Nitrogen removal in recirculating aquaculture water with high dissolved oxygen conditions using the simultaneous partial nitrification, anammox and denitrification system

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ABSTRACT

The efficient removal of nitrogen pollutants in the aquaculture systems is still a challenge due to the low concentration of organic carbon and high concentration of dissolved oxygen (DO) in the wastewater. The simultaneous partial nitrification, anammox and denitrification (SNAD) bioreactor was firstly used for the treatment of aquaculture wastewater in recirculating aquaculture system. The bioreactor operated for 180 days without adding extra organic carbon. After 60-day operation, the bioreactor reached the stable stage with the average concentration of ammonia/nitrate/nitrite/COD in the effluent with 0.26/0.75/0.47/0.27 mg/L. The *Pseudoxanthomonas* was the dominant genus in the biofilm samples. The typical nitrogen functional bacteria and genes for nitrification, anammox and denitrification were detected with different abundance in different procedures along the bioreactor. Network analysis revealed the significant correlations between nitrogen functional bacteria and genes. The SNAD bioreactor achieved the effective removal for nitrogen and COD under high DO conditions in recirculating aquaculture system.

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1. Introduction

Aquaculture, as one of the most important traditional industry, has been developed rapidly with the civil economy growth and the improvement of living standard (Wang et al., 2018a). However, severe pollutants have been generated from the aquaculture industry and discharged into natural environment, exerting a significant influence on environmental ecosystem and human health (Wang et al., 2018b, 2019a). The non-point pollution from aquaculture industry has been becoming a great issue for the water environment protection (Wang et al., 2018b). Therefore, efficient treatment for aquaculture wastewater is indispensable for the removal of contaminants (Kim et al., 2020). Typical aquaculture modes include the conventional (without recirculation unit) and recirculating systems (Wang et al., 2018b). The aquaculture wastewater in the recirculating aquaculture system (RAS) is circulated and reused after the systemic treatment while the wastewater in the conventional system without recirculation is discharged into coastal water directly or with simple treatment (Wang et al., 2018b). Recirculating aquaculture system has been an important approach for the control of the non-point pollution from aquaculture industry (Wang et al., 2019a). The accumulation of ammonium and nitrite which are high toxic substances for fish, shrimp and other aquatic organisms (Shao et al., 2019) will hinder the application of the RAS systems in the aquaculture industry. Therefore, the effective removal for nitrogen pollutants, especially ammonia and nitrite, is the key for the treatment of aquaculture wastewater. However, the efficient removal of nitrogen pollutants is still a challenge due to the low concentration of organic carbon and high concentration of dissolved oxygen (DO) of the wastewater in the aquaculture systems.

In traditional biotechnological process for decreasing nitrogen and chemical oxygen demand (COD), the nitrification and denitrification reactions were performed in separated reactors owing to the different demand of various functional microbial communities (Zhang et al., 2013). Therefore, a lot of advanced technologies have been developed for the removal of nitrogen with low carbon nitrogen ratio, such as SND (Simulation Nitrification and Denitrification) (Zhao et al., 2017), OLAND (Oxygen Limited Autotrophic Nitrification Denitrification) (Nhu Hien et al., 2017), SHARON (Single reactor for High Activity ammonia Removal Over Nitrite) (Claros et al., 2012) and CANON (Completely Autotrophic Nitrogen removal Over Nitrite) (Zhang et al., 2013). Meanwhile, anaerobic ammonium oxidation (anammox) have been widely applied to nitrogen removal (Zheng et al., 2019), which was an energy-saving process without extra organic electron donor that oxidized ammonium and nitrite to N_2 (Wang et al., 2019b). The simultaneous partial nitrification, anammox and denitrification (SNAD) process has been developed to treat the wastewater with high concentration of nitrogen and low concentration of COD simultaneously (Keluskar et al., 2013; Wang et al., 2016; Wen et al., 2016). SNAD has been proven to be an efficient, economical and environmentally friendly process. Meanwhile, the SNAD process could protect the anammox process from being inhibited by organic carbon and DO (Jiang et al., 2018). As the biological oxidation of ammonia to nitrite and nitrate, nitrification is an effective approach for the removal of ammonia and organic contaminants (Jin et al., 2017). Under oxygen limitation, ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB). The remaining ammonia and nitrite are removed by anammox process, followed by denitrification process contributed to the reduction of nitrite and nitrate to N_2 . As a consequence, nitrifiers, denitrifiers and anammox bacteria generally coexist and maintain harmony for the removal of nitrogen and organic carbon (Keluskar et al., 2013).

Aquaculture water often possesses high concentration of nitrogen (ammonia, nitrate and nitrite) and suspended particle as well as low concentration of COD (Shao et al., 2019). The SNAD process might be an effective technology for the treatment of wastewater with high-concentration nitrogen and low-concentration COD (Gao et al., 2013).

However, the anammox and denitrification processes could be inhibited under high-concentration DO conditions (Persson et al., 2014). DO concentration of aquaculture wastewater is usually high since oxygen is essential for the aquaculture animals, which is quite different from other wastewaters. Rare information is available on using SNAD system for treating water with high DO concentration in aquaculture systems. This study established a typical SNAD system for simultaneous removal of nitrogen and COD under high-concentration DO conditions in the recirculating aquaculture system. The removal efficiencies for nitrogen and COD were evaluated by daily monitoring the water quality parameters in the influent and effluent. In order to figure out the removal mechanism of contaminants, both the bacterial communities and the nitrogen functional bacteria (AOB, anammox and denitrifying bacteria) were further investigated.

2. Materials and methods

2.1. Reactor system and operation strategy

The SNAD bioreactor with the effective volume of 60 L was established using two parallel U-type tubes working as baffled biofilter and a reoxygenation tank serving as moving-bed biofilter (Fig. 1). The U-type tubes were filled by cylindrical plastic biofilter media (diameter of 25 mm), which were cleaned by tap water directly. The bioreactor was equipped with a peristaltic pump for the recirculation of water. The hydraulic retention time (HRT) was set as 5 h and the influent flux was maintained in 12 L/h. An aerator was connected with the reoxygenation tank to maintain the high concentration of DO in the reoxygenation tank. For the start-up of the bioreactor, the effluent of the fish pond was introduced into the bioreactor. Subsequently, the water was circulated in the bioreactor and the fish ponds to form the recirculating system.

2.2. Physicochemical analysis and removal performance

The content of organic matter in the wastewater was expressed as COD. The concentrations of ammonia, nitrite, nitrate and COD were measured daily in accordance with the Standard Methods for the Examinations of Water and Wastewater. The total nitrogen (TN) represented the summation of ammonia (NH_4^+-N), nitrite ($NO_2^- -N$) and nitrate ($NO_3^- -N$). The pH was analyzed using a pH meter (PHS-3C, INESA, China). DO level was measured by a portable DO meter (JPB-607A, INESA, China) and suspended solid (SS) was determined by a multi-parameter water quality tester (5B-2H, Lianhua, China). Prior to

Fig. 1. Schematic diagram of the simultaneous partial nitrification, anammox and denitrification (SNAD) bioreactor in recirculating aquaculture system.

the measurement of ammonia, nitrite and nitrate, water samples were filtered through 0.45 µm mixed cellulose esters membranes (Merck Millipore Ltd, Ireland). Removal efficiencies of ammonia, nitrite, nitrate, TN and COD in the bioreactor were calculated by equation as the following:

$$\text{Removal efficiency} = \frac{C_{\text{inf}} - C_{\text{eff}}}{C_{\text{inf}}} \times 100\% \quad (1)$$

C_{inf} and C_{eff} represented the concentration of nitrogen/COD in the influent and effluent of the bioreactor, respectively.

2.3. Quantification of the microbial activity of SNAD biofilm

The aerobic nitrite oxidation, anammox and denitrification activities of the SNAD biofilm in the biofilter were determined following the method of Zheng et al. (2016). Briefly, the plastic media with biofilm used in the batch tests were taken out from the SNAD bioreactor at the end of the experiment. The procedure for batch test was described in the methodology of Zheng et al. (2016). The batch tests were performed in 1000 mL flasks. $\text{NH}_4^+ - \text{N}$ (70 mg·L⁻¹) and NaHCO_3 (840 mg·L⁻¹) were added for the AOB activity assay. $\text{NH}_4^+ - \text{N}$ (70 mg·L⁻¹) and $\text{NO}_2^- - \text{N}$ (70 mg·L⁻¹) were mixed for the assay of anammox activity. $\text{NO}_2^- - \text{N}$ (70 mg·L⁻¹) and NaH_2CO_3 (520 mg·L⁻¹) were mixed for denitrification activity assay. In the anammox and denitrification activity measurement system, the flasks were pumped with high-purity N_2 gas (99.99%) for 15 min to remove the oxygen before being sealed. In the case of the AOB activity assay, the flasks were purged with air at the flow rate of 250 mL·min⁻¹, setting the DO > 5 mg·L⁻¹. All the assays were conducted at 30 °C.

2.4. Analysis of microbial communities

Biofilm samples on the surface of plastic biofilter media were collected for DNA extraction by TIANamp Soil DNA Kit (TIANGEN Biotech, Beijing, China) according to manufacturer's instructions. The concentration, purity and quality of DNA were measured by a UV-vis spectrophotometer (NanoDrop Lite, Thermo Scientific, Wilmington, USA) and 1% agarose gel electrophoresis. Purified microbial DNA extracted from biofilm samples were sent to Majorbio (Shanghai, China) for high-throughput sequencing. The V4-V5 regions of the bacterial 16S rRNA genes were selected for amplification by PCR system (GeneAmp 9700, ABI, USA), with a pair of primers 515F (5'-GTGCCAGCMGCCG CGG-3') and 907R (5'-CCGTCGAATTCMTTTRAGTTT-3'). The PCR program and Illumina MiSeq Sequencing were carried out as reported in our previous research according to the standard protocols in Majorbio (Shanghai, China) (Zhang et al., 2020). Raw reads were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession Number: SRP229760).

2.5. Quantification of nitrogen functional genes

Eight nitrogen functional gene fragments were quantified by qPCR, including ammonia mono-oxygenase gene (*amoA*), nitrite oxidoreductase gene (*nirK*), anammox bacteria (ANO 16S rRNA gene), membrane-bound nitrate reductase gene (*narG*), periplasmic nitrate reductase gene (*napA*), copper-containing nitrite reductase gene (*nirK*), nitric oxide reductase gene (*qnorB*) and nitrous oxide reductase gene (*nosZ*). All primer sequences have been listed in Table 1 as reported in previous publications (Braker and Tiedje, 2003; Ji et al., 2012).

The plasmid DNA was constructed by gene synthesis with the carrier of PMD-19 T. Calibration standard curves for absolute quantification were established by a ten-fold dilution series of plasmids DNA ranging from 10⁷ to 10⁰ copies/µL. All the plasmids DNA were run together with samples in triplicate. Each 10 µL mixture for qPCR contained 1.0 µL DNA template, 0.4 µL forward primers (0.4 µM), 0.4 µL reverse

Table 1
Primers for target nitrogen functional genes.

Targets	Primer Sequence (5'-3')	Amplicon size/bp
<i>amoA</i>	<i>amo598f</i> GAATATGTTTCGCTGATTG <i>amo718r</i> CAAAGTACCACCATACGCAG	120
<i>nirK</i>	<i>nirK</i> F1 CAGACCGACGTGTGCGAAAG <i>nirK</i> R1 TCYACAAGGAACGGAAGGTC	322
ANO 16S rRNA	AMX809F GCCGTAAACGATGGGCACT AMX1066R AACGTCTCACGACAGAGCTG	257
<i>narG</i>	<i>narG</i> 1960m2f TA(CT)GT(GC) GGGCAGGA(AG)AAACTG	100
<i>napA</i>	<i>napG</i> 2050m2r CGTAGAAGAAGCTGGTGTGTT <i>napA</i> V17F TGGACVATGGGYTYAAYC	152
<i>nirK</i>	<i>napA</i> 4R ACYTCRCGHGCVTRCCRCA <i>nirK</i> 583F TCATGGTGTGCTGCCGKGCACGG	326
<i>qnorB</i>	<i>nirK</i> 909R GAACTTGCCGGTGKCCACAGC <i>qnorB</i> 2F GGNCAYCARGGNTAYGA	262
<i>nosZ</i>	<i>qnorB</i> 5R ACCCANAGRTGNACNACCCACCA <i>nosZ</i> 1527F CGCTGTTCTTCGACAGYCA	250
	<i>nosZ</i> 1773R ATRTCGATCARCTGTCGTT	

primers (0.4 µM), 5.0 µL SYBR Premix Ex Taq II (Takara, Dalian, China), and 3.2 µL ddH₂O. The amplified reactions were conducted by Bio-Rad qPCR system (Bio-Rad CFX384 Touch, USA) with the following program: denaturation at 95 °C for 30 s, 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Melting curves were conducted with temperature ranging from 60 °C to 95 °C, and the melting curves with a single peak were considered as specific amplification. When the amplification efficiencies were in the range of 90%–110% and the R² values of standard curves were higher than 0.99, the reactions were taken into account for quantification of target genes as previous reports (Lu et al., 2020).

2.6. Statistical analysis

Statistical analysis and plotting were performed using Origin 2017 (Origin Lab Corporation, USA). Correlations were analyzed by SPSS 19 (IBM, USA) and the statistical tests were considered significant at $p < 0.05$. Network analysis was performed in R environment, and further visualized by Cytoscape 3.7.1.

3. Results and discussion

3.1. Performance of the SNAD bioreactor in the recirculating aquaculture system

The SNAD bioreactor in the recirculating aquaculture system was operated for 180 days. The temperature, pH and DO concentration of the effluent were controlled at 25–30 °C, 7.5–8.5, and 7.0 mg/L, respectively. The performance of COD, SS, DO, and C/N ratio of the reactor were showed in Fig. 2. The experimental period could be divided into three stages: Phase I, days 1–20; Phase II, 21–60; Phase III, 61–180. In the first 20 days, the concentrations of COD decreased significantly from 49.1 mg/L to 10.4 mg/L in the influent and from 39.1 mg/L to 6.9 mg/L in the effluent. The reactor showed high performance for the removal of COD. In the second phase, the COD level still presented obvious fluctuation. However, it demonstrated an overall decreasing tendency, with the maximum of 21.6 mg/L in the influent and 9.5 mg/L in the effluent. From day 61 to day 180 (Phase III), the lower concentration of COD varied from 0.9 mg/L to 9.7 mg/L in the influent and ranged from 0.6 mg/L to 6.4 mg/L in the effluent, indicating the effective removal for COD of the bioreactor. The performance of SS demonstrated that it gradually decreased from Phase I to Phase III and decreased from the influent to the effluent, at the average of 8.9 mg/L (6.3 mg/L), 8.0 mg/L (4.0 mg/L) and 6.2 mg/L (3.4 mg/L) in the influent (effluent). The DO concentration was maintained in the range of

Fig. 2. The water treatment performance of the SNAD bioreactor on basic water quality parameters.

5–7 mg/L, followed by gradient descent in the effluent of U-type tube I (2 m, 1–3 mg/L) and U-type tube II (5 m, 0–1 mg/L). Subsequently, the DO concentration increased to 7–8 mg/L in the effluent of the bioreactor, which could provide adequate oxygen for the fish growth. Additionally, the low C/N ratio in the system was appropriate for the removal of nitrogen in SNAD process.

In recent years, many countries and regions have enforced stringent discharge standards of nitrogen in aquacultural industry. It was urgent to develop eco-friendly and economical technologies for nitrogen removal. The SNAD process required the simultaneous activities of nitrification, anammox and denitrification, which both preferred to anoxic and anaerobic conditions (Gao et al., 2013). Therefore, the variation of DO created favorable conditions for nitrogen removal in the SNAD process. The SNAD bioreactor was constructed with the advantages of short process flow, fewer equipment, and lower energy consumption. It was performed in natural conditions without the addition of extra carbon. Wastewater from the fish ponds flowed through the aerobic zone, anoxic zone, anaerobic environment and re-aerobic environment in the SNAD bioreactor. The system provided suitable environment for the breeding of diverse functional bacteria and genes by the variation of aerobic-anoxic-anaerobic conditions. Additionally, the plastic biofilter media in the bioreactor were excellent carriers for

Fig. 3. The performance of ammonia (A), nitrate (B), nitrite (C) and total nitrogen (TN).

the breeding of bacteria, as plastic biofilter media was suitable for forming biofilm (Cimbritz et al., 2019). They were filled into the reactor, implementing a separation of gas-liquid-solid phase, maintaining biofilms for succession of microorganisms, and resulting in the coexistence of aerobic and anaerobic environment. As a result, the SNAD bioreactor provided the alteration of aerobic-anoxic-anaerobic environment and gas-liquid-solid phase for the breeding of bacteria. It was an efficient and economical approach for water treatment under high DO conditions in the recirculating aquaculture system.

3.2. Nitrogen removal of the SNAD bioreactor

The performance of nitrogen removal was exhibited in Fig. 3. In Phase I, ammonia concentration in the influent was up to 1.36 mg/L, and it decreased to 0.50 mg/L on day 20. In the effluent, the concentration of ammonia maintained in the range of 0.02 mg/L to 0.89 mg/L. The highest removal efficiency of ammonia reached 92%. For nitrate, the maximum concentration was 6.11 mg/L in the influent, while it decreased to 4.66 mg/L in the effluent. The removal rate of nitrate reached to 63%, resulting in the lower nitrate concentration in the effluent. The nitrite concentration in the influent decreased from

2.07 mg/L to 0.42 mg/L on day 20 and it varied from 0.01 mg/L to 1.92 mg/L in the effluent, indicating that the nitrite removal efficiency could reach 99%. After 20-day operation, the TN concentration in the influent decreased stepwisely to 5.41 mg/L, while that in the effluent was lower with 4.71 mg/L. In Phase II (from day 20 to day 60), the nitrogen descended by the time and tended to be stable. The influent ammonia concentration decreased gradually from 0.5 to 0.6 mg/L to 0.2–0.5 mg/L, while the effluent ammonia concentration ranged from 0.1 to 0.4 mg/L during day 55 to day 60. At the end of Phase II, the concentrations of nitrate and nitrite reduced to 2.50/0.73 mg/L and 0.29/0.11 mg/L in the influent/effluent, respectively. The removal efficiencies of ammonia, nitrate, nitrite and TN reached 67%, 100%, 100% and 92%. After running for 60 days, the bioreactors arrived at the stable stage (Phase III). The influent ammonia, nitrate and nitrite concentrations were almost lower than 0.8 mg/L, 3.0 mg/L and 1.5 mg/L, respectively. The bioreactor held significant effect for the removal of nitrogen at the stable state, maintaining the average concentration of ammonia, nitrate and nitrite in the effluent at 0.26 mg/L, 0.75 mg/L and 0.47 mg/L, which were suitable for the subsistence of fish.

In the B1 biofilm with high DO concentrations, the anammox activity, denitrification activity, and aerobic nitrite oxidation activity were 0.186 (kg TIN kg VSS⁻¹ d⁻¹), 0.143 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), and 0.241 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), respectively. In the B5 biofilm with moderate DO concentration, the anammox activity, denitrification activity, and aerobic nitrite oxidation activity were 0.217 (kg TIN kg VSS⁻¹ d⁻¹), 0.178 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), and 0.159 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), respectively. In the B9 biofilm with low DO concentrations, the anammox activity, denitrification activity, and aerobic nitrite oxidation activity were 0.269 (kg TIN kg VSS⁻¹ d⁻¹), 0.211 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), and 0.091 (kg NO₂⁻-N kg VSS⁻¹ d⁻¹), respectively. The concentration of DO has great influence on the nitrogen removal process. The anammox and denitrification activity increased while the aerobic nitrite oxidation decreased in the SNAD bioreactor where DO concentration decreased. However, the anammox and denitrification were not totally inhibited under high DO concentrations in this system. The inhibition of high DO on these anaerobic bioprocesses might be alleviated by the biofilm on the biofilter media (Zheng et al., 2016). Consequently, the SNAD bioreactor presented high performance for nitrogen removal to make the nitrogen contents meet the fishery water quality standards for fish growth.

3.3. Microbial community structure of the SNAD bioreactor

The microbial communities of the biofilm on the surface of SNAD bioreactor media were investigated (Table 2). The higher Shannon value and the lower Simpson value, the higher community diversity (Wen et al., 2016). The result demonstrated that community diversity in anoxic environment (B5 and B9) was higher than that in aerobic environment (B1 and B10). The Sobs, Ace and Chao1 estimators were used to estimate the community richness based on OTU number. These values demonstrated that the community richness of the biofilm samples collected from the bioreactor decreased stage by stage with the water flow direction, from the influent to the effluent (B1 to B10).

As shown in Fig. 4A, a total of 24 phyla were detected in these biofilm samples. Approximately 43.8%, 74.2%, 60.6% and 52.0% of the sequences were assigned into Proteobacteria in B1, B5, B9 and B10,

respectively. However, the relative abundance of other phyla varied significantly in different biofilm samples. In the biofilm sample of B1, Bacteroidetes accounted for 41.1%, followed by Chloroflexi (5.2%), Actinobacteria (2.8%) and Acidobacteria (2.3%). The difference was that Chloroflexi (12.5%) was the secondary abundant phylum in B5, followed by Actinobacteria (6.3%) and Acidobacteria (2.0%). The relative abundances of Chloroflexi (15.6%) and Bacteroidetes (12.2%) were second to the phylum of Proteobacteria in B9. On the contrary, there was obvious difference in the sample of B10, in which Firmicutes (21.3%) and Actinobacteria (19.3%) were detected with higher abundances. Similar dominant phyla were determined in previous reports (Zheng et al., 2019). Besides the DO concentration in different sampling points, the distribution and abundance of microorganisms, nitrogen, organics, etc., seemed also responsible for the bacterial community structure in the SNAD bioreactor. According to previous studies, the filamentous bacteria (Firmicutes, Bacteroidetes, Chloroflexi, etc.) with rapid growth rate were significant builders for backbones within bio-flocs, implying their competence in the removal of organic matter under substrate limiting conditions (Guo and Zhang, 2012).

A total of 298 genera were detected and identified in the biofilm samples, and the dominant genera were listed in Fig. 4B. *Pseudoxanthomonas*, *Acinetobacter*, *Lysobacter*, *Exiguobacterium*, *Pseudomonas*, *Gemmobacter* and *Cloacibacterium* were the dominant genera in the biofilm samples, with the average of relative abundances at 14.4%, 9.4%, 6.1%, 5.3%, 5.2%, 4.9% and 3.9%, respectively. However, the relative abundances of these genera differed in different samples. *Pseudoxanthomonas*, *Gemmobacter* and *Pseudomonas* were detected with higher relative abundance in B1, while *Lysobacter*, *Pseudoxanthomonas*, *Pseudomonas* and *Gemmobacter* accounted for higher percentage in B5. The top four dominant genera in B9 were *Pseudoxanthomonas*, *Acinetobacter*, *Cloacibacterium* and *Lysobacter*, while *Acinetobacter* and *Exiguobacterium* were the dominant genera in B10. Bacterial community structures were significantly different due to the different concentration of DO, nitrogen, COD, and so on. Different processes in the bioreactor presented great impact on bacterial communities, resulting in the shift of dominant bacteria following the wastewater treatment processes.

3.4. Functional bacteria of the SNAD bioreactor

Some nitrogen functional bacteria, including the nitrifying bacteria, anammox bacteria and denitrifying bacteria, were detected in this system. As shown in Fig. 5A & B, both AOB and NOB existed in the system, and NOB were detected with higher relative abundance in B10 (0.12%) and B1 (0.05%). However, the relative abundance of AOB was at the same level (0.04%) in different biofilm samples, which played important roles in the spatial nitrification process with limited oxygen. Nitrospirae was detected in B1 with the percentage of 0.04%, playing an important role in the ammonia oxidizing bacteria. Furthermore, the phylum of Planctomycetes, which was affiliated with anammox bacteria (Wang et al., 2019b), increased from the aerobic environment (1.0% of B1 and 1.0% of B10) to the anoxic environment (1.1% of B5 and 1.5% of B9). After 180-day operation, the order of *Planctomycetales* became dominant and played a vital role in the anammox process in the biofilter system, with higher abundance in B5 and B9 (1.0% and 1.4%) than B1 and B10 (0.9% and 0.8%). The result further confirmed that the anoxic condition was more suitable for the breeding and enrichment of anammox bacteria.

The presence of COD enabled the survival of denitrifiers, and the denitrifying bacteria were assigned to the phyla of Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. The impact of COD concentration in the influent on the shift of microbial communities was mainly mediated by the heterotrophic bacteria. The phylum of Bacteroidetes has been proven to be related to the degradation of organic carbon (Ding et al., 2018). It was detected with higher relative abundance in B1, in which the COD and DO concentrations were higher. The dominant phylum, Proteobacteria, was partially identified

Table 2
Diversity and richness estimators of bacterial communities in the bioreactor.

Sample	Shannon	Simpson	Sobs	Ace	Chao1	Coverage	Seq_num
B1	3.03	0.1144	410	458.09	463.37	0.9982	61,324
B5	3.19	0.0929	390	425.40	434.25	0.9985	55,045
B9	3.27	0.0938	384	405.67	411.50	0.9989	42,215
B10	3.10	0.1083	321	357.79	358.66	0.9987	45,036

Fig. 4. Bacterial communities on phylum level (A) and on genus level (B).

as nitrogen-fixation bacteria (Wen et al., 2016) and affiliated with AOB (*Nitrosomonadaceae*), NOB (*Nitrospira* and *Devosia*), and denitrifiers. Chloroflexi was speculated as scavengers of organic matter derived from anammox bacteria. Most members of Acidobacteria and Chlorobi were autotrophic bacteria and anaerobic bacteria. Furthermore, the bacteria responsible for heterotrophic denitrification and autotrophic denitrification were identified in Chlorobi and Chloroflexi, with higher

relative abundance in the anoxic environment (B5 and B9).

The genera of *Pseudoxanthomonas*, *Acinetobacter* and *Pseudomonas* were predominant as denitrifiers in this system. Other genera of denitrifiers were demonstrated in Fig. 5A & B. On the whole, the detected denitrifiers, including aerobic denitrifying bacteria and anaerobic denitrifying bacteria, presented a high relative abundance in the biofilm samples. The denitrifiers accounted for 40.2% and 37.6% in the anoxic

Fig. 5. The relative abundance of nitrogen functional bacteria (A & B) and absolute abundance nitrogen functional genes (C) of the SNAD bioreactor in the recirculating aquaculture system.

environment (B9 and B5), while the percentages of them in the aerobic environment were lower (B1 of 32.8% and B10 of 32.9%). To our knowledge, the genera of *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, *Paracoccus*, *Bacillus*, *Mesorhizobium*, *Stenotrophomonas*, *Halomonas*, *Thauera*, *Enterobacter* were important denitrifiers possessing abilities of heterotrophic nitrification and aerobic denitrification (HN-AD) as previous reports (Lu et al., 2008; Thakur and Medhi, 2019). Interestingly, most of the HN-AD bacteria were detected with higher relative abundance in the aerobic conditions (B1 and B10). Overall, the special bacterial structure provided an appropriate environment for the functional bacteria, and guaranteed the good performance for nitrogen removal.

In the SNAD process, the functional bacteria played important roles in the removal of nitrogen and COD (Zheng et al., 2019). Wastewater from the fish ponds was the source for the inoculation of functional bacteria to the bioreactor. As shown in the schematic diagram of the experimental equipment, B1, B5, B9 and B10 were located in different processes in the bioreactor to hold different functional microbial

communities. In addition, the plastic biofilter media filters filled in the bioreactor could be considered as micro-reactors, with different microbial communities in the inside and outside surface of the biofilm on the carriers. Consequently, the nitrifying bacteria, anammox bacteria and denitrifying bacteria, autotrophic bacteria and heterotrophic bacteria, as well as aerobic bacteria and anaerobic bacteria could be co-existent and synergistic in the SNAD system for the removal of nitrogen.

3.5. Nitrogen functional genes of the SNAD bioreactor

Real-time PCR quantified the absolute abundance of nitrogen functional genes, including genes involved in nitrogen transformation of nitrification (*amoA* and *nxrA*), anammox (ANO 16S rRNA gene) and denitrification (*narG*, *napA*, *nirK*, *qnorB* and *nosZ*) (Fig. 5C). In the nitrification process, *amoA* was a marker involved in ammonia oxidation to nitrite, and *nxrA* was a marker involved in nitrite oxidation to nitrate. ANO 16S rRNA gene, as a marker for anammox process, was involved in the transformation of ammonia and nitrite to nitrogen gas. As

previously reported, the denitrification process was divided into four steps. Both *napA* and *narG* were involved in the first step (nitrate to nitrite), *nirK* was a marker for the second step (nitrite to nitric oxide), *qnorB* was a marker involved in the third step (nitric oxide to nitrous oxide), and *nosZ* was involved in the last step (nitrous oxide to nitrogen gas) (Ji et al., 2012).

All the target functional genes were detected in the biofilm samples and the absolute abundances of these genes remained relatively stable from B1 to B4. The absolute abundance of *napA* was maintained at the highest level (4.6×10^6 copies/g– 8.7×10^6 copies/g), while that of *narG* was relatively lower (4.0×10^4 copies/g– 8.5×10^4 copies/g). The abundance of ANO 16S rRNA gene (1.2×10^6 copies/g– 2.5×10^6 copies/g) was second to *napA*, while the abundance of *nrxA* was the lowest. The gene of *amoA* was detected with the absolute abundance in the range of 2.0×10^3 copies/g– 4.9×10^3 copies/g from B1 to B4, while *nrxA* was detected with the absolute abundance ranging from 8.9 copies/g to 33.3 copies/g. The gene of *nrxA* exhibited a similar variation trend to *amoA* with lower abundance. In the biofilm samples of B5, the absolute abundance of target genes decreased significantly due to the reduction of substrates (organic carbon and N compounds) in the tailing water, while it increased to higher level in the biofilm samples from B6 to B9. The absolute abundance of *amoA* increased significantly in the samples from B6 to B9, as a result of the appropriate DO concentration for the partial nitrification reaction. It was important to note that the abundance of ANO 16S rRNA gene exceeded *napA* under strictly anaerobic conditions (B9). At B10, all the target genes reached equal level with the absolute abundance higher than 10^4 copies/g.

The simultaneous partial nitrification, anammox and denitrification systems were highly effective process to remove nitrogen. Nitrogen functional genes played important roles in the removal of nitrogen and they acted as important indicators for nitrogen removal ability. Despite the variation of DO concentration along the bioreactor, all the target nitrogen functional genes were detected in the biofilm samples. The different abundance of *narG* and *napA* might be attributed to the nitrogen functional bacterial communities, in which denitrifying bacteria accounted for the largest proportion. Additionally, *narG* played dominant roles under hypoxic conditions, while *napA* played significant roles both under aerobic and anoxic conditions (Huang et al., 2017). It was further confirmed that denitrification acted as a vital pathway for the removal of nitrogen in the SNAD process. In addition, owing to the appropriate enhancement of the anaerobic or anoxic environment in the bioreactor, the absolute abundance of denitrification functional genes increased slightly due to the shortage of extra organics. The abundance of ANO 16S rRNA gene was higher than the *nrxA* gene, indicating that the anammox reaction preceded the nitrite oxidation in the SNAD process. The similar environmental conditions and ecological interactions by AOB and NOB resulted in the similar variation pattern of *amoA* and *nrxA*. Furthermore, the lower abundance of *nrxA* might be attributed to the nitrification process and the competitive advantages of AOB (Wang et al., 2017).

3.6. Co-occurrence patterns of nitrogen functional bacteria and genes

Co-occurrence patterns between the dominant bacterial communities, nitrogen functional bacteria and nitrogen functional genes were investigated by network analysis. Connections with significant and strong correlations ($p < 0.05$, Pearson's correlation coefficient > 0.9) have been taken into account for further analysis. Fig. 6A exhibited the correlations between nitrogen functional genes and the bacterial communities on phylum level. There were close correlations between denitrification genes (*narG*, *napA*, *nirK*, *qnorB* and *nirK*), and the nitrification gene of *amoA* was closely related to *nrxA*. The phyla of *Bacteroidetes*, *Acidobacteria*, *RBG-1*, *Zixibacteria*, *Ignavibacteriae*, *Spiriochaetae* and *Nitrospirae* were assigned to *napA*, and *Ignavibacteriae* was assigned to *nosZ*. The phyla of *Firmicutes*, *Cyanobacteria* and *BRC1* were assigned to *amoA*, and *Actinobacteria*, *Firmicutes*, *Cyanobacteria* and

BRC1 were assigned to *nrxA*. Additionally, there were positive and significant correlations between the bacteria in different phyla, indicating the close connections between different functional bacteria for nitrogen removal.

The correlations between nitrogen functional genes and bacterial communities on genus level were demonstrated in Fig. 6B. The network analysis was performed based on the dominant bacteria (top 50 genera), nitrogen functional bacteria (30 genera) and nitrogen functional genes. A total of 391 pairs of significant correlations were identified involving 8 connections of gene & gene, 63 connections of gene & bacteria and 320 connections of bacteria & bacteria. Total 16 bacterial genera were assigned to *amoA* and 20 genera were assigned to *nrxA*. The representative nitrification bacteria *Devosia* have positive and significant correlations with *amoA* and *nrxA*. Furthermore, 26 genera were significantly correlated with the target denitrification genes, including amounts of typical denitrification bacteria, *Denitratisoma*, *Enterobacter*, *Flavobacterium*, *Inhella* and *Thauera*, for instance.

Co-occurrence patterns between target genes and microbial taxa could indicate the possible host information, if the target genes and the co-existed microbial taxa possessed the significantly similar abundance trends among different samples (Lu et al., 2020). Therefore, the network analysis has been utilized to reveal the correlations between the target nitrogen functional genes and bacteria, and the potential host bacteria of nitrogen functional genes. Complicated strong and significant correlations were identified between different nitrogen functional genes and bacteria on phylum and genus level, for example, gene & gene, gene & bacteria, and bacteria & bacteria. According to previous reports, the aggregated growth was a favorite way for anammox bacteria and the competing of denitrification and anammox could promote the agminate growth of anammox bacteria (Gao et al., 2012). Nitrification, anammox and denitrification reaction in this SNAD bioreactor were the dominant nitrogen removal pathways. Owing to the coexistence of aerobic, anaerobic and anoxic environment, the SNAD bioreactor achieved high performance of nitrogen removal for treating aquaculture wastewater in the recirculating aquaculture system. The combined actions of nitrogen functional bacteria and nitrogen functional genes were crucial and indispensable for the removal of nitrogen, resulting in the effective removal for ammonia, nitrite and nitrate in the SNAD process.

4. Conclusions

The SNAD system for simultaneous removal of nitrogen and COD under high DO conditions was established in the recirculating aquaculture system. The bioreactor achieved high-efficient removal for nitrogen and COD in spontaneous environment. The typical nitrogen functional bacteria and genes for nitrification, anammox and denitrification were detected with different abundances in different procedures along the bioreactor. Complicated strong and significant correlations between nitrogen functional bacteria and genes were identified. The SNAD bioreactor achieved effective removal for nitrogen and COD under high DO conditions in the recirculating aquaculture system with the cooperation of nitrogen functional bacteria and genes.

CRediT authorship contribution statement

Jian Lu: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Resources, Writing - original draft, Writing - review & editing. **Yuxuan Zhang:** Formal analysis, Investigation, Methodology, Writing - original draft. **Jun Wu:** Methodology, Investigation, Software, Writing - review & editing. **Jianhua Wang:** Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial

A. Phylum

B. Genus

● AOB ● NOB ● ANAMMOX ● Denitrification ● Nitrogen functional genes ● Other genera

Fig. 6. Network analysis between nitrogen functional bacteria and genes on phylum level (A) and on genus level (B).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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