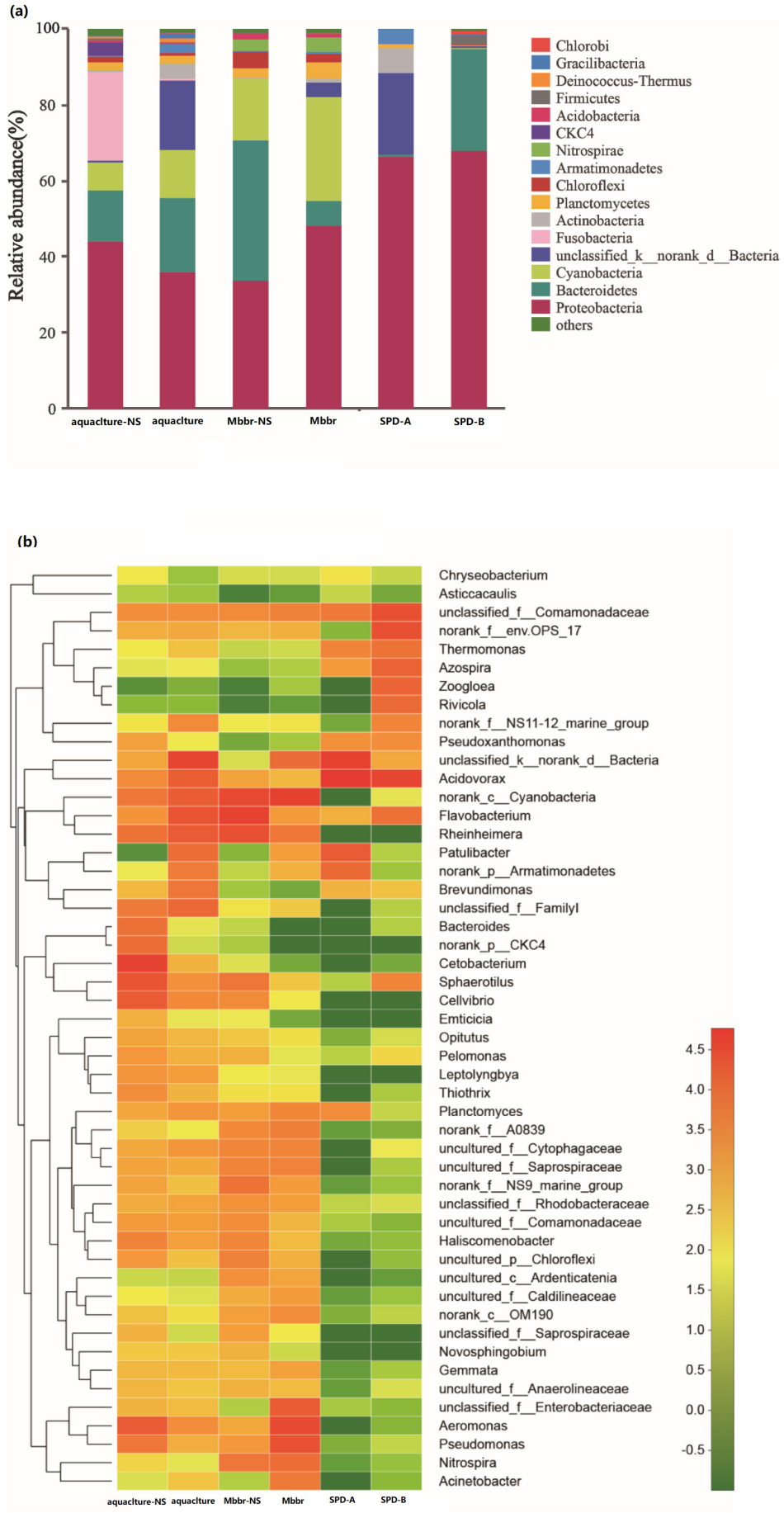

Fig. 6. Bacterial community compositions at a) the phylum and b) genus.

not change the microenvironment in the individual units of the RAS.

The hierarchically clustered heatmap at the genus level is presented in Fig. 6b). The effect of the SPD reactor on the structure of the MBBR biofilter and culture pond was also evident. It could be found that the SPD reactor created different dominant genera and microbial community structures in the units of the RAS. Before the SPD reactor was set up, the relative abundances of norank c_*Cyanobacteria*, *Flavobacterium*, *Rheinheimera*, *Sphaerotilus,* and *Nitrospira* were the most predominant in the MBBR biofilter. However, after the SPD reactor was established, norank c_*Cyanobacteria* and *Nitrospira* remain the predominant bacterial genera. Moreover, the other two genera, *Aeromonas* and *Pseudomonas,* were enriched in the MBBR. Heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria, *Pseudomona*s, are highly enriched in RAS, which provides a biological basis for nitrogen removal in the RAS [25].

In the aquaculture pond, *Bacteroides*, *Cetobacterium*, *Sphaterotilus*, *Celivibrio*, *Aeromonas,* and *Pseudomonas* became the dominant bacterial genera before the SPD reactor was set up, all related to substrate degradation and heterotrophic denitrification [6, 26]. The top genera with the highest relative abundance became *Flavobacterium*, *Acidovorax*, *Rheinheimera*, *Patulibacter,* and *Brevundimonasin* in the aquaculture pond after the SPD reactor had been established. *Flavobacterium* have also been reported as denitrifying polyphosphate accumulating organisms, which could achieve denitrification innovative biological phosphorus removal process [27]. Relative abundances of dominant bacteria indicate that the microbial community in the aquaculture pond changed greatly at the genus level after the SPD reactor was set up.

Conclusions

The SPD system supported by PHBV was applied to the pilot-scale RAS to remove the nitrate accumulated in the aquaculture tanks. After 90 days, the concentration of nitrate decreased from 100 mg/L to less than 40 mg/L, and the concentration of nitrite, ammonia nitrogen, and DOC in the aquaculture did not change significantly. High-throughput sequencing revealed that the SPD reactor increased microbial diversity in the RAS and significantly altered community abundance. This included a rise in the relative abundance of Cyanobacteria at the phylum level and an increase in *Acidovorax* (the dominant genus in the SPD reactor) within the RAS aquaculture ponds, indicating denitrification activity.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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